The subunit composition and dimeric species of tropomyosin with respect to its \( \alpha \) and \( \beta \) subunits have been analyzed from several physiologically different types of skeletal muscle of the rabbit using one- and two-dimensional gel electrophoresis. The findings indicate that there are two types of tropomyosin distributions in rabbit muscles: one in which \( \alpha \) is the prevalent species and another in which \( \beta \) is prevalent. While the \( \alpha \)-prevalent pattern is characteristic of some fast muscles, notably longissimus dorsi and psoas, it is not the only pattern found in fast muscles. Plantaris, which is histochemically indistinguishable from longissimus dorsi, exhibits an \( \alpha \)-prevalent distribution. This observation shows that there are at least two types of fast white muscle fibers which differ in their tropomyosin composition and species. The \( \alpha \beta \)-pattern is also found in fast red, slow, and mixed muscles. The fast and slow \( \alpha \beta \)-prevalent patterns can be distinguished electrophoretically because of heterogeneity of their subunits. Finally, the relative abundance of the different forms of tropomyosin in each of the muscles studied indicates that the tropomyosin dimer does not assemble randomly from \( \alpha \) and \( \beta \) subunits, but that \( \alpha \beta \) is assembled preferentially in vivo.

Tropomyosin is dimeric, coiled-coil protein which, because of its interaction with troponin and actin, is a key component of the thin filament calcium regulatory complex of skeletal muscle. In mammalian skeletal muscle it is composed of two polypeptides designated \( \alpha \) and \( \beta \) (Cummins and Perry, 1973) which have highly conserved amino acid sequences (Stone and Smillie, 1978; Mak et al., 1980). Studies on the distribution of the \( \alpha \) and \( \beta \) polypeptides in tropomyosin by Eisenberg and Kielley (1974) and Lehrer (1976) demonstrated the presence of \( \alpha \) and \( \beta \) subunits of tropomyosin in the fast, white skeletal muscle of the rabbit back. Although several studies have been done on tropomyosin subunit composition and assembly since that initial work, many questions remain unanswered. In particular, the suggestion by Dhoot and Perry (1979) that the \( \alpha \) tropomyosin subunit is specific to fast muscle and the \( \beta \) subunit is prevalent in slow muscle is in conflict with analyses of the molecular composition of the single mammalian muscle fibers (Schachat et al., 1980a; 1980b). This leaves the fast or slow specificity of the \( \alpha \) and \( \beta \) subunits in question. Also, the process of tropomyosin assembly, that is, whether its subunits assemble at random or in a directed manner, is yet to be determined. In addition, the results of studies on chicken muscle tropomyosin which show there are fast and slow skeletal muscle specific forms of \( \alpha \) subunits (Montarras et al., 1981) suggest a possible source of tropomyosin heterogeneity not yet investigated in mammals.

In an attempt to resolve these questions of specific expression, heterogeneity, and assembly of tropomyosin, we have determined the relationship between mammalian skeletal muscle fiber type and tropomyosin in several physiologically distinct kinds of rabbit skeletal muscles.

MATERIALS AND METHODS

Muscles—Muscles were dissected tenden to tendon from 3- to 4-kg female New Zealand White Rabbits. The rabbits were killed by injection of Nembutal (150 mg) into the heart following anesthetization with intramuscular injections of Ketaset (35 mg/kg), Rompun (5 mg/kg), and acepromazine (1 mg/kg). Following dissection all operations were done at 0–4 °C unless mentioned.

Preparation of Tropomyosin—Muscles were rinsed in 0.85% NaCl, minced, suspended in 10 volumes of rigor buffer (100 mM KCl, 20 mM sodium phosphate, 5 mM MgCl₂, 5 mM ethylene glycol bis(\( \alpha \)-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 1 mM diithiothreitol, \( \mathrm{pH} 7 \)), and homogenized in a Waring Blender in five 10-s bursts with 30-s cooling between homogenizations. The homogenate was centrifuged at 7500 × \( g \) for 5 min, the supernatant decanted, and the pellet was washed by resuspending it in 5 volumes of rigor buffer and the washing repeated twice more. This pellet was then resuspended in 5 volumes of rigor buffer supplemented with 39% Triton X-100, centrifuged as before, and then washed once more in rigor buffer.

