Electrophoretic analysis of proteins from single bovine muscle fibres

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(Received 14 November 1980/Accepted 1 December 1980)

A number of single fibres were isolated by dissection of four bovine masseter (ma) muscles, three rectus abdominis (ra) muscles and eight sternomandibularis (sm) muscles. By histochemical criteria these muscles contain respectively, solely slow fibres (often called type I), predominantly fast fibres (type II), and a mixture of fast and slow. The fibres were analysed by conventional sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and the gels stained with Coomassie Blue. Irrespective of the muscle, every fibre could be classed into one of two broad groups based on the mobility of proteins in the range 135 000–170 000 daltons. When zones containing myosin heavy chain were cut from the single-fibre gel tracks and 'mapped' [Cleveland, Fischer, Kirschner & Laemmli (1977) *J. Biol. Chem.* **252**, 1102–1106] with *Staphylococcus* proteinase, it was found that one group always contained fast myosin heavy chain, whereas the second group always contained the slow form. Moreover, a relatively fast-migrating α -tropomyosin was associated with the fast myosin group and a slow-migrating form with the slow myosin group. All fibres also contained β -tropomyosin; the coexistence of α - and β -tropomyosin is at variance with evidence that α -tropomyosin is restricted to fast fibres [Dhoot & Perry (1979) *Nature (London)* **278**, 714–718]. Fast fibres containing the expected fast light chains and troponins I and C fast were identified in the three ra muscles, but in only four sm muscles. In three other sm muscles, all the fast fibres contained two troponins I and an additional myosin light chain that was more typical of myosin light chain 1 slow. The remaining sm muscle contained a fast fibre type that was similar to the first type, except that its myosin light chain 1 was more typical of the slow polymorph. Troponin T was bimorphic in all fast fibres from ra muscles and in at least some fast fibres from one sm muscle. Peptide 'mapping' revealed two forms of fast myosin heavy chain distributed among fast fibres. Each form was associated with certain other proteins. Slow myosin heavy chain was unvarying in the three slow fibre types identified. Troponin I polymorphs were the principal indicator of slow fibre types. The myofibrillar polymorphs identified presumably contribute to contraction properties, but beyond cud chewing involving ma muscle, nothing is known of the conditions that gave rise to the variable fibre composites in sm and ra muscles.

In recent years much effort has been directed at the characterization of muscle fibre types. Nomenclatures vary, but all authorities recognize a slow-twitch fibre type, often called type I (Peter *et al.*, 1972), which can be distinguished histochemically (Padykula & Herman, 1955) from fast-twitch fibres, type II. Moreover, certain myofibrillar proteins in type I fibres can be distinguished immunologically from their fast-twitch analogues.

Abbreviations used: SDS, sodium dodecyl sulphate; sm, ma and ra, sternomandibularis, masseter and rectus abdominis (muscles) respectively.

Dhoot & Perry (1979), for instance, describe different forms of troponin and tropomyosin in type I and type II fibres, and slow and fast myosins have long been recognized. With classic histochemical techniques, type II fibres can be conveniently subdivided into those with both an oxidative and a glycolytic capacity, type IIA, and those with a low oxidative but a very high glycolytic capacity, type IIB (Brooke & Kaiser, 1970; Peter *et al.*, 1972). In contrast with type IIB, type I fibres have a high oxidative and a low glycolytic capacity. Brooke & Kaiser (1974) also distinguish an undifferentiated fast fibre, type IIC.

The above studies have been performed largely by optical-microscopic observations on cross-sections of muscle tissue. An alternative approach has been to isolate single muscle fibres, either for enzymic analyses (Lowry *et al.*, 1978) or for direct identification of proteins by SDS/polyacrylamide-gel electrophoresis (Weeds *et al.*, 1975; Pette & Schnez, 1977a,b). This latter technique has had only limited success, partly because of the small amount of protein in a rabbit muscle fibre. Bovine muscle fibres are, however, relatively large, and have been analysed by this technique in the present study.

Most fibres were dissected from bovine sterno-mandibularis, a ventral neck muscle used for lowering the head. By histochemical criteria this muscle contains a mixture of fibre types (Leet & Locker, 1973; Davey & Winger, 1979). By contrast, bovine cheek muscle, masseter, is a homogeneous slow muscle (R. H. Locker, personal communication) adapted in this species for cud chewing; masseter was used as a reference standard for slow-twitch fibres. Rectus abdominis is located in the lower belly and is involved in flexing the lower spine. It is composed predominantly of fast fibres (Leet & Locker, 1973) and was also used as a reference standard.

