Troponin I from Human Skeletal and Cardiac Muscles

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(Received 13 September 1977)

1. Myofibrils from human skeletal muscle contained regulatory proteins exhibiting similar electrophoretic behaviour to those present in rabbit skeletal muscle. 2. All human skeletal muscles examined contained two forms of troponin I corresponding to the forms already characterized in fast and slow rabbit muscle. 3. The ratios of the amounts of the two forms of troponin I in different human skeletal muscles were not identical with the ratios for the type 1 to type 2 fibres published in the literature. The ratios could, however, be arranged in the same rank order. 4. Primate heart contained a single form of troponin I different in molecular weight and amino acid composition from the skeletal forms. 5. A monospecific antiserum to human cardiac troponin I was prepared in the sheep and shown not to react with the fast or slow skeletal-muscle forms of troponin I from human and other species. 6. The anti-(human cardiac-muscle troponin I) reacted with the cardiac troponin I from the human, baboon, rabbit and rhesus monkey. Positive reactions were also obtained with urea extracts of whole cardiac tissue.

The proteins of the myofibril of striated muscle have been characterized with some precision in experimental animals, such as the rabbit. Indeed the state of knowledge of the myofibril of rabbit fast skeletal muscle is such that the primary structures of five of the six major proteins of which it is composed, together accounting for about 95% of the total protein, have been determined (Elzinga *et al.*, 1973; Collins, 1974; Stone *et al.*, 1974; Wilkinson & Grand, 1975; Pearlstone *et al.*, 1976). It is likely that proteins similar to those well characterized in animal skeletal and cardiac muscle are present in the corresponding human tissues. Nevertheless minor differences in the proteins isolated from the myofibrils of human muscle are likely to arise as a result of normal species variations and the differences in the relative proportions of the different fibre types that are known to exist between species. Differences in the activity patterns of human muscle compared with those of the equivalent tissue from experimental animals may also be reflected in the composition of the myofibrillar proteins. These proteins possess a number of features that it may be possible to exploit in the diagnosis of the diseases of muscle. In particular, myosin, tropomyosin, troponin I, troponin C and troponin T all exist in polymorphic forms that are characteristic of the muscle type from which the protein is derived, i.e. fast and slow skeletal, cardiac and smooth (see Perry, 1974, for review). The polymorphic forms of each protein are different gene products, and for troponin C (Hirabayashi & Perry,

1974) and the α - and β -subunits of tropomyosin (Cummins & Perry, 1974) can be distinguished by immunochemical means. Actin appears to be more strongly conserved, although differences between the primary sequences of skeletal and cardiac forms have been reported (Elzinga *et al.*, 1976).

The present investigation describes the characterization of one of the regulatory proteins, troponin I, with the aim of defining its distribution, the number of polymorphic forms present in human striated muscle and evaluating its use in the immunopathology of muscle. It has been shown that two forms of troponin I, which are chemically and immunochemically different from human cardiac troponin I, are present in all human skeletal muscles studied. The proportion of the two forms is constant for a given muscle, but varies between muscles.

Materials and Methods

Tissue sources

Samples of fresh human rectus abdominus muscle were obtained during routine abdominal surgery. Gastrocnemius and soleus muscle specimens were excised from healthy areas immediately after surgical amputation of limbs. Biceps and anconeus specimens were excised *post mortem*, usually within 1–2 h of death.

Myocardial tissue was excised during cardiac surgery or obtained within 1–2 h *post mortem* whenever possible. Cardiac and skeletal muscle, excised from the baboon and rhesus monkey immediately after

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death, were supplied by the Primate Colony, Department of Anatomy, University of Birmingham.

Unless samples were used immediately for protein preparations, they were deep frozen in either liquid N₂ or solid CO₂ and stored at -20°C.

Preparation of muscle proteins

Unless otherwise stated, all preparative procedures were carried out at 4°C.

Myofibrils and 'relaxing protein' fraction. Myofibrils from human skeletal muscle were prepared by the method of Perry & Zydowo (1959). 'Relaxing protein' fractions were prepared from the extract obtained by homogenizing minced muscle in 4 vol. of 50 mM-KCl/10 mM-Tris/15 mM-2-mercaptoethanol adjusted to pH 8.0 with 1 M-HCl. After standing for 30 min the homogenate was centrifuged for 20 min at 1500g, the residue dried with ethanol and diethyl ether and extracted with 1 M-KCl/25 mM-Tris/15 mM-2-mercaptoethanol, adjusted to pH 8.0 with 1 M-HCl as described for the preparation of tropomyosin (Cummins & Perry, 1973). The extract was dialysed against 0.2 M-KCl / 5 mM-Tris / 15 mM-2-mercaptoethanol, adjusted to pH 7.9 with 1 M-HCl, and the fraction precipitated between 40 and 60% saturation with (NH₄)₂SO₄ used as 'relaxing protein' preparations. Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate indicated that this fraction contained predominantly tropomyosin and the components of the troponin complex.

Tropomyosin and troponin components. Tropomyosin from rabbit fast skeletal muscle was prepared by the method of Cummins & Perry (1973). Troponin was prepared from rabbit skeletal muscle as described by Ebashi *et al.* (1971) and fractionated into troponins I, T and C by the method of Margossian & Cohen (1973). The method of Spudich & Watt (1971) was used to prepare rabbit skeletal actin.