Myofilaments were extracted by resuspending the pellet in 10 volumes of rigor buffer supplemented with 5 mM ATP, \( \mathrm{pH} 7 \), and 5 mM CaCl₂ and homogenized in a French Pressure Cell at 8,000 p.s.i. The resulting homogenate was centrifuged at 12,000 × \( g \) for 10 min to remove most thick filaments and then the supernatant was centrifuged at 100,000 × \( g \) for 90 min to pellet thin filaments. The thin filaments were resuspended in 2 volumes of rigor buffer supplemented with 1 mM ATP, \( \mathrm{pH} 7 \), and then the tropopinin-tropomyosin fraction prepared by ammonium sulfate fractionation as described by Hart-shorne and Mueller (1968). The tropomyosin was purified by hydroxyapatite chromatography using a modification of the procedure of Eisenberg and Kielley (1974). Ten to 20 mg of the tropomin-tropomyosin fraction were dialyzed against 100 mM NaCl, 20 mM Tris, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, \( \mathrm{pH} 8 \), and adjusted to 20 mM sodium phosphate, 0.5 mM NaCl, 0.1% Triton X-100, \( \mathrm{pH} 7 \), and loaded onto a hydroxyapatite column (1 × 23 cm) equilibrated in 0.5 mM NaCl, 20 mM sodium phosphate, 1 mM dithiothreitol, \( \mathrm{pH} 7 \), and washed with two column volumes of that buffer. Tropomyosin was eluted with a linear gradient formed with 100 ml each of 20 mM and 300 mM sodium phosphate in 0.5 mM NaCl, 1 mM dithiothreitol, 0.1% Triton X-100, \( \mathrm{pH} 7 \), and 1-mL fractions were collected.

Gel Electrophoresis—One- and two-dimensional gel electrophoreses were performed as described by Laemmli (1970) and O’Farrell (1975), respectively, except where modifications are described under “Results.” The tube gel used in the first dimension was 14 cm × 0.25 cm and the slab gel of the second dimension was 12 cm × 9.5 cm × 0.15
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Histochemical profiles of several rabbit skeletal muscles and their tropomyosin subunit composition

Muscles fibers were typed in serial sections as described by Dubowitz and Brooke (1973). In accord with common practice, Type I, Type IIa, and Type IIb fibers were equated with slow-twitch oxidative, fast-twitch oxidative-glycolytic, and fast-twitch glycolytic fibers described by Peter et al. (1972). Fibers were scored as fast-twitch glycolytic if they stained positively in the routine ATPase stain and negatively in a stain for succinic dehydrogenase, fast-twitch oxidative-glycolytic if they stained positively in both histochemical tests, and slow-twitch oxidative if they were negative in the routine ATPase stain (all slow-twitch oxidative fibers were positive in the succinic dehydrogenase stain). Results are based on the scoring of at least 250 fibers from each muscle. Tropomyosin subunit compositions were determined as described under "Results."

**TABLE I**

| Muscle       | Distribution of fiber types (± SD) | Tropomyosin subunit composition |
|--------------|-----------------------------------|---------------------------------|
|              | Fast-twitch oxidative              | Fast-twitch oxidative-glycolytic | Slow-twitch oxidative           |
|              | glycolytic                         | glycolytic                      | fₐ                        |
| Longissimus dorsi | 0.79 ± 0.03                        | 0.12 ± 0.01                     | 0.09 ± 0.01 | 0.93 | 0.07  |
| Plantaris    | 0.76 ± 0.05                        | 0.17 ± 0.02                     | 0.08 ± 0.02 | 0.52 | 0.48  |
| Tongue       | ND                                 | 0.99 ± 0.03                     | 0.01 ± 0.00 | 0.47 | 0.53  |
| Diaphragm    | 0.45 ± 0.02                        | 0.29 ± 0.02                     | 0.26 ± 0.02 | 0.49 | 0.51  |
| Soleus       | ND                                 | 0.01 ± 0.00                     | 0.99 ± 0.03 | 0.48 | 0.52  |

ND, not detected.