In the present study, fibre types have been characterized by myofibrillar protein composition. The definitive variations were seen in the light and heavy chains of myosin, tropomyosin, troponin and a group of proteins around 160 000 daltons. Among other results, the study demonstrates two kinds of fast myosin heavy chain, and fibre types where a fast myosin heavy chain is associated with light chains one of which is more characteristic of slow fibres.

Experimental

Dissection of muscles

Table 1 summarizes the origins of the muscles used. The fibres were usually dissected from the muscles within 6 h of slaughter, but in some cases up to 24 h after slaughter. Such post-slaughter delays did not affect gel-staining patterns. Sm fibres were dissected from the core and the periphery of the mid-region. The fibres from ma and ra muscles were dissected from the central region of the muscles.

The dissection medium was phosphate-buffered saline (137 mM-NaCl/3 mM-KCl/2 mM-KH₂PO₄/8 mM-Na₂HPO₄, pH 7.3), containing 5 mM-EGTA (sodium salt), pH 7.3, to minimize contraction. The fibre bundles were teased apart under a stereo-microscope with zoom facility, and only fibres at least 8 mm long were selected; a code number was assigned (A1, A2 etc.) and any peculiar characteristics were noted.

Wide differences were encountered in the ease of isolating single fibres, presumably through variations in amounts of endomysial collagen and in fibre diameter. The largest fibres isolated were from sm muscles of old and large animals. Such fibres were typically between 2 and 3 cm long and about 70 µm in diameter. In no case could a complete muscle cell be isolated, but each fibre was part of a single cell, except for M7 and M12 (ra muscle) and 20 of the 28 ma fibres. These were selected as clumps of two or three fibres because of dissection difficulty. Each resulting gel pattern could, however, be clearly equated with a particular single fibre type from its respective muscle.

Fibres were transferred to the bottom of Pyrex

Table 1. *Sources of bovine muscle fibres*

All animals were bulls, but muscles M, O and R were from castrated animals. Muscles H and I were from the same animal, as were muscles J and K. —, information not available.

Muscle	Code	Breed	Animal age (years)	Fibres sampled
sm	A	Angus	4	14
	C	Angus	>1	18
	I	Friesian	1.5	12
	L	Friesian?	2.5	7
	J	Friesian	5	23
	E	—	2	16
	B	Hereford × Angus	1	8
	G	Hereford	4 or 7	16
	H	Friesian	1.5	11
ma	K	Friesian	5	7
	N	Hereford	>1	6
	Q	—	—	4
	M	—	—	10
ra	O	Angus	1	9
	R	Friesian	5	6

test tubes (4 cm × 4 mm inside diameter) by being picked up with a finely drawn glass rod. The tubes were stoppered, frozen in liquid N₂ and kept at -70°C for up to 3 months before analysis. The time between fibre selection and freezing was typically 2 min.

SDS/polyacrylamide-gel electrophoresis

Slab gels (0.8 mm thick) were prepared as described by Laemmli (1970), except that 0.5 mM-EDTA was included in all gels. Care was taken to ensure that the interface between the stacking and the resolution gels was flat. This was achieved by performing polymerization within 3 min of casting, followed by removal of unpolymerized supernatant with an aspirator. Attempts at layering the resolution gel were unsuccessful. For routine analysis of single fibres, a 12.5% (w/v) polyacrylamide resolution gel (1.2% cross-linked) was overlaid with a 3%-polyacrylamide stacking gel (3% cross-linked) containing 24 sample tracks each 3 mm wide.

Gel sample buffer [20% (v/v) glycerol/2% (w/v) SDS/1% (v/v) 2-mercaptoethanol/2 mM-EDTA/50 mM-Tris-HCl, pH 6.8] was added (usually 20 µl) to each single-fibre tube; they were flame-sealed, then incubated at 110°C for 15 min. The sample was stirred with a microsyringe needle, and a 17.5 µl sample, containing on average an estimated 20 µg of protein, was then transferred to the gel. Electrophoresis was performed at a current density of 9 mA · cm⁻² until the dye boundary had crossed the gel interface, then increased to 16 mA · cm⁻².

Peptide 'mapping' of heavy-chain myosin purified on the above gels was performed by the method of Cleveland *et al.* (1977), with minor modifications in the run procedure. The stacking gel and the resolution gel contained respectively 3 and 15% acrylamide (both 3% cross-linked). *Staphylococcus aureus* (V8) proteinase was obtained from Miles Laboratories and papain (type IV) from Sigma Chemical Company.