Troponin I was isolated directly from whole primate skeletal and cardiac muscle by using the affinity-chromatographic method of Syska *et al.* (1974). Approx. 10g wet wt. of muscle was homogenized in 90 ml of 9 M-urea/1 mM-CaCl₂/15 mM-2-mercaptoethanol/75 mM-Tris, adjusted to pH 8.0 with 1 M-HCl. After centrifugation at 8000g for 20 min, the supernatant was decanted through glass wool and applied to columns (1 cm × 10 cm) of rabbit fast-skeletal-muscle troponin C linked to Sepharose 4B. Approx. 2 mg of troponin I was isolated per 10g (wet wt.) of cardiac and skeletal muscle. Troponin T and troponin C were isolated from rabbit white muscle as described by Margossian & Cohen (1973).

Troponin C-like protein from bovine brain. This protein, kindly supplied by Dr. T. C. Vanaman of the Department of Microbiology and Immunology, Duke University, Durham, NC, U.S.A., was prepared by the method of Watterson *et al.* (1976).

Electrophoresis

Electrophoresis was carried out in vertical slab assemblies (Cummins & Perry, 1973; Perrie *et al.*, 1973) under the following conditions: (1) In 0.1 M-sodium phosphate buffer, pH 7.0, 0.1% sodium dodecyl sulphate as described by Weber & Osborn (1969) in 10% (w/v) polyacrylamide gels; (2) on 20% (w/v) polyacrylamide gel in the presence of 82.5 mM-Tris/400 mM-boric acid buffer (pH 7.0)/0.1% sodium dodecyl sulphate/5 M-urea as described by Cummins & Perry (1973); (3) at pH 8.5 by the method of Schaub & Perry (1969) with 6 M-urea/25 mM-Tris/80 mM-glycine buffer (pH 8.5)/15 mM-2-mercaptoethanol in 8.0% (w/v) polyacrylamide gels; (4) on 15% (w/v) polyacrylamide gel at pH 3.2 as described by Panyim & Chalkley (1969) by using 0.9 M-acetic acid, and 6 M-urea in the gels.

Determination of ratios of polymorphic forms of troponin I

The relative amounts of the two forms of troponin I present in the protein isolated by affinity chromatography from different human muscles were determined by estimating the relative amounts of the complexes formed by each form of troponin I in the presence of an excess of troponin C from rabbit fast skeletal muscle as described by Amphlett *et al.* (1975). Densitometric scanning of polyacrylamide gels was carried out as described by Cummins & Perry (1973). Determination of protein weight ratios by pyridine extraction of strained electrophoretic bands was carried out as follows. Gel slices containing protein bands stained with Coomassie Brilliant Blue R were homogenized in 2.3 ml of 25% (v/v) pyridine in water and stirred for 2 h. After centrifugation at 500g for 5 min the A₆₀₀ of the supernatant was measured against 25% (v/v) pyridine blanks. Correction was made for background staining of the gel. Dye uptake was assumed to be equal for both the fast and slow forms of troponin I-troponin C complex.

CNBr digestion

This was carried out at troponin I concentrations of 0.5–2 mg/ml in 70% (v/v) formic acid for 24 h at 21°C as described by Wilkinson (1974). A 100-fold molar excess of CNBr over total methionine residues was used. After digestion, the reaction mixture was diluted 20-fold with water and freeze-dried. The molecular weights of the CNBr bromide peptides were estimated by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate as described by Weber & Osborn (1969). The molecular weights of the CNBr peptides of troponin I from rabbit fast skeletal muscle, determined by Wilkinson & Grand (1975) by sequence studies, were used to calibrate the gels.

Amino acid analysis

Amino acid analysis was carried out as described by Wilkinson *et al.* (1972).

Determination of protein concentrations

Protein was determined either by the Nesslerization procedure (Strauch, 1965) assuming a nitrogen content of 16%, or by determination of the A_{280} , with the following specific extinction coefficients: tropomyosin, 3.30; troponin C, 2.14; troponin T, 5.00; troponin I, 3.97.

Preparation of antisera to human cardiac troponin I

The purified protein (1 mg/ml) dissolved in 10 mM-HCl was diluted with an equal volume of Freund's Complete Adjuvant (Difco Laboratories, Detroit, MI, U.S.A.). Sheep were immunized by multisite injection into the back and hind limbs (four sites per dose) with 100 µg doses of troponin I at weekly intervals. After 4 weeks, 3–5 ml of blood was withdrawn and preliminary examination of the antisera carried out on agar plates by the Ouchterlony (1953) procedure.

When the antiserum was found to produce a satisfactory response, larger volumes (100–200 ml) of blood were withdrawn, the blood clot was removed and the antisera were stored at -20°C .

Immunodiffusion

Ouchterlony (1953) immunodiffusion tests were carried out in general as described previously (Cummins & Perry, 1974): 1.5% (w/v) agar containing 145 mM-NaCl/10 mM-sodium phosphate buffer (pH 7.1)/2.0% (w/v) poly(ethylene glycol) 6000 (BDH Chemicals, Poole, Dorset, U.K.) and 0.1% NaN_3 was used, and 35 µl of antigen or antisera solution was added to wells.

Antigen concentrations were adjusted to 1 mg/ml in 145 mM-NaCl/10 mM-sodium phosphate buffer, pH 7.1.