**RESULTS**

Tropomyosin Subunit Composition in Fast and Slow Skeletal Muscle of the Rabbit—In order to test the proposition that the α and β subunits are specific to or preferentially found in fast and slow skeletal muscles, respectively (Dhoot and Perry, 1979), the fraction of each subunit in tropomyosin from physiologically different types of skeletal muscle was determined. Longissimus dorsi and plantaris were chosen as examples of fast white muscles (those that are composed primarily of fast-twitch glycolytic fibers), tongue was selected as an example of a fast red muscle (one composed primarily of fast-twitch oxidative glycolytic fibers), and soleus was selected as a representative slow red muscle (one composed of slow-twitch oxidative fibers). The histochemical analysis and fractions of α and β subunits for each muscle are shown in Table I. Fiber typing was performed as described under "Materials and Methods" and Table I. The fα and fβ were determined by densitometric analysis of Coomassie brilliant blue stained urea-NaDodSO₄ polyacrylamide gels (Fig. 1) of purified tropomyosin and troponin-tropomyosin preparations (Fig. 2). Urea-NaDodSO₄ gels were used to avoid interference by troponin-T in the determination of fα and fβ. The fα and fβ measurements did not differ regardless of whether they were made on thin filaments, troponin-tropomyosin, or purified tropomyosin preparations.

Since fα was approximately 0.5 for the fast muscles, plantaris and tongue, and the slow muscle, soleus, it follows that neither α nor β subunits are expressed exclusively in either fast or slow muscles. We find, as others have (Cummins and Perry, 1974; Eisenberg and Kielley, 1974; Lehrer, 1975), that longissimus dorsi is an exception to this approximately equal distribution of α and β subunits. Its prevalence of α does not correlate with its speed of contraction (its fastness) or with its histochemical fiber type. This is clearly demonstrated by comparing it with the other fast white muscle in this sample, plantaris, which has equal amounts of each subunit (fα = 0.52). These muscles are histochemically indistinguishable. Each is composed of greater than 90% fast fibers and between 75 and

\[\text{ND} \]
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Fig. 2. Polyacrylamide-NaDodSO₄ gel electrophoresis of tropomyosin preparations from several rabbit skeletal muscles. The 10% polyacrylamide-NaDodSO₄ gel electrophoretic analysis of the preparations used in this study: troponin-tropomyosin (Tm) from longissimus dorsi (a), plantaris (b), tongue (c), diaphragm (d), and soleus (e); purified tropomyosin from diaphragm (f) and longissimus dorsi (g); and thin filament preparations from soleus (i), semitendinosus (h), diaphragm (j), tongue (k), plantaris (l), medial gastrocnemius (m), psoas (n), and longissimus dorsi (o). The positions of the major structural proteins, myosin heavy chain, actin, and the tropomyosin subunits are indicated.

80% fast-twitch glycolytic fibers. So the differences in their subunit compositions cannot be accounted for by differences in fiber type. This suggests that longissimus dorsi belongs to a class of fast white muscles different from that represented by plantaris. Of other fast muscles studied psoas, which like longissimus dorsi is a fast white muscle from the rabbit back, exhibits a preponderance of a subunit (Fig. 1, slot h), and medial gastrocnemius has approximately equal amounts of both subunits (Fig. 2, slot m) like plantaris.

Comparison of the protein composition of thin filament preparations by NaDodSO₄-polyacrylamide gel electrophoresis reveals that the similarity of longissimus dorsi (Fig. 2, slot o) and psoas (Fig. 2, slot n) extends beyond their high f₃ to other myofibrillar proteins. The slow muscles soleus and semitendinosus, likewise, appear similar (Fig. 2, slots i and h), as do the three fast muscles, tongue, plantaris, and medial gastrocnemius (Fig. 2, slots k, l, and m). This indicates that each of the muscles chosen for detailed analysis, longissimus dorsi, plantaris, tongue, and soleus, is representative of other skeletal muscles.

Identification of α₃, αβ, and β₃ Species of Tropomyosin in a Mixed Muscle by Two-dimensional Electrophoresis—Previous studies on the distribution of the α and β subunits in tropomyosin dimers were conducted with preparations from the fast white muscles of the rabbit back and hindlimb (Eisenberg and Kielley, 1974; Lehrer, 1975). We felt that these studies suffered from several limitations. First, the studies were carried out on a preparation from fast white muscles of the rabbit back and hindlimb which had f₃ values of between 0.67 and 0.8. These values, as shown in the preceding section, are not characteristic of most muscles. Second, no β₃ species of tropomyosin was detected and it was not possible to determine whether this was because β₃ was lacking or because it was not resolved. Together, these limitations prevented a comprehensive perspective of the distribution of tropomyosin species from being presented. Without knowledge of the distribution of dimeric species, no conclusions could be made about the process of tropomyosin assembly from its subunits.

