The multiplicity of combinations of myosin light chains and heavy chains in histochemically typed single fibres

Rabbit soleus muscle

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1. Six adult rabbit soleus muscles were analysed by isolating histochemically identified fibre pieces from freeze-dried serial cross-sections. 2. By the use of this method, four fibre types (I, IC, IIC and IIA) were identified and analysed micro-electrophoretically. 3. Type I fibres contained the slow myosin heavy chain HCl and the slow myosin light chains LC1s and LC2s. 4. Type IIA fibres contained the fast myosin HCIIa with the fast light chains and, in addition, either LC1s or both LC1s and LC2s. 5. The C fibres (IC and IIC) represented intermediate populations between types I and IIC (IC) and between IC and IIA (IIC). They contained varied ratios of HCl/HCIIa with both sets of fast and slow light chains. With regard to myosin composition and isoforms of other myofibrillar proteins (M- and C-proteins, α -tropomyosin, troponin I), IC fibres resembled type I and IIC fibres resembled type IIA. 6. The presence of various myosin light and heavy chains within a specific fibre suggests a multiplicity of isomyosins. Without consideration of LC1s_a and LC1s_b differences, at least 54 possible isomyosins can be derived: type I fibres contain one isomyosin, types IC and IIC 54 possible isomyosins, and type IIA up to 18.

INTRODUCTION

Electrophoresis under non-denaturing conditions has demonstrated several fast and slow isomyosins in adult skeletal muscle (d'Albis *et al.*, 1979; Hoh & Yeoh, 1979). Fast myosin, with two possible heavy chain (HC) homodimers (HCIIa)₂ and (HCIIb)₂ and three possible light chain (LC) combinations (LC1f)₂(LC2f)₂, (LC3f)₂(LC2f)₂ and (LC1f)(LC3f)(LC2f)₂ (Lowey *et al.*, 1979), may exist in six fast isomyosins. Assuming the existence of a heavy chain heterodimer (HCIIa)(HCIIb), a total of nine fast isomyosins would be possible. Slow myosin, containing the heavy chain homodimer (HCl)₂ and three possible light chain combinations (LC1s_a)₂(LC2s)₂, (LC1s_b)₂(LC2s)₂ and (LC1s_a)(LC1s_b)(LC2s)₂ should exist in three slow isomyosins. However, perhaps because of the limited resolution of electrophoresis under non-denaturing conditions, only three adult fast and two adult slow isomyosins have, as yet, been detected in mammalian muscle (d'Albis *et al.*, 1979; Hoh & Yeoh, 1979; Pinter *et al.*, 1981).

Additional information on the diversity of myosin has come from electrophoretic analyses under denaturing conditions and from single-fibre analyses. By use of these methods, a relationship between histochemically defined fast (type II) and slow (type I) fibres and the distribution of fast and slow myosin subunits has been established (Weeds *et al.*, 1975; Pette & Schnez, 1977a). Subsequently, a coexistence of fast and slow myosin light chains was demonstrated in single fibres undergoing fast-into-slow conversions (Pette & Schnez, 1977b) and under normal conditions (Gauthier & Lowey, 1979; Lutz *et al.*, 1979; Pierobon-Bormioli *et al.*, 1981).

The co-expression of fast and slow myosin subunits appears to be a general property of a normally minor fibre population designated as type IIC fibres (Billeter *et al.*, 1981; Salviati *et al.*, 1982, 1983; Baumann *et al.*, 1984). However, mixtures of fast and slow myosin components have also been observed in human slow fibres (Billeter *et al.*, 1981; Salviati *et al.*, 1983; Schantz *et al.*, 1982; Baumann *et al.*, 1984) and in some fast fibres of the rat soleus (Mizusawa *et al.*, 1982), the rabbit soleus, diaphragm and masseter (Salviati *et al.*, 1982; Mabuchi *et al.*, 1984; Reiser *et al.*, 1985) and selected bovine muscles (Young & Davey, 1981; Young, 1982). Taken together, these various studies indicate that an even greater number of isomyosins is possible.