Urea extracts were prepared by homogenizing 1 g of cardiac or skeletal muscle in 9 vol. of 9 M-urea/75 mM-Tris (adjusted to pH 8.0 with 1 M-HCl)/1 mM- CaCl_2 /15 mM-2-mercaptoethanol. After centrifugation at 8000g for 20 min and decanting through glass wool, the supernatants were applied directly to the antigen wells.

Results

Identification of troponin components in human muscle

On electrophoresis of extracts of myofibrils prepared from freshly excised human gastrocnemius muscle, at pH 7.0 in the presence of 0.1% sodium

dodecyl sulphate, bands migrating with mobilities identical with those of actin, tropomyosin, troponin I and troponin C isolated from rabbit fast skeletal muscle were clearly observed (Plate 1a). Although components migrating with mobilities similar to those of troponin T and the three light-chain components of myosin of rabbit fast skeletal muscle could also be distinguished, the poor resolution from neighbouring bands and the relatively small amounts of these components present made precise identification difficult. Essentially similar patterns were obtained with myofibrils isolated from other human skeletal muscles, such as soleus, rectus abdominis, biceps, diaphragm and erector spinae. Unless the myofibrils were prepared either from freshly excised muscle or from muscle frozen or cooled within 2 h of removal, the electrophoretic pattern was less clearly resolved and extra bands, especially those corresponding to the lower-molecular-weight range, appeared on electrophoresis in sodium dodecyl sulphate. Myofibrils isolated from tissue that had been deep frozen immediately after excision and stored for several months gave a similar electrophoretic pattern to those isolated from fresh muscle.

To identify troponin I and troponin C more clearly, whole myofibrils were electrophoresed at pH 8.5 in 6 M-urea in the presence of Ca^{2+} . Under these conditions, troponin I and troponin C form a complex that migrates with a velocity intermediate between those of the individual proteins (Perry *et al.*, 1972; Head & Perry, 1974). The complex is dissociated when the free Ca^{2+} is removed with EGTA, and troponin I and troponin C migrate with their normal mobilities. A component migrating faster than tropomyosin was detected on electrophoresis of extracts of whole human gastrocnemius myofibrils in the absence of EGTA. This component, presumed to be a complex of troponin I with troponin C, disappeared when such extracts were electrophoresed in the presence of EGTA and a faster-migrating component, presumably troponin C, appeared (cf. Plate 1b). As human gastrocnemius is histochemically a mixed muscle (Johnson *et al.*, 1973), troponin I preparations obtained from it would be expected to contain two forms of troponin I and hence form two types of troponin I-troponin C complex, by analogy with the situation in rabbit skeletal muscle (Syska *et al.*, 1976). With isolated human skeletal muscle troponin I preparations, two types of complex (Plate 1b, gel iii; see below) could be identified. As the slower complex migrated with a velocity identical with that of tropomyosin, it could not be resolved in extracts of whole myofibrils, which normally contain the latter protein (see below; cf. Plate 1b, gels i and ii).

Similar results were obtained when 'relaxing-protein' fractions from muscle (see the Materials and Methods section) were electrophoresed in 6 M-

urea at pH 8.5. With the 'relaxing-protein' fraction the main bands observed were those corresponding to the faster of the two troponin I-troponin C complexes, which in the presence of EGTA was replaced by troponin C, the troponin I remaining at the origin, and tropomyosin (Plate 1b).

The fast component, produced on electrophoresis of these extracts in the presence of EGTA, migrated with the same mobility as did the Ca^{2+} -free form of troponin C isolated from rabbit fast skeletal muscle. The troponin C isolated from human muscle gave a single band on electrophoresis in 6M-urea, pH 8.6, and in sodium dodecyl sulphate, pH 7.0. Its mobility in the presence of Ca^{2+} was greater than that of the Ca^{2+} -free form and was identical with that of troponin C from rabbit fast skeletal muscle in the presence of Ca^{2+} .

Owing to the poor resolution obtained on electrophoresis of whole extracts of myofibrils in sodium dodecyl sulphate, troponin T was not readily identified. In the whole troponin complex prepared from human or baboon skeletal muscle the only other major component in addition to troponin I and troponin C observed on electrophoresis was a protein of mol.wt. approx. 37000. This was considered to represent the troponin T component of primate muscle.

Troponin I from human skeletal muscle

The yield of troponin I isolated by the affinity-chromatographic method from freshly excised human gastrocnemius, rectus abdominis, biceps, diaphragm, soleus and cardiac muscles was about the same as that obtained from rabbit longissimus dorsi muscle, i.e. about 2mg from 10g of muscle. Although the sterically available troponin C bound to Sepharose was estimated to be in about a 4-fold molar excess over the yield of troponin I, only about 20–30% of the total troponin I in the muscle applied to the column was extracted by this procedure. Similar yields have been reported previously with troponin I and troponin C affinity columns (Syska *et al.*, 1976; Head *et al.*, 1977). Troponin I isolated from human gastrocnemius muscle migrated on electrophoresis in the presence of sodium dodecyl sulphate with a velocity identical with that of troponin I from rabbit fast skeletal muscle (Plate 1c). Preparations from freshly excised muscle specimens migrated as a single component, but if tissue was left for more than 1–2h at 18°C before either deep-freezing or extraction, minor, faster-migrating components, presumably the results of proteolytic degradation by endogenous enzymes, were observed (see, e.g., Plate 1c). In its susceptibility to proteolytic modification, human troponin I resembled troponin I from other species (Wilkinson *et al.*, 1972).