Destained gels were photographed through an orange filter by using Pan F 120 film developed in Perceptol (Ilford). Microdensitometry of photographic negatives was performed with a Nikon projection microscope (Model 6c) adapted for this purpose (O. A. Y. may be contacted for details).

Sources and preparation of marker proteins

Myosin was purified from bovine sm and ma muscles (Szent-Györgyi, 1951), and tropomyosin was purified from ra and ma muscles (Bailey, 1951; Cummins & Perry, 1973). Troponin was prepared from ra and ma muscles by the method of Ebashi *et al.* (1971) and by a modification to this method; to increase the yield of troponin, the (NH₄)₂SO₄

fractionation range was extended from the reported 50–55% to 35–55% saturation. Desmin was purified from sm muscle (Young *et al.*, 1981).

Sigma Chemical Co. supplied horse heart myoglobin and the following rabbit muscle enzymes: creatine phosphokinase, glycerol 3-phosphate dehydrogenase, phosphorylase *a* and a number of glycolytic enzymes. A marker mix from Pharmacia Fine Chemicals containing phosphorylase (94 000 daltons), serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), trypsin inhibitor (20 100) and α-lactalbumin (14 400) was routinely used.

Results

Identification of major myofibrillar proteins

Each gel track in Figs. 1–5 displays a marker(s) or the proteins from usually a single muscle fibre. Actin and the heavy chain of myosin were easily and unequivocally identified. α-Actinin was identified by its staining intensity, ubiquity, relative migration and solubility properties (Young *et al.*, 1981). Tropomyosin was identified by comparisons with the migrations of purified bovine tropomyosins; as the study progressed, two forms of bovine α-tropomyosin (Cummins & Perry, 1973) were identified (Fig. 1). Each was coincident on electrophoresis with its respective form purified from slow (ma) and fast (ra) muscles. Troponin components were similarly identified and are considered in detail below.

The light chains of myosin were identified by using markers of purified myosin and by the migrations of light chains relative to troponins I and C from fast and slow muscles. Troponin I was particularly useful as a reference, since both troponin I fast (TI_f) and slow (TI_s) (Dhoot *et al.*, 1979) were turquoise when stained, whereas other proteins were purple-blue. Thus it was possible to distinguish between TI_s and light chain 1 fast, which had identical migrations (Fig. 1a). By such criteria, three so-called fast and two slow light chains analogous to those described by Lowey & Risby (1971) were identified (Figs. 1 and 2). For example, fibre C10 (Fig. 2) contains light chain 1 fast (LC1_f), LC2_f and lesser amounts of LC3_f, whereas in fibre A1, LC3_f is more strongly expressed.

An overview of fibre types

In an analysis of the protein patterns of single fibres, one is faced with the problem of what importance to attach to each character. Leaching of sarcoplasmic proteins, which could otherwise be considered in analysis, compounds the problem. In this respect, myoglobin, which migrated just below LC3_f (Fig. 1a), was completely absent, or present only in traces, yet is found in significant con-

Fig. 1. SDS/polyacrylamide-gel electrophoresis of single *sm* muscle fibres and of slow and fast tropomyosins
 (a) SDS/polyacrylamide-gel electrophoresis with 12.5% (w/v) polyacrylamide was performed as described in the text. The markers were myoglobin (MYG) and enolase (EN). When myoglobin was re-run at a lower concentration (result not shown), its migration relative to myosin light chain 3 fast (LC3_f) was unchanged. The other two fast myosin light chains (LC1_f, LC2_f) are also indicated, as are the two slow light chains (LC1_s, LC2_s). Note that muscle G fast fibres (G12, G2) lack LC1_f but contain a protein more typical of LC1_s. Other myofibrillar proteins are: myosin heavy chain (MHC) (gel zones have been removed for peptide 'mapping'), α -actinin (α -AC), actin and α - and β -tropomyosin (α/β -TM). Slow and fast troponins I and C (TI_s, TC_s, etc.) are indicated, whereas troponin T polymorphs are examined in Fig. 5. A turquoise band is indicated by 't' and a purple-blue band by 'p'. LC1_f in fibre A4 and TI_s in G7 have identical migrations, but can be distinguished by colour, respectively purple-blue and turquoise. (b) A close-up view of slow (from a *ma* muscle) and fast (from a *ra* muscle) tropomyosins analysed in alternate fashion on a 12.5%-polyacrylamide gel. Each track contained 0.4 μ g of tropomyosin. The tracks f and s display the tropomyosin regions of a fast and a slow fibre dissected from the two muscles before tropomyosin purification. (The relatively broad β -tropomyosin band in f also contains a troponin T polymorph). Slow α -tropomyosin has a relatively slow migration. This difference is also observed between slow (G7) and fast (A4, G2) *sm* fibres in (a) above.

