MYOFIBRILLAR PROTEINS OF DEVELOPING AND DYSTROPHIC SKELETAL MUSCLE

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1. Introduction

It has now been established that several myofibrillar proteins occur in different isoenzymatic or polymorphic forms, and some of the factors that determine which genes are expressed have been described. Comparison of myofibrillar proteins from fast and slow-twitch skeletal muscle showed differences in myosin light chains [1], tropomyosin [2,3], the inhibitory [4] and tropomyosin-binding components [5] of troponin (TN-I and TN-T) and α-actinin [6,7]. Less is known about the extent to which foetal forms of myofibrillar proteins are replaced or modified during development of skeletal muscle. Mammalian foetal myosin has been reported to have a lower specific Ca^{2+}-ATPase activity [8–10], a lower amount of the light chain LC_3 [9,10] and a heavy chain containing a different amino acid sequence [11]. Dystrophic skeletal muscle myosin also showed a reduced Ca^{2+}-ATPase activity and myosin light chain LC_3 content [9,12]. However, little is known about the other myofibrillar proteins in foetal and dystrophic muscle.

Cross-reinnervation of fast- and slow-twitch muscles indicated that the type of innervation determined the type of myosin light chain [13,14], heavy chain [15] and TN-I [16] found in the muscle. Fast-twitch muscle subjected to chronic low-frequency stimulation acquired myosin light [17] and heavy chains [15,18] similar to those of slow-twitch muscle myosin. However, cross-reinnervation did not affect the proportion of the polymorphic forms of tropomyosin [16], implying that nerve mediated factors are not the only factors involved in regulating the genes for the proteins of the myofibril.

In the present investigation, myofibrils were prepared from the skeletal muscle of adult, dystrophic and foetal mice and the myofibrillar protein subunit composition analyzed by gel electrophoresis and densitometry. Differences in the subunit composition were related to the specific Mg^{2+}-ATPase activity of the myofibrils and to the inhibition of Mg^{2+}-ATPase due to EGTA which reflects the functioning of the troponin–tropomyosin complex. Myofibrils were used so as to minimize the possibility of loss of proteins and enzymatic activity during extraction of actomyosin.

The comparison of adult with foetal myofibrils indicated that during normal muscle development in mice:

(1) Specific Mg^{2+}-ATPase activities of myofibrils increased markedly.

(2) The inhibition of myofibril Mg^{2+}-ATPase due to EGTA increased and this was related to the replacement of a foetal form of troponin with an adult form.

(3) The total content of tropomyosin increased and this was accounted for by an increase in the relative amount of α-tropomyosin*.

(4) The relative amount of myosin light chain LC_3 increased.

(5) The weight ratio of myosin heavy chain-to-actin did not alter.

Myofibrils from adult dystrophic muscle had a Mg^{2+}-ATPase activity and LC_3 content at an intermediate level between normal adult and foetal myofibrils. In contrast, the content of α-tropomyosin, the type of troponin and the inhibition of Mg^{2+}-ATPase due to EGTA did not differ from normal.

* The two forms of tropomyosin are named α-tropomyosin and β-tropomyosin as proposed by Cummins and Perry [2].

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Table 1
Specific Mg^{2+}-ATPase activities of myofibrils from skeletal muscle of adult, dystrophic and foetal mice

	Specific Mg^{2+}-ATPase activities (μmoles ADP/mg myofibril protein/min)								
	Adult			Dystrophic			Foetal		
	Ca^{2+}	EGTA	% Inhibition	Ca^{2+}	EGTA	% Inhibition	Ca^{2+}	EGTA	% Inhibition
1	0.664	0.192	71.1	0.216	0.076	64.8	0.044	0.024	45.5
2	0.640	0.096	85.0	0.368	0.072	80.4	0.084	0.032	61.9
3	0.840	0.112	86.7	0.544	0.096	82.4	0.080	0.024	70.0
4	0.720	0.192	73.3	0.544	0.136	75.0	0.168	0.056	66.7
Mean	0.716	0.148	79.0%	0.418	0.095	75.7%	0.094	0.034	61.0%
% of adult Mg^{2+}–Ca^{2+}-ATPase	100%			58%			13%		
Statistical treatment of data (Paired comparisons *t*-test									
Difference from Mg^{2+}–Ca^{2+}-ATPase activity of adult			$\bar{D}$	0.298 ± 0.056			0.622 ± 0.049		
			t_3	5.321			12.694		
			p	< 0.01			< 0.01		
Difference in percentage inhibition due to EGTA from adult			$\bar{D}$			3.38% ± 1.75			18.0% ± 4.24
			t_3			1.93			4.245
			p			> 0.15			< 0.05