When troponin I from human gastrocnemius muscle was electrophoresed with excess rabbit fast-skeletal-muscle troponin C, at pH 8.5 in the presence of 6M-urea, two bands corresponding to troponin I complexes with troponin C were observed (Plate 1b, gel iii). The faster of these complexes migrated with a mobility similar to that of the complex formed when both proteins were isolated from rabbit fast skeletal muscle, and the troponin I component was therefore considered to be the equivalent of that present in rabbit fast skeletal muscle, i.e. the fast-muscle polymorph. The slower of the complexes migrated with a velocity identical with that of tropomyosin and was not distinguishable from the latter when whole myofibrils or 'relaxing-protein' preparations were electrophoresed under the same conditions (Plate 1b, gels i and ii). The mobility of this complex was identical with that of the major component obtained when the troponin I from rabbit soleus muscle was examined under the same conditions and that contained the slow form of rabbit skeletal-muscle troponin I (Amphlett *et al.*, 1975). The form of human skeletal-muscle troponin I giving rise to the complex of lower electrophoretic mobility was therefore considered to be the slow polymorph. As with troponin I preparations from rabbit soleus muscle (Syska *et al.*, 1974), the fast and slow forms of troponin I from human skeletal muscle were not resolved on electrophoresis in sodium dodecyl sulphate, indicating that they possess very similar molecular weights.

Proportion of fast and slow muscle forms of troponin I in different human muscle types

The relative amounts of the fast and slow forms of troponin I in preparations isolated from human skeletal muscle were determined by a method involving the determination of the relative amounts of the two complexes formed on electrophoresis in the presence of an excess of rabbit skeletal-muscle troponin C (cf. Plates 1 and 2a; see the Materials and Methods section). Values obtained by densitometric gel scanning of the bands or by direct measurement of dye absorbed by pyridine extraction of the bands were essentially the same. The ratios of fast and slow forms of troponin I for six different human skeletal muscles are presented in Table 1.

It was possible to obtain values for the ratio of the amounts of the two forms of troponin I on a number of different samples of rectus-abdominus-muscle troponin I, as small samples of this muscle were frequently available during abdominal surgery. The ratios obtained with different samples of the same muscle were in good agreement if fresh tissue was used for troponin I preparations. Table 1 shows that, except for rectus abdominus muscle (Plate 2a, gel i), the human muscles studied contained

EXPLANATION OF PLATE I

Polyacrylamide-gel electrophoresis of myofibrillar proteins and cardiac- and skeletal-muscle troponin I from human muscle

(a) Whole myofibrils: 75 μ g of human gastrocnemius myofibrils was applied to a 10% (w/v) polyacrylamide gel in 0.1 M-sodium phosphate buffer (pH 7.0)/0.1% sodium dodecyl sulphate. (b) 'Relaxing-protein' extracts: samples of protein (75–100 μ g) were applied to 8.0% (w/v) polyacrylamide gels in 6 M-urea/25 mM-Tris/80 mM-glycine (pH 8.5)/15 mM-2-mercaptoethanol. (i) Human gastrocnemius 'relaxing protein'+0.1 mM- CaCl_2 . (ii) Human gastrocnemius 'relaxing protein'+0.1 mM-EGTA. (iii) Human gastrocnemius troponin I+2-fold molar excess of rabbit fast skeletal-muscle troponin C (TN-C)+0.1 mM- CaCl_2 . (c) Skeletal- and cardiac-muscle troponin I: samples of troponin I (20–30 μ g) were applied to 10% (w/v) polyacrylamide gels in 0.1 M-sodium phosphate buffer (pH 7.0)/0.1% sodium dodecyl sulphate. Sources of troponin I: (i) human gastrocnemius; (ii) rabbit fast skeletal muscle; (iii) baboon cardiac muscle; (iv) human cardiac muscle, isolated from fresh myocardium; (v) human cardiac muscle, isolated from myocardium 3 h *post-mortem*; (vi) rabbit cardiac muscle.

EXPLANATION OF PLATE 2

Polyacrylamide-gel electrophoresis of human cardiac- and skeletal-muscle troponin I

(a) Skeletal and cardiac troponin I-troponin C complexes. Samples of troponin I (20–30 μ g) were applied to 8.0% (w/v) polyacrylamide gels in 6 M-urea/25 mM-Tris/80 mM-glycine buffer, pH 8.5, in the presence of 80 μ g of rabbit fast-skeletal-muscle troponin C. Sources of troponin I: (i) human rectus abdominis; (ii) human erector spinae; (iii) rabbit fast skeletal muscle; (iv) rabbit cardiac muscle; (v) human cardiac muscle. Bands due to an excess of troponin C are marked with arrows. Other bands are due to troponin I-troponin C complexes. (b) CNBr peptides of cardiac troponin I. Proteins were treated with CNBr as described in the Materials and Methods section and samples of digests (20–40 μ g) were applied to 20% (w/v) polyacrylamide gels in 5 M-urea/82.5 mM-Tris/400 mM-boric acid buffer (pH 7.0)/0.1% sodium dodecyl sulphate. (i) Undigested human cardiac-muscle troponin I (approx. 30 μ g); (ii) rabbit cardiac-muscle troponin I; (iii) human cardiac-muscle troponin I; (iv) baboon cardiac-muscle troponin I.