To increase the likelihood of finding a β₃ species, our initial study was conducted on tropomyosin from a mixed muscle, diaphragm. Diaphragm’s diverse fiber composition (composed of significant amounts of each of the three major histochemical fiber types) and approximately equal amounts of both subunits (Table 1 and Fig. 1, slot c) suggested it would exhibit more diversity than any of the other muscles chosen for detailed analysis. A minor modification of the two-dimensional electrophoretic procedure of O’Farrell (1975) was developed to resolve the different tropomyosin dimers and determine their subunit composition.

Diaphragm tropomyosin was intramolecularly cross-linked by oxidation of opposing cysteines using 5,5'-dithiobis(2-nitrobenzoic acid) as described by Lehrer (1975). The cross-linked sample was then subjected to isoelectric focusing as a first dimension with β-mercaptoethanol omitted from the lysis buffer (O’Farrell, 1975) preventing dissociation of tropomyosin to its subunits. Electrophoresis in the second dimension, NaDodSO₄-polyacrylamide gel electrophoresis, was then performed on identically prepared first dimension tube gels in each of two ways: first, with β-mercaptoethanol omitted from the equilibration buffer (O’Farrell, 1975) so that tropomyosin dimers were resolved (Fig. 3, A and C); and second, with β-mercaptoethanol present (Fig. 3, B and D) so that the subunit composition of the tropomyosin species resolved by the two-dimensional procedure could be determined. The two types of electrophoreses were designated oxidized-oxidized and oxidized-reduced, respectively, where oxidized or reduced refers to the state of tropomyosin cysteine residues during each electrophoretic dimension.

On the basis of the studies by Lehrer (1975), we expected to find three dimeric species of tropomyosin on oxidized-oxidized gels: one corresponding to α₂ tropomyosin; a second with a higher Mₐ, corresponding to the αβ species; and, a third species, not previously observed, corresponding to β₁ tropomyosin. Although the pattern observed (Fig. 3, A and C) is more complex, its general features conform to expectation. There is a cluster of two spots labeled α₂, four spots labeled αβ, and a single spot labeled β₁ (see Fig. 4 for a schematic representation) which correspond to the three species we had expected to resolve. The labeling of the α₂ cluster and the β₁ spot is based on their composition. As shown in the oxidized-reduced gels of Fig. 3, B and D, the cluster labeled α₂ contains only α subunit and the spot labeled β₁ contains only β subunit. Confirming the designation of each of the four spots labeled αβ tropomyosin is more complex. The presence of α and β subunits at a constant ratio on oxidized-reduced gels throughout the region containing those species suggests that each spot represents an αβ species. Their pl values are between those of α₂ and β₁, consistent with their designation. Further, spots 3 and 4 can be unequivocally identified as αβ species without the possibility of cross-contamination in the studies on tropomyosin from fast muscles described below. Similarly, spot 1 can be identified as αβ in the analysis of tropomyosin from slow muscle. The identification of spot 2 is then firmly established from the constant subunit ratio throughout that region.

Heterogeneity of the α Subunit and the Heterogeneity of Tropomyosin Species—The heterogeneity of α₂ and αβ species complicated the analysis of the distribution of tropomyosin species. Since it seemed possible that some of the heterogeneity was due to phosphorylation of a fraction of the α subunits, the effect of alkaline phosphatase treatment prior to electrophoresis was studied. As can be seen by inspection of Fig. 3, which shows the results of electrophoretic analysis of untreated and phosphatase-digested diaphragm tropomyosin,
In order to confirm that tropomyosin had been dephosphorylated by the alkaline phosphatase incubation, diaphragm tropomyosin samples were analyzed by the standard two-dimensional gel electrophoresis procedure of O'Farrell (1975). This analysis revealed that there is a a subunit heterogeneity which is not due to phosphorylation. Fig. 5A shows that there are two major species of a subunit in diaphragm which differ in pI by 0.07 pH unit prior to phosphatase incubation. Incubation with phosphatase does not affect either of the major species (Fig. 5B), but it does eliminate minor, more acidic species of subunit associated with each of the major species. On the basis of the analysis by Montarras et al. (1981), the difference in pI between each major species of subunit and the minor, more acidic species associated with it, corresponds to the difference between unphosphorylated and phosphorylated tropomyosin subunits. This confirmed the efficacy of the phosphatase treatment and the existence of at least two species of a subunit.