Fig. 2. SDS/polyacrylamide-gel electrophoresis of single *sm* muscle fibres

Fibres from three animals were analysed on a 12.5%-polyacrylamide gel. All were fast (see later), but those from muscle G lacked LC1_f. When LC3_f was strongly expressed, as in muscle A fibres, certain other proteins including phosphorylase (PE), creatine phosphokinase (CPK), aldolase (AL) and glyceraldehyde 3-phosphate dehydrogenase (GPDH) were likewise expressed. Other abbreviations are defined in Fig. 1.

centrations in *sm*-muscle extracts (Young *et al.*, 1981). The present study therefore considers mainly the myofibrillar proteins, which remain insoluble in the dissection medium.

All fibres from the three muscles could be placed into one of two broad classes based on a cluster of proteins identified on 12.5%-polyacrylamide gels between 135 000 and 170 000 daltons (Fig. 3). One class is characterized by sets of bands at each end of this range, whereas in the other class, bands in this region are closer together, although it is not often clear that each set of bands is comprised of more than one protein. However, numbers of proteins were always observed when the gel composition was changed to 7.8% polyacrylamide (Fig. 4). At both gel concentrations, classification was complicated by variations in loading and, concomitantly, intensity of staining, but fibres could always be consistently classed into the two broad groups. The usefulness of this somewhat arbitrary classification became evident when gel zones incorporating myosin heavy

Fig. 3. *SDS/polyacrylamide-gel electrophoresis of bovine muscle fibres*

Fibres from a number of animals and muscles were analysed on a 12.5%-polyacrylamide gel. Fibres can be classed into two groups on the basis of a cluster of proteins between 135000 and 170000 daltons (135 and 170k). The class characterized by two sets of bands separated by some 35000 daltons, e.g. J5, contained slow myosin heavy chain, whereas the other class, e.g. M12, contained fast heavy chain. It is not always clear on 12.5% gels that numbers of proteins occur in this molecular-size range, but it is evident on gels designed to resolve this range more clearly (Fig. 4). Myosin light chains and troponins are indicated, as is the migration position of desmin (DS). MIX refers to Pharmacia marker mix, its heaviest component protein being phosphorylase (PE). The relatively broad bands of, respectively, slow and fast troponins I in J5 and J11 are presumed to be doublets and are indicated thus: =t. In fibre J11, but not in J2, LC3_f and certain glycolytic enzymes identified in Fig. 2 are strongly expressed. Comparisons between other fibres are made in the text. Other abbreviations are defined in Fig. 1.

chain were 'mapped' with *Staphylococcus* proteinase (see below); it was clear that the widely spaced type (e.g. fibre J5, Fig. 3) always contained slow myosin heavy chain, whereas the other class (e.g. M12, Fig. 3) always contained fast heavy chain. Fibres will be referred to as 'slow' or 'fast' by these criteria.

No attempt has been made to identify the proteins in the 135000–170000-dalton range, which are likely to include C-protein (Offer *et al.*, 1973), M-protein (Trinick & Lowey, 1977) and others (Etlinger *et al.*, 1976).

Considering now the tropomyosin bands, two forms of α -tropomyosin (Cummins & Perry, 1973) were recognized, one form exemplified by the slow fibre G7 and the other by the fast fibres A4 and G2 (Fig. 1a). (It should be noted that light chain 1 fast is absent from muscle G fast fibres). The G7 α -tropomyosin has a slightly slower migration. In routine analysis the difference between the two forms was clear only when a slow and a fast fibre of similar staining intensity were adjacent. In all instances the slower-migrating form of α -tropomyosin was associated with slow fibres and the faster form with fast fibres. Tropomyosin purified

from slow (ma) and fast (ra) muscles clearly showed this difference (Fig. 1b).

In contrast with the usually clear correlations observed above, the relationships between fast and slow fibres, myosin light chains and troponins were more complex. On the basis of these relationships the fibres have been broadly classed into three fast and three slow types (Scheme 1). The scheme is intended only as a general guide to the most obvious differences observed in the present study and should be referred to as the various types are now examined in detail.