EXPLANATION OF PLATE 3

Immunochemical reactions of sheep antisera raised against human cardiac-muscle troponin I

For details of agar diffusion procedure see the Materials and Methods section. Antigen or antisera solution (35 μ l) was added to wells. Antigen protein concentrations were adjusted to 1 mg/ml with 145 mM-NaCl/10 mM-sodium phosphate buffer, pH 7.1. In all cases the centre well contained sheep anti-(human cardiac-muscle troponin I) serum. Abbreviations: HCl, human cardiac-muscle troponin I; HRAI, human rectus abdominis troponin I; HBicI, human biceps troponin I; RCI, rabbit cardiac-muscle troponin I; BCI, baboon cardiac-muscle troponin I; RSC, rabbit skeletal-muscle troponin C; CCC, bovine cardiac-muscle troponin C; CBC, bovine brain 'troponin C-like' protein; CCT, bovine cardiac-muscle troponin T; HCEx, human cardiac-muscle urea extract; BCEx, baboon cardiac-muscle-urea extract; RCEx, rabbit cardiac-muscle urea extract; RhCEx, rhesus-monkey cardiac-muscle urea extract; BSEx, baboon skeletal-muscle urea extract; RSEx, rabbit-skeletal-muscle urea extract. (a) Effect of troponin C on reaction between human cardiac-muscle troponin I and corresponding sheep anti-(human cardiac-muscle troponin I) serum. (b) Effect of cardiac-muscle troponin C and troponin T on reaction between human cardiac-muscle troponin I and sheep anti-(human cardiac-muscle troponin I) serum. (c) Reaction of sheep anti-(human cardiac-muscle troponin I) serum with troponin I from human and rabbit skeletal muscles. (d) Reaction of sheep anti-(human cardiac-muscle troponin I) serum with cardiac-muscle troponin I from primates and the rabbit (e) Reaction of sheep anti-(human cardiac-muscle troponin I) with urea extracts of whole skeletal and cardiac muscle.

Table 1. *Relative amounts of the fast and slow forms of troponin I in human skeletal muscle*

Troponin I was isolated by affinity chromatography and electrophoresed in the presence of an excess of troponin C from rabbit fast skeletal muscle. Values for the weight ratios of the amounts of protein present in each of the two bands corresponding to the complexes formed by the two troponin I polymorphs and troponin C were determined as described in the Materials and Methods section.

Muscle	No. of samples	No. of determinations	Ratio fast/slow	Ratio type II/type I
Rectus abdominis	8	10	4.27 ± 0.14	1.17*
Biceps	2	4	2.07 ± 0.18	0.84–1.36*†
Diaphragm	2	4	1.95 ± 0.25	
Soleus	2	4	1.30 ± 0.20	0.12–0.30*‡
Erector Spinae	2	4	1.03 ± 0.14	0.15–0.82*‡
Anconeus	2	2	1.08 ± 0.02	

* On the basis of myofibrillar adenosine triphosphatase staining activity at pH 9.5 (Johnson *et al.*, 1973).

† On the basis of staining activity of myofibrillar adenosine triphosphatase at pH 9.4 and mitochondrial NADH-tetrazolium reductase (Kleine & Heene, 1971).

‡ On the basis of acid- and alkali-stable myosin adenosine triphosphatase-staining activity (Taylor *et al.*, 1974).

approximately equal proportions of the two forms of troponin I.

When troponin I preparations from different muscles were mixed and excess rabbit fast-skeletal-muscle troponin C was added, the fast- and slow-moving complexes from the different muscles co-migrated. Usually two bands corresponding to the complexes of fast and slow forms of troponin I with troponin C were observed when troponin I from human muscles was electrophoresed in the presence of an excess of troponin C from rabbit fast skeletal muscle. For troponin I isolated 4 h *post mortem* from two samples of erector spinae muscle, however, a component was observed migrating between the bands corresponding to the fast and slow troponin I–troponin C complexes (Plate 2a). This electrophoretic pattern was not seen in samples of troponin I isolated from other muscles immediately *post mortem*. The polymorph ratio for erector spinae muscle in Table 1 does not include an allowance for this band, which was thought to be a complex formed from a product of partial proteolysis of the troponin I. With troponin I preparations isolated from other human skeletal muscles at a similar time *post mortem*, a far more complex and variable pattern of troponin I–troponin C complexes was observed such as would be expected if the troponin I had undergone proteolytic breakdown by endogenous cathepsins.

Troponin from primate heart

Yields of troponin I, similar to those obtained from skeletal muscle, were obtained from freshly pooled surgically excised samples of infundibulum and auricular appendage of human myocardium. Yields were significantly decreased from tissue samples left for some time *post mortem* without chilling, and breakdown products of lower molecular weight were observed on electrophoresis at pH 7.0 in the presence of sodium dodecyl sulphate (Plate 1c). Degradation *post mortem* was observed more frequently with cardiac than with skeletal-muscle troponin I, and degraded preparations gave rise to several bands corresponding to complexes of the degradation products of troponin I with troponin C when electrophoresed in 6 M-urea at pH 8.5 in the presence of rabbit skeletal-muscle troponin C. Troponin I, isolated from freshly excised specimens of human myocardium, migrated on electrophoresis in sodium dodecyl sulphate as a single component identical in mobility with troponin I isolated from rabbit cardiac muscle (Plate 1c).