While the molecular basis for the subunit heterogeneity cannot be assigned with certainty, it is not phosphorylation. Also, it cannot be due either to the preparation procedure, since fast muscle tropomyosin prepared in the same way at the same time yields only a single major a subunit (Fig. 6A), or to the age of the preparations, since the heterogeneity is not altered by storage of tropomyosin. So, we believe that differences in primary structure are the most likely bases for the heterogeneity.

The heterogeneity of the a subunit appears to be responsible for the heterogeneity of a2 species observed in the two-dimensional oxidized-oxidized gel analysis. As described in the following sections, tropomyosin from fast muscles exhibits only the a2 spot labeled 1 in Fig. 4, while tropomyosin from slow and mixed muscles, which has two a species, exhibits both a2 species. However, the heterogeneity of aβ species cannot be explained solely by a heterogeneity since there are four aβ species.

Three Different Tropomyosin Distributions in Fast Muscles—As expected from the differences in their subunit composition, longissimus dorsi and plantaris exhibit different distributions of tropomyosin species. Analyzed by the oxidized-oxidized and oxidized-reduced electrophoresis procedures, longissimus dorsi (Fig. 7, A and B) exhibits a pattern in which a2 is the prevalent tropomyosin species, while plantaris (Fig. 7, C and D) shows a prevalence of aβ tropomyosin. The a2 species in both muscles corresponds in mobility to the a2 spot labeled 1 in Fig. 4 and the aβ species to those labeled 3 and 4. The oxidized-reduced gels unambiguously demonstrate the

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**Fig. 3.** Modified two-dimensional electrophoretic analysis of diaphragm tropomyosin. The dimeric forms of diaphragm tropomyosin are analyzed by oxidized-oxidized (A) and oxidized reduced (B) two-dimensional gel electrophoresis as described under "Results." Phosphatase-treated diaphragm tropomyosin is analyzed by oxidized-oxidized (C) and oxidized-reduced (D) gels and shows no significant change in pattern. In all figures trace quantities of uncross-linked a and b subunits can be seen at more acidic pI values than the dimeric species. The 9% polyacrylamide- NaDodSO4 electrophoresis was used in the second dimension.

**Fig. 4.** A schematic representation of the pattern of diaphragm tropomyosin on oxidized-oxidized two-dimensional gels. The M, and pI values of all aα, aβ, and bβ species of diaphragm tropomyosin are indicated on this representation of Fig. 3A. The M, assignments are based on the mobility of actin, C protein, myosin, and the subunits of tropomyosin. The pI values were determined from first dimension isoelectric focusing (IEF) tube gels as described by O'Farrell (1975). SDS, sodium dodecyl sulfate.

**Fig. 5.** Heterogeneity of the a subunit from diaphragm tropomyosin. Two-dimensional analysis by the procedure of O'Farrell (1975) revealed the presence of two major species of a tropomyosin subunit in diaphragm tropomyosin (A). Phosphatase treatment prior to analysis left the two major species unaffected but did eliminate minor, more acidic species associated with each of the major species (B). The positions of a and b subunits in the 9% polyacrylamide-NaDodSO4, second dimension are indicated.
composition of each of these species. Since this difference in tropomyosin distribution between two fast white muscles is not explicable on the basis of differences in fiber type distributions (Table I), it must mean that there are at least two types of fast white (fast-twitch glycolytic) fibers: one in which \(a_2\) is the prevalent tropomyosin species and another having \(\alpha \beta\) as its prevalent species.

The tongue, a fast red muscle, exhibits a distribution different from either of the fast white muscles studied (Fig. 8, A and B). Although its \(f_t\) is similar to plantaris', and it has the same \(a\) subunit found in the other fast muscles, it differs in having substantial amounts of all three dimeric species. Its distribution implies that \(\beta_2\) tropomyosin as well as \(a_2\) and \(\alpha \beta\) can be found in fast fibers.

\(\alpha \beta\) Is the Prevalent Tropomyosin in Slow Muscles—Fig. 9, A and B, shows the results of oxidized-oxidized and oxidized-reduced two-dimensional electrophoresis of tropomyosin from the slow muscle soleus. The pattern of tropomyosin dimers is composed of species that correspond to \(a_2\) species 1 and 2, \(\alpha \beta\) species 1, 2, and 4, and the \(\beta_2\) species (see Fig. 4). As in diaphragm, the heterogeneity of \(a_2\) and \(\alpha \beta\) species correlates with heterogeneity of the \(a\) subunit revealed by two-dimensional gel electrophoresis (Fig. 6B). Also, like diaphragm, tongue, and plantaris, the prevalent species of tropomyosin is \(\alpha \beta\). Analysis of a second slow muscle, semitendinosus, revealed a similar pattern (see the following section, Fig. 10, slot i), suggesting that the observations are not peculiar to soleus. The presence of \(a_2\), \(\alpha \beta\) and \(\beta_2\) species in slow and fast muscles shows that no dimeric form is specific for any muscle type.