Fast fibres from bovine muscles

Fast fibres from the three ra muscles were closely similar in the distribution of their myosin light chains and their troponins I and C. Light chain 1 fast (LC1_f) and LC2_f were clearly identified, whereas LC3_f was either absent or faintly expressed; TI_f and TC_f were clearly expressed (see, for example, M7, Fig. 3). Sm muscles A, C, I and L yielded fast fibres qualitatively similar to those from ra muscles. Comparison between the four fibres at the left of Fig. 3 suggests that the proteins in question are identical.

Fig. 4. Montage of single-fibre analyses from a gel of lower acrylamide concentration

The 135 000–170 000-dalton (135 and 170k) cluster of proteins described in Fig. 3 and the text is more clearly resolved in this gel, which contained 7.8% polyacrylamide (1.2% cross-linked). The fibres were classed as fast (f) or slow (s) on the basis of this cluster. When the myosin heavy-chain zones were 'mapped', all putative slow fibres were found to contain slow myosin heavy chain, whereas putative fast fibres contained fast myosin heavy chain (Fig. 6). Variations not apparent on 12.5% gels were evident on this gel; in slow fibres the pattern was essentially unchanging in the specified molecular-size range, but differences were observed between 170 000 daltons and the position of myosin heavy chain (compare Q4, R4 and R7). That this variation relates to slow fibre type is examined in the text. With respect to fast fibres, peptide 'mapping' (Fig. 6) revealed that differences in the specified range between fibres J15, J21 and O6 on one hand, and O8 on the other, probably relate to the existence of two myosins heavy chain fast. Other abbreviations are defined in Fig. 1.

This fibre type will be referred to as fast₁, only for convenience of discussion.

The light chain and troponin distributions of other fast fibres from sm muscles J, E and B were distinctly different from the fast₁ type. The fibres of muscles B and E were thin and difficult to dissect out, producing only faint staining patterns. The patterns were, however, sufficiently clear to be identified with the larger muscle-J fast fibres exemplified by J11, J2 and J4 (Fig. 3) fast₂ fibres. Present were two light chains 1, two troponins I (both turquoise), one troponin C, one light chain 2 and various amounts of light

chain 3. Comparisons between appropriate fibres in Fig. 3 and other results not shown suggested that all but one form of troponin I and the slowest-moving light chain had their equivalents in fast₁ fibres. Other comparisons suggested that the latter chain was equivalent to light chain 1 of slow fibres (LC1_s).

The light-chain distribution of muscle-G fast fibres (Figs. 1 and 2) was distinct from both fast₁ and fast₂ fibres. In these fast₃ fibres, the slowest-moving light chain appeared identical with LC1_s, and a comparable amount of LC2_f was present. In other respects these fibres were similar to the fast₁ set.

* As is explained in the text, TT_f may be present in a number of forms.

Scheme 1. An outline of the fibre classification

The key characteristics of the six fibre types broadly identified in the three muscles (sm, ma, ra) are summarized. The drawing approximately locates the relative migrations of the myosin light chains and troponin components associated with each type. The abbreviations LC1_f, LC2_s etc. refer to the fast and slow light chains, whereas TT_f, TI_s etc. refer to the fast and slow troponin components. A bar (——) represents a protein and a broken bar (-----) indicates variable expression of a protein; (2) means a protein is bimorphic, and a question mark indicates uncertain identity.

The overall staining pattern of fast fibres dissected from a particular muscle was normally distinctive and was irrespective of whether sampling was delayed *post mortem* or whether, in sm muscles, the core or the periphery was sampled. One notable exception was fibre J11, which among 18 fast fibres from muscle J was alone in its high content of LC3_f (Fig. 3).

The data do not indicate a relationship between fast fibre type and animal breed or age (Table 1).

Slow fibres from bovine muscles

Within a given sm muscle there was a rough bimodal distribution of fibre diameters; although the gel tracks of thinner fibres (as judged by eye) did not stain strongly, their characterization according to protein banding was unequivocally slow. Out of a total of 114 sm-muscle fibres, 21 (18%) were classed as slow. The proportions varied among muscles; from muscles A, C, I and L (the source of fast₁ fibres) 8% of the total were slow, with the highest

proportion in L (two of seven fibres). In contrast, 34% of fibres from muscles J, E and B (the source of fast₂ fibres) were slow, the lowest proportion being 22% in J. From muscle G only one out of 16 was slow. Out of 25 ra fibres, 36% were slow, significantly more than expected from the composition of tropomyosin (Fig. 1) and troponin (see below) purified from ra muscle. This selection bias may have arisen from the different diameters of ra fast and slow fibres; in contrast with the situation in sm muscles, ra slow fibres were markedly wider than fast fibres and were perhaps more readily isolated. In sm muscles, similar pressures may have biased fibre selection.