Undegraded preparations of troponin I could be obtained by affinity chromatography of extracts of baboon hearts excised immediately after death. Preparations of baboon cardiac-muscle troponin I migrated on electrophoresis in sodium dodecyl sulphate as a single component identical in mobility with human and rabbit cardiac troponin I (Plate 1c).

On electrophoresis at pH 8.5 in 6 M-urea, the complexes formed by baboon or human cardiac troponin I with rabbit fast-skeletal-muscle troponin C migrated with identical mobilities that were slightly less than that of the complex formed between human slow-skeletal-muscle troponin I and rabbit fast-skeletal-muscle troponin C (Plate 2a).

CNBr digestion of cardiac troponin I

Preparations of troponin from rabbit, baboon and human heart were digested with CNBr as described in the Materials and Methods section and the resulting digests compared by electrophoresis in 82.5 mM-Tris/400 mM-borate (pH 7.0)/0.1% sodium dodecyl sulphate/5 M-urea (Plate 2b). In all cases only occasional trace amounts of undigested protein could be detected. Nevertheless, the electrophoretic pattern of rabbit cardiac-muscle troponin I digests differed significantly from those obtained with human and baboon cardiac-muscle troponin I. So far as could be determined from their appearance on electrophoresis, the CNBr digests of baboon and human cardiac-muscle troponin I appeared to be identical. Both contained four peptides the apparent molecular weights of which differed from those present in CNBr digests of rabbit cardiac-muscle troponin I (Table 2) (Grand *et al.*, 1976). The total of

Table 2. *Molecular weights of CNBr peptides from primate cardiac troponin I*

Cyanogen bromide digestion and electrophoresis in sodium dodecyl sulphate was carried out as described in the Materials and Methods section. Molecular weights were determined by calibration of the gel with the CNBr peptides from rabbit cardiac-muscle troponin I with the molecular weights reported by Grand *et al.* (1976).

	Molecular weights	
	Human and baboon cardiac-muscle troponin I	Rabbit cardiac-muscle troponin I
Whole protein	23 000	23 350
CNBr peptides identified on electrophoresis in sodium dodecyl sulphate	13 200 10 700 8 500 5 400	16 500 11 220 5 280 4 950

Table 3. *Amino acid composition of troponin I isolated from cardiac muscles*

Values are expressed as the nearest whole number of amino acid residues/23 500 g of protein. Amino acid analyses were carried out as described by Wilkinson *et al.* (1972).

Amino acid	Baboon cardiac	Human cardiac	Rabbit cardiac*
Lys	24	20	24
His	3	3	3
Arg	23	23	23
Asx	19	17	17
Thr	8	9	10
Ser	11	15	8
Glx	30	32	31
Pro	9	10	5
Gly	12	12	11
Ala	23	26	23
Val	7	6	8
Met	3	2	4
Ile	9	8	7
Leu	22	22	23
Phe	4	4	4
Tyr	3	4	2
No. of preparations	3	4	

* Data of Grand *et al.* (1976).

the molecular weights of the bands identified in CNBr digests of primate cardiac-muscle troponin I suggested that partial cleavage products were present, as after digestion of rabbit cardiac-muscle troponin I with CNBr (Grand *et al.*, 1976).

Electrophoresis at pH 3.2 in the presence of 6M-urea confirmed that peptide patterns obtained with CNBr digests of baboon and human cardiac-muscle troponin I were identical, but different from those of digests of rabbit cardiac-muscle troponin I.

Table 4. *Amino acid composition of troponin I from human skeletal muscles*

Values are expressed as the nearest whole number of amino acid residues/21 000 g of protein. Analyses were carried out as described by Wilkinson *et al.* (1972).

Amino acid	Rectus abdominus	Biceps	Soleus	Rabbit fast skeletal*
Lys	22	24	21	24
His	6	5	6	4
Arg	16	17	16	16
Asx	18	17	16	15
Thr	7	6	6	3
Ser	11	11	12	10
Glx	28	29	32	33
Pro	8	7	8	5
Gly	10	8	10	8
Ala	14	15	15	14
Val	11	11	9	7
Met	7	8	7	9
Ile	5	4	6	5
Leu	18	19	19	17
Phe	4	3	3	3
Tyr	2	2	2	2
No. of estimations	2	2	2	
Ratio of fast/slow troponin I	4.27	2.07	1.30	All fast

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

Amino acid compositions of troponin I

The similarity of the electrophoretic patterns obtained with CNBr digests suggested that methionine contents were identical in the human and baboon cardiac-muscle troponin I. If this is indeed the case, the value obtained for the methionine content of human cardiac-muscle troponin I (Table 3) would appear to be too low. It is possible that the analytical data reflect the errors inherent in the determination of low amounts of methionine in the proteins rather than a real difference. Except for proline, the content of which was twice as high in the primate, only minor differences existed in the amino acid compositions of primate and rabbit cardiac troponin I preparations (Table 3).

Although the troponin I preparations of human rectus abdominus, biceps and soleus muscles (Table 4) consisted of variable proportions of the fast and slow polymorphic forms, the amino acid analyses of the four human skeletal troponin I preparations were very similar. The fact that the molar ratio of the fast and slow forms of troponin I ranged from 4.3:1 in rectus abdominus muscle to 1.3:1 for biceps muscle suggests that both the fast and slow forms of troponin I from human muscle have similar amino acid compositions. They also had a similar composition to rabbit fast-skeletal-muscle troponin I, although

human skeletal-muscle preparations contained significantly more proline per mol, as with troponin I from primate compared with the rabbit heart.