\(\alpha \beta\) Is the Preferred in Vivo Form of Tropomyosin—The difficulty of measuring the relative amounts of the tropomyosin species by two-dimensional gel electrophoresis led us to consider a one-dimensional electrophoretic procedure. The separation of tropomyosin dimers on oxidized-oxidized gels indicated that covalently cross-linked \(a_2\), \(\alpha \beta\), and \(\beta_2\) species would be resolved by one-dimensional NaDodSO\(_4\)-polyacrylamide gel electrophoresis. Using 6.5% polyacrylamide gels, the electrophoresis was performed as described by Laemmli (1970) on covalently cross-linked samples from the muscles studied (Fig. 10) and the distribution of dimeric species determined. The identification of species on the gel was based on their relative abundance and mobilities on the oxidized-oxidized two-dimensional gels. The \(\alpha \beta\) species migrated as a single band and was identified as the prevalent band in diaphragm, plantaris, soleus, and tongue. \(a_2\) was identified as the prevalent species in longissimus dorsi and it appeared as two bands in

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**Fig. 6.** \(\alpha\) subunit species in fast and slow muscle tropomyosins. Two-dimensional gel electrophoretic analysis according to the procedure of O’Farrell reveals a single major \(\alpha\) subunit (the minor, more acidic species is also seen) in longissimus dorsi tropomyosin (A) and two species in soleus tropomyosin (B). The positions of \(a\) and \(\beta\) subunits in the 9% polyacrylamide-NaDodSO\(_4\), second dimension are indicated.

**Fig. 7.** Electrophoretic analysis of tropomyosin species in fast white muscles. The tropomyosin species in two fast white muscles are compared by two-dimensional electrophoresis. Oxidized-oxidized electrophoresis of longissimus dorsi (A) and plantaris (C) reveals differences in their distributions. The subunit composition of the species seen in the oxidized-oxidized gels were determined on oxidized-reduced gels of longissimus dorsi (B) and plantaris (D). The positions of the major oxidized tropomyosin species and reduced tropomyosin subunits in the 9% polyacrylamide-NaDodSO\(_4\) gel electrophoretic second dimension are indicated. The additional \(\alpha\) spot in B results from tropomyosin not oxidized by the 5,5'-dithiobis(2-nitrobenzoic acid) treatment.

**Fig. 8.** Electrophoretic analysis of tropomyosin from a fast red muscle. The tropomyosin species present in tongue are analyzed by oxidized-oxidized (A) and oxidized-reduced (B) two-dimensional electrophoresis. The positions of the major oxidized tropomyosin species and reduced tropomyosin subunits in the 9% polyacrylamide-NaDodSO\(_4\) gel electrophoretic second dimension are indicated.
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A

β

α

α₂

β₂

B

those tropomyosins which showed heterogeneity of the subunit. β₂ was identified as the most rapidly migrating tropomyosin species. Since the objective of this analysis was to determine the distribution of α₂, αβ, and β₂ tropomyosins, the inability to resolve αβ species was not a serious limitation.

Table II shows the distribution of tropomyosin dimers from different rabbit skeletal muscles as determined by densitometry of the gel presented in Fig. 10. The distribution expected from random assortment of subunits (calculated from the binomial distribution) is also shown in Table II. Comparison of the distribution shows that αβ is the preferred in vivo form. It is present at levels in excess of or equal to those expected from random assortment in all muscles; its presence at levels greater than those expected by random assortment of subunits suggests it is either preferentially formed or differentially stable.

In order to exclude the possibility that subunit exchange during the preparation or storage of samples was responsible for the distribution observed, several precautions were taken. First, any potentially denaturing step in the preparation of samples was avoided. For that reason we preferred to work with troponin-tropomyosin preparations (even though, when tested in longissimus dorsi, diaphragm, and soleus, the distributions in purified tropomyosins were the same). Second, distributions were checked over the course of several months after isolation of samples and were found to be unchanged.