Each row represents the values obtained using myofibrils prepared on the same day from normal adult mice, dystrophic littermates and normal foetuses. Mg^{2+}-ATPase activity of myofibrils was determined in 0.6 mM $MgCl_2$, 0.6 mM ATP, 0.025 M Tris-HCl, pH 7.2, in the presence of either 0.01 mM $CaCl_2$ or 1.0 mM EGTA at 25°C. The ADP released was determined as described previously [9]. Specific ATPase was measured with myofibrils at concentration of 0.125 mg protein/ml of incubation mixture. Protein concentration was determined by the Lowry procedure [27].

The results indicate that, of the genes (myosin and troponin) whose expression is known to be regulated by nerve mediated factors, only the expression of the myosin genes is affected in murine muscular dystrophy.

2. Materials and methods

Myofibrils were prepared [19] from mixed skeletal muscle dissected from the diseased hind limbs and pelvic region of 6–10 week-old adult dystrophic mice and from the same area in normal litter mates. Foetuses (19 day in utero) were taken from Banded-Dutch mothers and the whole eviscerated hind regions of the carcasses used to prepare foetal myofibrils. All solutions contained 0.5 mM dithiothreitol. The use of solutions containing the proteolytic enzyme inhibitor phenyl methyl sulphonyl fluoride (10 μM) did not affect the results obtained.

3. Results

The specific Mg^{2+}-ATPase activities of dystrophic and foetal myofibrils were on average 58% and 13% respectively of the adult level, the difference being significant at the 1% level when tested as paired comparisons (table 1). The presence of 1 mM EGTA inhibited the Mg^{2+}-ATPase activity of normal adult, dystrophic and foetal myofibrils by 79%, 76% and 61% respectively. The difference between the inhibition of foetal and adult myofibril Mg^{2+}-ATPase tested as paired comparisons was significant ($D = 18.0\% \pm 4.2\%$, $t_3 = 4.245$, $p < 0.05$) suggesting a quantitative or qualitative difference in the troponin-tropomyosin complex. There were no grounds for supposing that inhibition of dystrophic myofibril Mg^{2+}-ATPase differed from adult ($D = 3.4\% \pm 1.8\%$, $t_3 = 1.93$, $p > 0.15$).

Comparison of myofibrils by gel electrophoresis (fig.1) indicated that foetal myofibrils contained a