All fibres from ma muscles were uniformly thin and gave remarkably similar patterns (compare H and K fibres, Fig. 3), which were characteristically slow-type in their distribution of light chains and troponin components. This slow₁ type contains light chain 1 slow (LC1_s), LC2_s, troponin T slow (TT_s), TI_s and TC_s (see Q5, Fig. 5). The claim from

histochemistry that bovine ma muscle is homogeneous and slow is clearly justified by the present analyses.

Three slow fibre types were represented in sm muscles. Only one example of a slow₁ fibre was identified, the sole slow fibre isolated from muscle G (G7, Fig. 1). Slow₂, which was widespread in sm muscles, was closely similar to slow₁, but its troponin I slow was present as a doublet. This doublet, which was consistently observed in slow₂ fibres, is indicated in J5 (Fig. 3); slow₁ and slow₂ also differ in the relative staining intensities of LC1_s and troponins I.

Slow₃ was encountered only once in sm muscle (J12, Fig. 3). J12 might have been overlooked as a distinct type had slow₃ not been clearly identified in ra muscles, where it was the only slow fibre type. The slowest-moving light chain 1 (nominally LC1_s) was more typical of LC1_f, though not identical (compare, for example, O5 and O10, Fig. 5). Light chain 2 of slow₃ appeared identical with LC2_s, common to the other slow fibre types. Similarities and differences relating to the troponins are examined below. (Heavier proteins also provide some interesting comparisons. In Fig. 4, Q4, which is a slow₁ fibre, is different from ra slow fibres R4 and R7 in the region between 170000 daltons and myosin heavy chain. By contrast, H11, also a slow₁ fibre, is identical with J10, a slow sm fibre. Since R4 and R7 are probably slow₃ fibres, it is likely that J10 is more akin to a slow₁ type and is not a second example of a slow₃ fibre in sm muscle.)

The specific expression of the troponins

Troponin of ma muscle was resolved into troponin T slow (TT_s), TI_s and TC_s (Dhoot *et al.*, 1979; Harrington, 1979) and thus presented no surprises (Fig. 5). Comparison of ma troponin with a slow₁ fibre (Q5) and a slow₃ fibre (R1) in Fig. 5 shows that troponins I of the two fibre types are distinct. Other comparisons throughout the present study suggested that these two troponins I comprise the doublet troponin I of slow₂ fibres. As judged by relative migration, all slow troponins C are identical, and in all slow fibres a protein equivalent to TT_s from ma muscle was identified between α - and β -tropomyosin.

Fast troponins I and C from ra muscle were clearly resolved between LC1_f and LC2_f as expected, but three major bands were present in the general region of TT_f (Fig. 5). The relative staining intensities of the two lower bands were the same in the two ra troponin preparations (RAT1, RAT2), whereas the upper band varied. In contrast with the upper band, which might be aldolase, the two lower bands have clear equivalents in the fast ra fibre O4, and are presumed to represent two troponins T fast coexisting in ra-muscle cells. In fast sm-muscle fibres there was sometimes clear evidence for a troponin T

Fig. 5. Analysis of bovine troponins

The two tracks labelled MAT display a troponin preparation from the ma muscle Q. Two loadings (2 μ g, 5 μ g) are represented. Troponin T slow (TT_s), TI_s and TC_s are indicated. (The protein migrating just below actin in these tracks is probably not a troponin component, since its relative concentration was much higher in a preparation where the extraction steps were poorly controlled owing to a coarse muscle mince). Comparison of tracks Q5, MAT and R1 shows that slow troponins I of slow₁ and slow₃ fibres are distinct, but, as judged by migration, their troponins C are identical. The tracks labelled RAT 1 and RAT 2 display troponin preparations from the ra muscle R. (Each was derived from a different (NH₄)₂SO₄-fractionation range, respectively 50–55 and 35–55% saturation). Troponin I fast (TI_f) and TC_f are indicated in track RAT 1. That RAT tracks contain two troponins T fast (TT_f) and a contaminant all in the region of β -tropomyosin is examined in the text. Other abbreviations are defined in Fig. 1.

doublet akin to that from fast ra fibres, exemplified in J20 (Fig. 5). However, in other cases the doublet was poorly resolved from β -tropomyosin; doublet troponin T may therefore be present in other sm fast fibres, being obscured in some by β -tropomyosin. In certain fast fibres, such as L4 (Fig. 3), however, there is no evidence for a doublet, and a possible troponin T is identified just below α -tropomyosin.