To more directly assess the effects of subunit exchange, tropomyosin from diaphragm at 0.3 mg/ml was denatured and renatured and the initial distribution compared with the resulting one. Denaturation was accomplished by dialysis against 8 M urea, 20 mM Tris, 5 mM dithiothreitol, pH 8, for 2 h at room temperature and renaturation by dialysis for 2 to 6

Table II

Distribution of tropomyosin species in rabbit skeletal muscles

The distribution of tropomyosin species was determined by one-dimensional gel electrophoresis as described under "Results." The distribution of tropomyosin species expected from random assortment is based on the binomial expansion.

| Muscle                  | Determined by electrophoresis | Expected from random assortment |
|-------------------------|-------------------------------|---------------------------------|
|                         | α₂   | αβ   | β₂   | α₂   | αβ   | β₂   |
| Longissimus dorsi       | 0.84 | 0.16 | ND   | 0.86 | 0.13 | 0.005|
| Plantaris               | 0.13 | 0.85 | 0.02 | 0.27 | 0.50 | 0.23 |
| Tongue                  | 0.24 | 0.59 | 0.17 | 0.22 | 0.50 | 0.28 |
| Soleus                  | 0.07 | 0.91 | 0.02 | 0.23 | 0.50 | 0.27 |
| Diaphragm               | 0.07 | 0.90 | 0.03 | 0.24 | 0.50 | 0.26 |
| Denatured-renatured     | 0.33 | 0.36 | 0.31 | 0.24 | 0.50 | 0.26 |
| Diaphragm               |      |      |      |      |      |      |

* ND, not detected.

Fig. 9. Electrophoretic analysis of tropomyosin species from the slow skeletal muscle, soleus. The tropomyosin species present in soleus are analyzed by oxidized-oxidized (A) and oxidized-reduced (B) two-dimensional electrophoresis. The positions of the major oxidized tropomyosin species and reduced tropomyosin subunits in the 9% polyacrylamide-NaDodSO₄ gel electrophoretic second dimension are indicated.

Fig. 10. One-dimensional analysis of tropomyosin species from several rabbit skeletal muscles. The 6.5% polyacrylamide gel electrophoresis in the presence of NaDodSO₄ is used to measure the distribution of tropomyosin species in preparations from several rabbit skeletal muscles: longissimus dorsi tropomyosin (a) and troponin-tropomyosin (b), plantaris troponin-tropomyosin (c), tongue troponin-tropomyosin (d), diaphragm troponin-tropomyosin (e), soleus troponin-tropomyosin (f), phosphatase-treated diaphragm troponin-tropomyosin (g), denatured-renatured diaphragm tropomyosin (h), and semitendinosus (i), medial gastrocnemius (j), and psoas (k) thin filaments. The positions of the dimeric species were assigned as described under "Results." The additional band in α is alkaline phosphatase.
h at room temperature against either 50 mM Tris, 1 mM dithiothreitol, pH 8, or phosphate-buffered saline (0.15 M NaCl, 20 mM sodium phosphate, 1 mM dithiothreitol, pH 7). The resulting distributions were the same; that from the phosphate-buffered saline is shown in Fig. 10, slot h. αβ accounted for only 36% (significantly less than expected from random assortment) of the renatured species. The failure to obtain either the in vivo distribution or a random distribution by this procedure complicates its interpretation, but it does indicate that subunit exchange, at least by the mechanisms induced by this method of denaturation-renaturation, are not responsible for the distributions observed.

**DISCUSSION**

The objective of these studies is to present a comprehensive analysis of the relationship between mammalian skeletal muscle fiber type and tropomyosin subunit composition and species distribution. To do this, we have studied several physiologically different kinds of rabbit skeletal muscles. This was necessary because previous studies on mammalian skeletal muscle tropomyosin usually were limited to preparations from the fast white muscles of the rabbit back and hindlimb, muscles which single fiber analysis (Schachat et al., 1980a, 1981) indicated were not typical even of other fast white muscles.

Analysis of the tropomyosin subunit composition of the fast muscles, plantaris and tongue, and the slow muscles, soleus and semitendinosus, shows that, contrary to the proposal of Dhoot and Perry (1979), neither subunit is strictly specific for either fast or slow muscles. Longissimus dorsi and psosas, fast white muscles from the rabbit back, do exhibit α₂ prevalence. However, this is peculiar to these fast white muscles and is not due to a restriction of α to fast muscles.