Immunochemical properties of troponin I

Unlike troponin I from human skeletal muscle, human cardiac troponin I was isolated as a single component and was therefore suitable for production of a monospecific antibody. When the reaction between human cardiac troponin I and its antisera raised in the sheep was tested by immunodiffusion in agar under a variety of different conditions, the precipitin lines formed were often very weak, although occasionally more intense, broader lines were obtained with some antisera preparations (Plate 3a). A doublet also occasionally appeared, but this correlated with the age of the tissue specimen from which troponin I was isolated and was therefore presumed to be due to the presence of a fragment produced by proteolysis. The low intensity of the precipitin reaction was considered to be due to the relatively insoluble nature of cardiac troponin I under the conditions of pH and ionic strength used for immunodiffusion. If the cardiac troponin I suspended in 145 mM-NaCl/10 mM-sodium phosphate buffer, pH 7.1, was solubilized by the addition of either rabbit fast-skeletal-muscle or bovine cardiac troponin C in a 1:1 molar ratio, a stronger precipitin line was routinely obtained when it was tested against sheep anti-(human cardiac-muscle troponin I) serum (Plates 3a and 3b). The troponin C-like protein isolated from bovine brain (Watterson *et al.*, 1976) was equally as effective in promoting a strong precipitin line between human cardiac troponin I and its antisera (Plate 3a).

When bovine cardiac-muscle troponin T was added to human cardiac troponin I plus bovine cardiac troponin C a similar increase in the intensity of the precipitin line occurred (Plate 3b). Surprisingly, bovine cardiac-muscle troponin T, which is not usually considered to be able to solubilize troponin I, was equally as effective as troponin C in intensifying the precipitin line given by human cardiac-muscle troponin I when tested against anti-(human cardiac-muscle troponin I) serum. Under the same conditions, neither bovine cardiac-muscle troponin C nor bovine cardiac-muscle troponin T gave any reaction against the serum (Plate 3b).

The effect of troponin C on troponin I in agar immunodiffusion was not dependent on the presence of Ca^{2+} ions. Rabbit skeletal-muscle troponin C plus human cardiac-muscle troponin I gave strong precipitin lines when tested against anti-(human cardiac-muscle troponin I) in both the presence and absence of EGTA. The action of troponin C in intensifying the precipitin reaction was not specific

to this protein, as polyanions such as heparin, which are able to solubilize troponin I, also intensified the precipitin reaction at weight ratios ranging from 1:10 to 1:1 for troponin I/heparin.

The anti-(human cardiac-muscle troponin I) serum was specific for cardiac troponin I and failed to react with other troponin components and tropomyosins from human, baboon and rabbit cardiac muscle and human and rabbit skeletal muscle. Troponin T isolated from rabbit fast skeletal muscle and bovine cardiac muscle also failed to give a precipitin line in the Ouchterlony procedure. Confirmation that the antiserum was monospecific for human cardiac-muscle troponin I, and not some unidentified impurity associated with it, was obtained by further purifying the protein. Human cardiac-muscle troponin I isolated by affinity chromatography was electrophoresed as described in the Materials and Methods section at pH 3.2 in the presence of 6 M-urea. The troponin I band, located by staining a thin slice of the polyacrylamide gel, was homogenized in 6 M-urea for 5 min and centrifuged at 500g for 5 min to remove the acrylamide. The supernatant was freeze-dried and a sample dissolved in water (approx. 0.1 mg/ml). When tested against anti-(human cardiac-muscle troponin I) sera, a weak precipitin band was obtained, which was considerably enhanced by the addition of rabbit skeletal-muscle troponin C. Extracts from all other portions of the gel failed to give any reaction.

Skeletal-muscle troponin I preparations isolated from human biceps, soleus, diaphragm and rectus abdominus muscles failed to give a positive reaction when tested against anti-(human cardiac-muscle troponin I) sera (Plate 3c) in the presence or absence of troponin C. Human, baboon and rabbit cardiac-muscle troponin I, however, all gave a common single precipitin line when tested against anti-human cardiac-muscle troponin I) sera. Rabbit cardiac-muscle troponin I, however, invariably gave a weaker precipitin line than the primate antigens under the same conditions (Plate 3d).

The presence of antigen could be conveniently detected in extracts of whole muscle prepared in 9 M-urea/75 mM-Tris adjusted to pH 8.0 with 1 M-HCl. The sample (35 μl) of urea extract added to the antigen well of the Ouchterlony plate was such that the final concentration of urea in the agar was too low to affect precipitin formation. Urea extracts of human, baboon, rabbit and rhesus-monkey cardiac muscle prepared in this way all gave single precipitin lines that fused into a reaction of identity (Plate 3). Rabbit, human or baboon skeletal-muscle extracts, however, failed to give any reaction under similar conditions. The absence of reaction with the skeletal-muscle extracts was not due to the insolubility of troponin I, as troponin C is present in whole muscle extracts.