In spite of a considerable number of single-fibre studies, many questions concerning fibre 'types' and myosin composition remain unanswered. In our opinion, the histochemical typing of microdissected fibres is subject to methodical artifacts and misinterpretations. This procedure (Essén *et al.*, 1975) involves cutting small pieces from dissected fibres for subsequent myosin ATPase histochemistry. Although staining intensities depend on the myosin ATPase activity, they are heavily influenced by the sample thickness. Moreover, classification is done without the aid of surrounding fibres for comparison, such as is typical in an ATPase-stained cross-section of uniform thickness. As a consequence, only major fibre types can be distinguished and, owing to the ambiguity of the procedure, some of the fibres may be misclassified.

To investigate further by microanalyses the multiplicity of myosin light and heavy chain combinations, we have isolated pieces of single fibres from serial freeze-dried cross-sections that were matched with serial cross-sections

Abbreviations used: HC, heavy chain; LC, light chain.

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processed for myosin ATPase histochemistry. Instead of random fibre sampling, this method made it possible to select specific fibres. Since several pieces of the same fibre were dissected from serial sections, enough material was obtained for separate analyses of myosin light and heavy chains. Indeed, the results indicate a correlation between the ATPase-based histochemistry and myosin composition, and, in addition, point to a much greater isomyosin multiplicity than previously demonstrated.

MATERIALS AND METHODS

Muscles

Soleus muscles ($n = 6$) were dissected from adult male New Zealand White rabbits after cervical fracture and exsanguination. Muscle strips (approx. 3 mm thick) were immersed in a slightly stretched position in melting isopentane (-159°C) and subsequently stored in liquid N_2 . Pieces (8 mm long) of the frozen muscle strips were mounted and sectioned on a freeze-microtome at -25°C . Six thin ($12\ \mu\text{m}$) serial cross-sections were cut, followed by six to eight thick ($200\text{--}300\ \mu\text{m}$) cross-sections, six thin sections, six to eight thick sections and finally, six thin sections. The thin sections were mounted on glass coverslips, air-dried and processed for myosin ATPase histochemistry. The thick sections were carefully transferred, in series, to special aluminium holders and freeze-dried at -38°C as previously described (Spamer & Pette, 1977). This method of preparing serial cross-sections for combined histochemical and biochemical analyses has been used previously (Rehunen & Härkönen, 1980; Toyoshima *et al.*, 1983; Hintz *et al.*, 1984; Nemeth *et al.*, 1986; Staron & Pette, 1986).

Histochemical fibre typing

The thin cross-sections were stained for myofibrillar actomyosin ATPase (myosin ATPase) by using a modification (Staron *et al.*, 1983) of the method of Brooke & Kaiser (1970). Classification of fibre types was performed according to the staining intensities after preincubation pH values of 4.3, 4.5 and 9.6 (Fig. 1). Type I fibres were dark following preincubation at pH values of 4.3 and 4.5 and light after pH 9.6. The reverse was true for type IIA fibres. Although variable, the C fibres were stable at all three pH values, with the IIC fibres staining similarly to the IIA and the IC fibres staining similarly to the type I fibres. Subsequently, sections were either photographed by using a Polaroid attachment to a Leitz photomicroscope or traced at 120-fold magnification by using a camera lucida. Specific fibres (50 per muscle) were numbered for subsequent dissection.

Microdissection

The thick freeze-dried serial sections were transferred to room temperature and dissected at 40-fold magnification in a humidity- and temperature-controlled room. The selected fibre pieces were dissected, placed separately in specially designed micro-sample holders (Teutsch, 1986) and stored under vacuum at -38°C until further analysed. Because dissection was performed on 12–16 serial sections, a maximum of 16 cross-sectional pieces of each fibre was collected. To obtain approximate weights, freeze-dried fibre pieces were dissected from a section of known thickness and pooled according to their type. These pieces were weighed on a quartz-fibre fishpole

balance. Mean dry-weight values were used to estimate the amounts of protein applied for electrophoresis.