Expression of light chain 3 fast (LC3_f)

Many fast and slow fibres show a band in the region of LC3_f. This was unexpected in slow fibres, but whatever its true identity in slow fibres, it was

never a dominant protein in this group, unlike in certain fast fibres. Despite leaching of sarcoplasmic proteins, fast fibres rich in the putative LC3_f were also rich in proteins migrating in the same way as phosphorylase, creatine phosphokinase, aldolase and glyceraldehyde 3-phosphate dehydrogenase (muscle A fibres, Fig. 2; J11, Fig. 3). The coincidence of LC3_f, creatine phosphokinase and these glycolytic enzymes probably confirms these identities. However, the glycolytic enzymes phosphoglycerate mutase, phosphoglucose isomerase, enolase, pyruvate kinase and lactate dehydrogenase could not be positively identified for a number of reasons, such as co-migration of proteins or possible leaching. For instance, rabbit muscle phosphoglucose isomerase (Scopes & Penny, 1971) was coincident with bovine desmin, so the relatively intense desmin band in J11 (Fig. 3) might contain this enzyme. The strongly staining band immediately above desmin in J11 is unidentified.

Peptide 'mapping' of myosin heavy chains

The identification of myosin light-chain heterogeneity in a number of fibre types prompted a study

of myosin heavy-chain composition by peptide 'mapping'.

Fig. 6 shows typical results obtained by *Staphylococcus* proteinase digestion of myosin heavy chains judged slow or fast by criteria described above. Within each of several experiments, all heavy-chain zones from slow-fibre myosins produced identical peptide 'maps'. Since myosin from ma fibres was included in these experiments, it was clear that this type of 'map' represented slow myosin heavy chain. As a group, fast myosin 'maps' were distinct from the slow type, but there was minor variation within the group. For instance, the 'map' of ra fibre O8 myosin is subtly different from the other fast 'maps' in Fig. 6, which are identical with each other irrespective of staining intensity. Similar subtle differences were observed in other 'mapping' experiments involving fast myosin heavy chain from sm muscles I, J and L, and ra muscles M, O and R. Considered collectively, it was concluded that one of two myosin heavy-chain forms was associated with certain ra fast fibres, but never with the sm fast fibres, whereas the other form had the converse distribution. Myosin from ra fibre O6, for instance,

Fig. 6. Peptide 'maps' of slow and fast myosins heavy chain from Fig. 4

Gel zones containing myosin heavy chain from single fibres were loaded on top of a stacking gel so that putatively slow and fast fibres were grouped. The protein in each gel zone was 'mapped' with 0.25 μ g of *Staphylococcus* proteinase as described in the text. Both the slow (H8 to Q4) and fast myosin 'maps' (O8 to I5) were identifiable as groups, but whereas the slow 'maps' were identical, O8 was outstanding among the fast 'maps'. Densitometric traces of 'maps' O8, O6 and J21 are shown. The trace of O6 was recorded at a higher sensitivity than that for O8 and J21.

was identical with the type in sm J and I fibres (Fig. 6).

The difference between 'maps' of O6 to J23 on the one hand, and O8 on the other, is reflected in Fig. 4, where of the fast fibres, O8 alone is clearly distinct, particularly in the region between 135 000 and 170 000 daltons.

In another series of 'mapping' experiments (results not shown) with *Staphylococcus* proteinase and papain, it was established that fast₂ and fast₃ fibres contained fast myosin heavy chain. Since both these fibre types contain a myosin light chain more typical of slow fibres, it follows that at least some fast₂ and fast₃ fibre myosins are heterogeneous with respect to this light chain(s) (Lowey, 1979).

Discussion

Scheme 1 outlines the classification determined in the present study. It is a functional classification, since it centres on proteins that are intimately associated with the contraction mechanism, although not all have been considered, and the detailed contraction characteristics of the various types are not known. Nonetheless, the fibre types broadly identified here may be compared with previously described types.

By comparison with the considerable literature on muscle fibre types, it is clear that at least fast₁ fibres are equivalent to the previously described type II fibres (Peter *et al.*, 1972). Fast₂ and fast₃ fibres are previously undescribed, but, as judged by the characteristics of all but one of their myofibrillar proteins, are presumably also type II fibres. Further, it is likely that fibres where LC3_f and certain glycolytic enzymes are strongly expressed represent type IIB, although it is not known whether oxidative capacity is concomitantly low in these instances. All other fast fibres might represent type IIA.