If there is fast and slow specificity with regard to tropomyosin subunits, it most likely results from expression of different genes for those subunits in different muscle types. This is suggested by the heterogeneity of the α subunit observed in two-dimensional electrophoretic analysis. While the molecular basis of that heterogeneity is unknown, our results show that it is not due to phosphorylation, which is the only known reversible post-translational modification of tropomyosin (Mak et al., 1978; Montarras et al., 1981). This makes it likely that the heterogeneity reflects differences in the primary structure of subunits. This conclusion is at odds with the report of a single major α tropomyosin sequence (Stone and Smillie, 1978), but is consistent with the observation in earlier studies by the same group (Hodges et al., 1972) which reported that there might be as many as four tropomyosin subunit sequences.

The heterogeneity of the α subunit appears to explain the two α species observed by two-dimensional oxidized-oxidized gel electrophoresis. It also may account for some of the multiplicity of αβ species. However, other factors must be considered since two αβ species seem to be associated with each α species. Heterogeneity of the β subunit due either to the presence of two β subunit species or to partial reaction of the second β subunit cysteine (the only other cysteine in either subunit) at position 36 with 5,5'-dithiobis(2-nitrobenzoic acid) (Mak et al., 1980) might be responsible for the additional αβ species observed in this analysis.

In comparing the α subunit heterogeneity in rabbit with that reported in chick (Montarras et al., 1981), the mammalian case appears to be more complicated. Chick has fast and slow forms of α. We find one slow specific form and one that appears to be expressed in both fast and slow fibers. However, analysis of single fibers from rabbit and human skeletal muscles indicates that the shared species may represent two forms, one fast specific and another slow specific, which differ slightly in Mr.

Analysis of the distributions of tropomyosin dimers reveals that there are two distributions of the dimeric species of tropomyosin in these muscles, one in which α₂ is prevalent, and another in which αβ is prevalent. The αβ prevalent distribution is found in both fast and slow muscles. Lesser quantities of α₁ and β₂ are also present. The histochemical purity of tongue and soleus shows that these species must be present in both fast and slow fibers. So there is no restriction of any dimeric form of tropomyosin to fast or slow muscles.

Again, in this study as in the subunit composition analysis, longissimus dorsi and psosas exhibit a pattern different from other muscles. They have a prevalence of α₂ (as would be expected from their high f). The distribution of dimeric species found in plantaris, soleus, and diaphragm indicates that αβ is the preferred in vivo form of tropomyosin since it is present at levels greater than those expected by random assortment of subunits. While this might arise from either differential turnover of α₁ and β₂ species or subunit exchange coupled with differential stability of the α species, we believe it is more likely that αβ assembles preferentially. This suggestion is made because it seems unlikely that a catabolic mechanism would be used to determine the distribution of a major structural protein in muscle fibers and because our in vitro studies suggest that subunit exchange would not necessarily favor the formation of αβ.

If αβ is preferentially formed, then the α₁ and β₂ observed in distributions may result from an excess of α or β subunits, respectively, in individual fibers. This scheme would account for the observations on longissimus dorsi and tongue as well as those on plantaris, soleus, and diaphragm.

The prevalence of αβ over α₁ and β₂ species was unexpected in a coiled-coil protein like tropomyosin since myosin heavy chains which have extensive coiled-coil domains form homodimeric species (Schachat et al., 1977, 1978). This means that assembly of all coiled-coil molecules cannot be explained by a single, simple mechanism, such as assembly on polyribosomes during synthesis (which would generate homodimeric species).

Finally, these observations show that at the molecular level fiber diversity is far more diverse than previous biochemical studies and histochemical analysis suggested. Comparison of tropomyosin distributions in different fast white muscles demonstrates that there must be at least two different types of fast-twitch glycolytic fibers: one represented by fibers from longissimus dorsi in which α₁ is prevalent; the other similar to those found in plantaris where αβ is prevalent. That this does not represent the full extent of fast fiber heterogeneity with respect to tropomyosin is apparent from the levels of β₂ tropomyosin in the fast red muscle, tongue. Further, the two forms of α observed in slow muscle may be a basis for slow-twitch oxidative fiber diversity in addition to that generated by differences in myosin light chain complements (Schachat et al., 1980a, 1980b). These results suggest that it will be necessary to supplement histochemical analysis with molecular characterization, if skeletal muscle fiber diversity is to be understood; that diversity will only be appreciated when observations are made on more heterogeneous groups of muscles than are usually studied.

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