Electrophoresis for myosin light chains

Fibre pieces (equivalent to a total of $1\text{--}1.5\ \mu\text{g}$ dry wt.) were transferred to a glass capillary under the stereomicroscope. Subsequently, $15\ \mu\text{l}$ of a pre-extracting medium (80 mM-KCl/0.1 mM-dithiothreitol/0.1 mM-phenylmethanesulphonyl fluoride/1 mM-Tes, pH 7.0) was added and the capillaries were sonicated for 60 min in an ice bath. The supernatant, containing the bulk of the 'soluble' proteins, was then carefully decanted under the stereomicroscope by using a fine polyethylene tube attached to a micrometer-controlled syringe.

For electrophoresis, pre-extracted fragments were lysed for 10 min at 60°C in $10\ \mu\text{l}$ of a medium containing 10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol and 2.3% (w/v) SDS in 62.5 mM-Tris/HCl buffer, pH 6.8. Each completed incubation medium was applied to one slot of a 3% stacking gel. Micro-electrophoresis was performed at 80 V for 160–170 min in 0.8 mm-thick slab gels (1 cm-high stacking gel, 4 cm-high separating gel) with the Laemmli (1970) system.

Electrophoresis for myosin heavy chains

The procedure of Rushbrook & Stracher (1979) and of Carraro & Catani (1983) was applied, with a 0.75 mm-thick 4% stacking and 5% separating gel. Fibre pieces (equivalent to a total of approx. 300 ng dry wt.) were lysed, as above, without pre-extraction, and the extract was loaded for electrophoresis. Electrophoresis was run at 120 V for 19 h.

Identification of protein bands

Gels were stained by the silver staining method of Oakley *et al.* (1980). Protein bands were tentatively identified according to their apparent molecular masses compared with those of marker proteins. In addition, myosins purified from muscles composed predominantly of type I (soleus) or type II fibres (psoas) were used to supplement myosin subunit identification.

RESULTS

The soleus muscle contained four histochemically defined fibre types: I, IC, IIC and IIA (Fig. 1). All slow (type I) fibres appeared to be homogeneous with regard to their myosin ATPase staining and contained the slow myosin heavy chain HCl and the slow myosin light chains LC1s_a , LC1s_b and LC2s . Although not quantitatively analysed, differences were observed in the LC1s/LC1s ratio, both between soleus muscles from different rabbits and within the same muscle (Fig. 2). Likewise, the histochemically defined IIA fibres (Fig. 1) stained homogeneously. They contained the fast myosin HClIIa and the three fast myosin light chains LC1f , LC2f and LC3f . In addition, these fast fibres contained small amounts of slow myosin LC forms. They either displayed LC1s or the combination of LC1s plus LC2s (Fig. 3). However, within a given muscle the myofibrillar protein profile of the IIA fibres was homogeneous and all fibres contained either LC1s_b (Fig. 3b) or LC1s_b plus LC2s (Fig. 3a).

The IC and IIC fibres were histochemically heterogeneous and displayed a whole spectrum of staining intensities (Fig. 1). Their heterogeneity was likewise

Fig. 1. Histochemically defined fibre types of rabbit soleus muscle

The Figure shows serial cross-sections of rabbit soleus muscle stained for myosin ATPase after preincubation at various pH values. Four fibre types (I, IC, IIC and IIA) can be distinguished. Key: I, type I; IC, type IC; IIC, type IIC; A, type IIA. Magnification $\times 160$.

Fig. 2. Myofibrillar peptide patterns of single type I fibres from rabbit soleus muscle

The Figure shows single-fibre analyses of five histochemically defined type I fibres from the same rabbit soleus muscle. Differences can be seen in the ratio $LC1s_a/LC1s_b$. Abbreviations: AC, actin; CP, C-protein; HC, myosin heavy chain; TM, tropomyosin; TNC, troponin C; TNI, troponin I; X, X-protein; $1s_a$, $1s_b$ and 2s represent slow myosin light chains.

reflected by a coexistence of fast and slow myosins in various ratios (Fig. 4). The IC fibres were histochemically more like type I fibres and showed a predominance of the slow LC forms and the slow HC. The IIC fibres appeared histochemically to be more similar to IIA fibres and had a predominance of the fast LC forms and the fast HC. The two myosin HC forms existing in IC and IIC fibres were clearly distinct (Fig. 4) and corresponded to type I and type IIA HC forms, as demonstrated by co-electrophoreses (results not shown). Thus all IC and IIC fibres were identified by the coexistence of slow HCI and fast HCIIa.