Fast₂ and fast₃ fibres both contain a light chain 1 that is more typical of, and may be identical with, LC1_s, whereas myosin heavy chain from these fibres is fast. Lutz *et al.* (1979) and Gauthier & Lowey (1979) have demonstrated the simultaneous presence of fast and slow myosins in single muscle fibres. In their studies, a proportion of fibres reacted with both fast and slow anti-myosins, whereas in the present study all the fast fibres in question contained the mixture of light chains. This result and the immunological results are thus qualitatively different and their relationship is not clear.

Recent experiments (Wagner & Weeds, 1977; Hoh, 1978; Lowey, 1979) have demonstrated a lack of specificity in the interaction between the alkali light chains (LC1_f, LC3_f) and fast heavy chain of myosin. The question of specificity is now compounded by the hybrids, fast₂ and fast₃, described here. In fibre J11 (Fig. 3), for instance, a large number of

myosin isoenzymes are possible; is the distribution of light chains in complete myosin molecules random or otherwise? Whatever the answer, it is clear that some flexibility of association extends to hybrids between fast-type heavy chains and the aberrant light chain(s) of fast₂ and fast₃ fibres.

In their staining patterns, bovine slow fibres were less variable than fast fibres, although three types were distinguished; histochemically, however, all three might well be classed as slow-twitch oxidative, often called type I.

The present study has identified a number of troponin, tropomyosin and myosin polymorphs by the criteria of relative migration, stain colour and peptide 'mapping'. Two troponins I fast were identified (fast₂ fibres). At least two troponins I slow were distributed among slow fibres, and troponin T was bimorphic in some fast fibres. α -Tropomyosin was present in two forms that presumably represent the bovine equivalent of two of the four or more rabbit tropomyosin polypeptides recognized from sequence data (Hodges *et al.*, 1972). The α - and β -forms of tropomyosin were both present in all fibres examined, fast or slow. This finding is at variance with the results of Dhoot & Perry (1979), who have shown by immunological tests on human semispinalis capitis muscle that α -tropomyosin is probably restricted to fast fibres, and have suggested that β -tropomyosin is associated only with slow fibres. Moreover, considerable reported evidence supports the notion that fast muscles are richer in α -tropomyosin. In contrast, the α/β ratio of cross-innervated rabbit soleus muscle remained unchanged, even though troponin I slow was largely replaced by troponin I fast (Amphlett *et al.*, 1975), a finding more consistent with the present results. In resolving these differences, single-fibre analysis techniques might be very useful.

Turning now to the polymorphs of myosin, the five previously described myosin light chains (Lowey & Risby, 1971) have been identified in the present study, along with a probable sixth (in slow₃ fibres). Moreover, the exact identities of the aberrant light chains in fast₂ and fast₃ fibres are unknown. Of further interest has been the identification of an additional fast myosin heavy chain in certain fast fibres from *ra* muscles. From amino-acid-sequence data, Starr & Offer (1973) have demonstrated two fast myosins heavy chain in rabbit muscle. The two variants observed in the present study may represent the bovine equivalent of this polymorphism. It is clear that they can coexist in one muscle, and there is an indication (Figs. 4 and 6) that each variant occurs with certain other proteins. However, the two variants do not appear to dictate the expression of myosin light chains, since several instances were observed where the light-chain composition was identical in fibres containing either one heavy chain

or the other. This suggests there is no restriction on association between each variant and the light chains. In support of this hypothesis, Pope *et al.* (1977) and d'Albis *et al.* (1979) have reported that there is no selectivity in the interaction between individual (alkali) light chains and the two heavy-chain variants in rabbit muscle.

In view of the known specificity of *Staphylococcus* proteinase, it is likely that the fast heavy-chain variants of bovine myosin differ at least in some of their glutamyl or aspartyl environments. Had another enzyme been used, the variants might not have been detected. Conversely, another enzyme might expose variants, but would they be coincident with those discovered here? In this respect single-fibre analysis combined with the 'mapping' method of Cleveland *et al.* (1977) should provide fertile ground for experimentation.

The present study has demonstrated myofibrillar polymorphs that presumably contribute, as do the metabolic enzymes, to the characteristic physiological properties of individual fibre types. As a variable composite of these fibre types, each muscle is evidently fine-tuned to a particular role under a given set of conditions.

Beyond cud chewing involving ma muscle, however, nothing is known of the conditions that gave rise to the various fibre composites in sm and ra muscles.

We thank Dr. R. H. Locker for his critical comments on the manuscript.

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