Discussion

The electrophoretic properties of the myofibrillar proteins actin, tropomyosin, myosin and the troponin components appear to be similar in human and rabbit skeletal and cardiac muscles. In the human, two forms of troponin I are present in skeletal muscle and one in cardiac muscle. Their apparent molecular weights, as determined by electrophoresis in sodium dodecyl sulphate, are very similar to those reported for the corresponding proteins from the rabbit, i.e. 23000 and 29000 for the skeletal and cardiac forms respectively (Syska *et al.*, 1974). Indeed it seems likely that the molecular weights of the different forms of troponin I in all primates are similar to those of the equivalent proteins from the rabbit. Unlike the rabbit, in which the fast and slow forms of troponin I are often concentrated in different skeletal muscles, all the human muscles studied contained substantial amounts of both forms of troponin I. A similar distribution probably applies to the fast and slow forms of troponin T (Hitchcock, 1973; Perry & Cole, 1974; Clarke *et al.*, 1976).

The two forms of human skeletal-muscle troponin I are not readily distinguishable by electrophoresis of the purified proteins, but can be separated by electrophoresis of their respective complexes with troponin C, the mobilities of which are independent of the troponin C used (Head *et al.*, 1977; Syska *et al.*, 1974). The wide distribution of both forms of troponin I in human muscles probably reflects the fact that most are of mixed fibre type.

In rectus abdominus muscle, 81% of the troponin I was in the fast form, whereas in the other muscles studied it represented between 50 and 66% of the total. If fast-muscle troponin I is derived from fast type-II muscle cells and slow-muscle troponin I from the slow type-I cells, and the two types of cell contain approximately equal amounts of troponin I, these values should correspond to the published histochemical fibre-typing ratios. This does not appear to be the case; for example, human soleus muscle has been demonstrated to possess between 77 and 95% slow type-I fibres on the basis of histochemical fibre staining of both myosin adenosine triphosphatase and myofibrillar adenosine triphosphatase activities at pH 9.4 and 9.5 respectively (Table 1) (Johnson *et al.*, 1973; Taylor *et al.*, 1974).

It might be expected that the histochemical fibre type that reflects the enzymic composition of individual fibres would correlate with the particular contractile and regulatory-protein polymorph compositions of the myofibril. Certainly a similar correlation has been established in rat diaphragm muscle between myosin antisera staining and localization of alkali-stable adenosine triphosphatase staining (Gauthier & Lowey, 1975). One reason for the apparent discrepancy in skeletal muscle may be the

generally poor agreement between the published histochemical data on human muscle. Differences in the histochemical fibre-type ratios and the ratio of the two forms of troponin I present may also be due to sampling errors, i.e. the histochemical staining may be carried out on a more restricted region of the muscle than the protein isolation, which in general involved relatively larger amounts of the tissue. The final explanation of the apparent discrepancies will hopefully come from careful immunochemical investigation of the distribution of the two forms of troponin I among the different fibre types of human skeletal muscle.

Although the ratios of cell type and the amounts of the troponin I polymorphs for a given muscle do not agree in absolute values there is, however, good correlation when the two ratios are arranged in numerical order (Table 1).

The immunochemical studies provide further evidence of the tissue specificity of the regulatory proteins (Hirabayashi & Perry, 1974; Cummins & Perry, 1974). The finding that troponin C often enhanced the precipitin line obtained with antisera to human cardiac troponin I and its corresponding antigen contrasts with the finding of Hirabayashi & Perry (1974) with antisera to troponin C. They reported that the specific interaction that occurs between troponin C and troponin I weakened the immunochemical reaction between chicken skeletal-muscle troponin C and its monospecific antiserum raised in the guinea pig. In the latter case, the antigenic sites on troponin C appeared to be masked by interaction with troponin I. Such a masking of the antigenic sites on troponin I in the presence of troponin C does not occur with the anti-(cardiac-muscle troponin I) sera. The effect of troponin C did not appear to be specific for this protein, but was possibly due to its polyanionic properties, which helped to solubilize the troponin I. The polyanionic properties are probably independent of the presence of Ca^{2+} , which is known to enhance the binding of troponin C to troponin I (Head & Perry, 1974).

Although the anti-(human cardiac-muscle troponin I) sera did not react either with isolated skeletal-muscle troponin I preparations or extracts of whole muscle, both of which contained fast and slow forms of troponin I, it reacted with all samples of cardiac-muscle troponin I tested. The reaction with rabbit cardiac-muscle troponin I was not as intense as that with primate cardiac-muscle troponin I preparations. This was probably related to the differences in structure between the primate and non-primate antigens, which were reflected in the appearance of CNBr digests of the troponin I preparations on electrophoresis.

It can be concluded that of the three forms of troponin I present in human striated muscle, namely cardiac, fast skeletal and slow skeletal, the cardiac

form has been demonstrated to give rise to a highly specific antiserum that does not cross-react under the conditions of the Ouchterlony procedure with the skeletal-muscle forms of the protein. There is preliminary evidence (G. K. Dhoot, P. G. H. Gell & S. V. Perry, unpublished work) that fast-skeletal-muscle troponin I may be equally immunochemically specific. If similar immunochemical specificity is associated with all forms of troponin I isolated, antisera to them could provide a valuable tool for the detection of those diseases of cardiac and skeletal muscle that are characterized by a leakage of myofibrillar proteins into the bloodstream.

We are grateful to Miss Carol Bryant for skilled technical assistance and to Miss Susan Brewer for carrying out the amino acid analyses. This work was supported by the Muscular Dystrophy Group of Great Britain.

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