The polymorphism observed at the level of myosin extended to other myofibrillar proteins as well. Differences were observed in the tropomyosin between types I and IC on the one hand and types IIC and IIA on the other hand. Types I and IC contained a distinct slow form of the α -tropomyosin subunit, whereas all type II fibres (IIA and IIC) exhibited the fast α -tropomyosin (Fig. 4). There were no striking differences between fast and slow fibres with regard to the α/β subunit ratio. In addition, the slow troponin I was found in the IC, whereas the fast troponin I was present in the IIC fibres (Fig. 4). This may apply to the other troponin subunits (troponin T and troponin C), but was not clearly resolved by the method used.

Differences also existed in the C- and M-protein regions. Three bands were observed in the 135–145 kDa C-protein region (Fig. 5). The type I and IC fibres showed a predominance of the central band, whereas all type II fibres had three prominent bands. In addition,

Fig. 3. Myofibrillar peptide patterns of two different type IIA fibres in rabbit soleus muscles

The Figure shows single-fibre analyses on IIA fibres from soleus muscles of two different rabbits. One muscle (a) contains IIA fibres displaying, in addition to the fast myosin light chains LC1f, LC2f, LC3f, the slow light chain LC1s and LC2s. The other muscle (b) contains IIA fibres that lack LC2s. Key: I, type I; C, type IIC; A, type IIA. Abbreviations: 1f, 2f and 3f represent fast myosin light chains; for others see Fig. 2 legend.

slow (I and IC) and fast (IIC and IIA) fibres differed in the M-protein region (165 kDa), slow fibres being characterized by a slightly-slower-migrating M-protein (Fig. 5). Therefore the distinction of IC and IIC fibres was not only based on the predominance of either slow or fast myosin light and heavy chains but extended to slow and fast isoforms of other myofibrillar proteins.

Interestingly, all fibres in the soleus muscle contained a 29–30 kDa peptide, designated X-protein. The intensity of this band varied according to $\text{IIA} < \text{IIC} < \text{IC} < \text{I}$ (Fig. 4).

DISCUSSION

The rabbit soleus muscle contains I, IC, IIC and IIA fibres that can be distinguished histochemically by their myosin ATPase activity and biochemically by their myofibrillar protein composition. The present study demonstrates a direct correlation between the histochemical appearance of these fibre types and their myofibrillar protein profiles. The establishment of such correlations depends on the ability to distinguish unambiguously between discrete myosin ATPase staining intensities and to retrieve and analyse pieces of defined fibres from serial cross-sections.

The main results of this study are summarized in Table 1, showing the myosin LC and HC composition of the four major fibre types. Types I and IIA in the rabbit soleus contain distinct heavy chains and, therefore, are

considered as separate populations. The IC and IIC fibres represent intermediate populations with coexisting slow and fast myosins in various proportions. Since this applies to both light and heavy chains, it appears that a continuum of C fibres exists spanning the range from type I to type IIA.

By applying these data to the basic formula of the myosin molecule, a large number of possible isomyosins can be derived (Table 2). If the $\text{LC1s}_a/\text{LC1s}_b$ heterogeneity is not taken into account, the myosin of type I fibres has only one possible combination (Table 2, 1A). The myosin of IIA fibres has either six (Table 2, 13C–18C) or 18 combinations (Table 2, 1C–18C), depending upon the absence or the presence of LC2s respectively. For the IC and IIC fibres, a maximum of 54 isomyosins results from combining 18 possible LC combinations with the three HC combinations (Table 2, 1A,B,C–18A,B,C). The HC combinations include both slow and fast HC homodimers $(\text{HC1})_2$ and $(\text{HCIIa})_2$ and the theoretical heterodimer $(\text{HC1})(\text{HCIIa})$. If the HC heterodimer is excluded, the maximum possible number of isomyosins in the C fibres would amount to 36 (Table 2, 1A,C–18A,C). It is presently unknown whether the heterodimer exists. Regardless of whether there exist 36 or 54 possible isomyosins, this is an extreme example of the myosin multiplicity encountered within single muscle fibres.

The majority of the soleus fibres are pure type I fibres. These fibres appear to have a limited number of isomyosins, since they contain only the slow HC homo-

Fig. 4. Single-fibre analyses of myosin heavy chains and other myofibrillar peptides in the histochemically defined four fibre types of rabbit soleus muscle

Segments of the same fibre were isolated and separately analysed for their myosin heavy-chain composition (upper panel) and for their myofibrillar protein composition (lower panel). Note the higher intensity of the X-protein band in types I and IC. Key: I, type I; IC, type IC; IIC, type IIC; A, type IIA. Abbreviations: see Fig. 2 legend.

Fig. 5. M- and C-protein isoforms in single histochemically defined fibres from rabbit soleus muscle

Segments of specific fibres were analysed for their myofibrillar peptide patterns. Only the upper, high-molecular-mass, region of the gel is shown. Three bands are evident in the C-protein region. The types I and IC have a prominent middle C-protein band, whereas types IIA and IIC display three equally prominent bands. The M-protein band of types I and IC migrates slightly more slowly than that of types IIA and IIC. Key: I, type I; IC, type IC; A, type IIA; C, type IIC; I + A, co-electrophoresis of types I and IIA; A + C, co-electrophoresis of types IIA and IIC; I + C, co-electrophoresis of types I and IIC. Arrowheads point to double bands of the M-protein produced by co-electrophoresis.

Table 1. Myosin light and heavy chains in histochemically typed fibres from rabbit soleus muscle

The distribution of myosin light chains (LC) and heavy chains (HC) was determined electrophoretically in fibre pieces isolated from thick serial cross-sections, according to myosin-ATPase-stained thin serial cross-sections.

Fibre type	Light chains					Heavy chains	
	LC1s	LC2s	LC1f	LC2f	LC3f	HCl	HClIa
I	+	+				+	
IC	+	+	+	+	(+)	+	+
IIC	+	+	+	+	+	+	+
IIA	+	(+)	+	+	+		+

dimer and only the slow light chains. However, the slow fibres, not only in rabbit soleus, but also in other muscles of other species, contain various ratios of the slow alkali light chains LC1s_a and LC1s_b (Schachat *et al.*, 1980; Pinter *et al.*, 1981; Biral *et al.*, 1982; Salvati *et al.*, 1982, 1983; Mabuchi *et al.*, 1984). This phenomenon has now been observed within the same muscle. Type I fibres varying in their LC1s_a/LC1s_b ratio

Table 2. Theoretical combinations of myosin light chains (LC) and heavy chains (HC) according to their presence in histochemically defined fibre types of rabbit soleus muscle

Type I contains combination 1A. Types IC and IIC contain combinations 1A, B, C–18A, B, C. Type IIA contains combinations 13C–18C or 1C–18C.

	Light chains	Heavy chains		
		A	B	C
1	(LC1s) ₂ (LC2s) ₂	(HCI) ₂	(HCI)(HCIIa)	(HCIIa) ₂
2	(LC1s)(LC1f)(LC2s) ₂	(HCI) ₂	(HCI)(HCIIa)	(HCIIa) ₂
3	(LC1f) ₂ (LC2s) ₂	(HCI) ₂	(HCI)(HCIIa)	(HCIIa) ₂
4	(LC3f) ₂ (LC2s) ₂	(HCI) ₂	(HCI)(HCIIa)	(HCIIa) ₂
5	(LC1s)(LC3f)(LC2s) ₂	(HCI) ₂	(HCI)(HCIIa)	(HCIIa) ₂
6	(LC1f)(LC3f)(LC2s) ₂	(HCI) ₂	(HCI)(HCIIa)	(HCIIa) ₂
7	(LC1s) ₂ (LC2s)(LC2f)	(HCI) ₂	(HCI)(HCIIa)	(HCIIa) ₂
8	(LC1s)(LC1f)(LC2s)(LC2f)	(HCI) ₂	(HCI)(HCIIa)	(HCIIa) ₂
9	(LC1f) ₂ (LC2s)(LC2f)	(HCI) ₂	(HCI)(HCIIa)	(HCIIa) ₂
10	(LC3f) ₂ (LC2s)(LC2f)	(HCI) ₂	(HCI)(HCIIa)	(HCIIa) ₂
11	(LC1s)(LC3f)(LC2s)(LC2f)	(HCI) ₂	(HCI)(HCIIa)	(HCIIa) ₂
12	(LC1f)(LC3f)(LC2s)(LC2f)	(HCI) ₂	(HCI)(HCIIa)	(HCIIa) ₂
13	(LC1s) ₂ (LC2f) ₂	(HCI) ₂	(HCI)(HCIIa)	(HCIIa) ₂
14	(LC1s)(LC1f)(LC2f) ₂	(HCI) ₂	(HCI)(HCIIa)	(HCIIa) ₂
15	(LC1f) ₂ (LC2f) ₂	(HCI) ₂	(HCI)(HCIIa)	(HCIIa) ₂
16	(LC3f) ₂ (LC2f) ₂	(HCI) ₂	(HCI)(HCIIa)	(HCIIa) ₂
17	(LC1s)(LC3f)(LC2f) ₂	(HCI) ₂	(HCI)(HCIIa)	(HCIIa) ₂
18	(LC1f)(LC3f)(LC2f) ₂	(HCI) ₂	(HCI)(HCIIa)	(HCIIa) ₂

are histochemically (myosin ATPase) homogeneous. Because neural input plays an important role in regulating phenotype-specific properties of muscle fibres and because it is also believed that the motor unit represents a homogeneous population (for review see Pette & Vrbová, 1985), fibres with varied LC1s_a/LC1s_b ratios may reflect different motor units.

The soleus IIA fibres display a muscle-specific myofibrillar peptide profile. Contrary to the exclusive fast LC composition of tibialis-anterior IIA fibres (Staron & Pette, 1987), they additionally contain either LC1s or both LC1s and LC2s. Thus there exist at least three populations of IIA fibres, one in the tibialis anterior and two in the soleus muscle. These last two appear to be selective within soleus muscles of different rabbits, because type IIA fibres from a given soleus muscle display identical peptide patterns of either one or the other type. This may reflect the homogeneity of fibres belonging to the same motor unit. Thus we assume that the minor IIA population in the soleus muscle makes up a single motor unit.

In view of the small number of C fibres, it is difficult to explain their heterogeneity within a given soleus muscle. As evidenced histochemically and biochemically, these fibres display a complete spectrum. The IIC fibres are generally considered to represent transient states (Jansson *et al.*, 1978; Billeter *et al.*, 1980, 1981; Salvati *et al.*, 1983), but their heterogeneity may be due to several small motor units. We have now added an additional group of transient fibres, designated as IC, which can be regarded as a link between the slow type I and the IIC fibres. This is based on the finding that IC fibres display a higher HCI/HCIIa ratio, whereas the IIC fibres are characterized by a higher HCIIa/HCI ratio. This is supported by the histochemical appearance of these fibres, the IC fibres resembling type I and the IIC fibres resembling IIA. Thus a correlation appears to exist

between the HC composition of muscle fibres and their histochemical myosin ATPase staining intensity (Staron & Pette, 1986). Histochemical evidence for the existence of type I subtypes has been previously demonstrated in rat (Karpatis *et al.*, 1975; Khan, 1978) and human (Askanas & Engel, 1975; Jansson *et al.*, 1978) muscle.

In conclusion, the four histochemically defined fibre types of normal rabbit soleus muscle represent a continuum with a high number of possible isomyosins. This phenomenon further reflects the dynamic nature and plasticity of muscle fibres.

Note added in proof (received 5 March 1987)

Recently Danieli Betto *et al.* (1986) demonstrated three distinct myosin heavy chains corresponding to types I, Ila and Iib by using one-dimensional SDS/polyacrylamide-gel electrophoresis on single rat muscle fibres. Some of the fibres co-expressed two myosin heavy-chain isoforms (types I and Ila or types Ila and Iib).

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