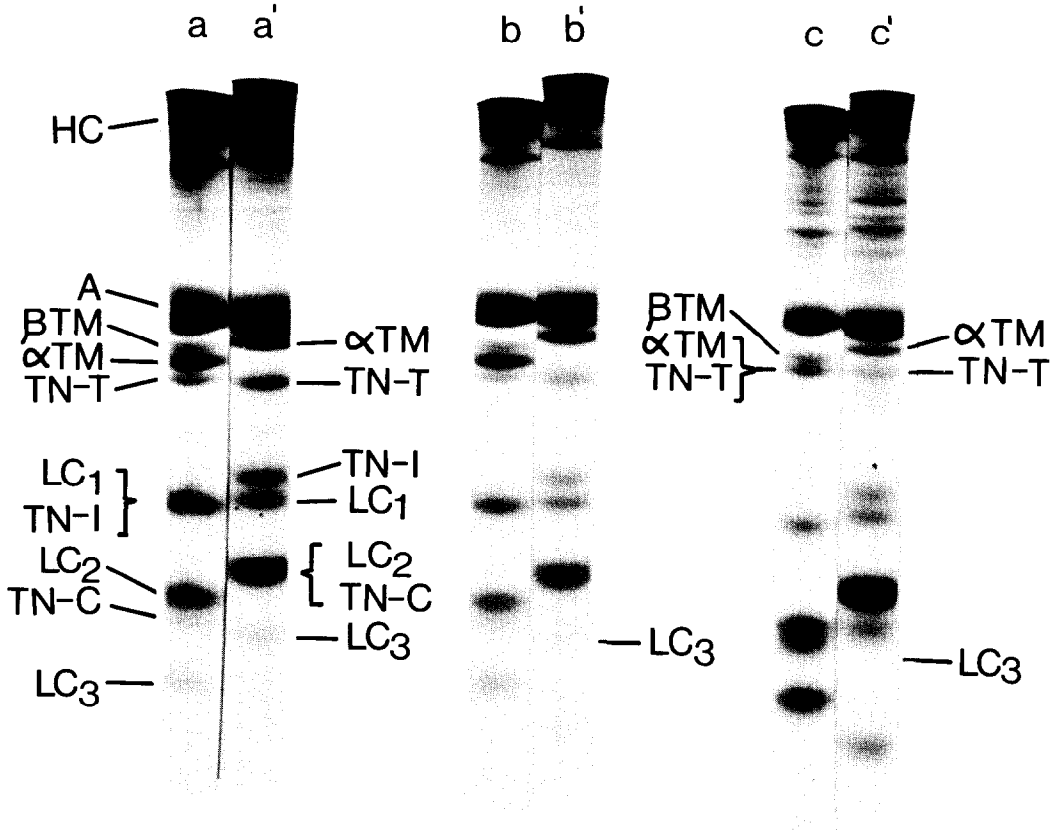

Fig.1

Table 2
Stoichiometry of myofibrillar proteins

Protein	Mol. wt.	Mol/7 mol actin		
		Adult	Dystrophic	Foetal
β-TM	37 400	0.61 ± 0.14	0.72 ± 0.08	0.62 ± 0.10
α-TM	36 100	1.77 ± 0.09	1.72 ± 0.13	1.77 ± 0.14 [0.86] [1.13]
TN-T	34 250	0.94 ± 0.10	0.77 ± 0.05	
LC_1 TN-I	24 500	2.10 ± 0.16 [1.23] [0.90]	1.90 ± 0.13 [1.07] [1.05]	2.04 ± 0.12 [1.05] [1.16]
LC_2	19 700	2.38 ± 0.33	2.19 ± 0.38	n.d.
TN-C	18 400	0.94 ± 0.08	n.d.	n.d.
LC_3	15 850	0.62 ± 0.15 [0.60]	n.d. [0.30]	n.d. [0.20]

Fast-Green stained gels were analyzed using a Joyce-Loebl densitometer with the red filter 204. The recorded peaks from the chart paper were cut out and weighed as described by Potter [22]. The linearity of staining intensity (peak weight or area) to protein concentration was previously demonstrated over a wide range of protein concentration [28] and applies to myofibrillar proteins [22]. The staining intensity of actin, myosin light chain LC_2 and TN-C are lower than the other proteins and correction factors have been determined [22]. The peak weights were expressed as a ratio with respect to actin = 7.0. The mole of each chain per 7 mol of actin were determined using mol. wts. determined by SDS-electrophoresis. The molar ratios of chains LC_1, TN-I and LC_3 and foetal α-tropomyosin and TN-T were determined in the SDS–5 M urea pH 7.0 gels (values in parentheses). Foetal and dystrophic myofibrils contained contaminating proteins which migrated close to LC_2, TN-C and LC_3 in SDS-electrophoresis. Attempts to remove these by treatment of myofibrils with sucrose solutions and Triton X-100 [32] or glycerination [21] were not successful. Molar ratios of chains were not determined (n.d.) when contaminating proteins interfered. The values obtained represent the means ± standard error of the mean of six experiments using SDS, pH 7.0, gels and the mean of two experiments using SDS–5 M urea, pH 7.0 gels.

different polymorphic form of TN-T. When electrophoresis was carried out in the presence of SDS-pH 7.0, the foetal form of TN-T comigrated with α-tropomyosin (fig.1c), but in the presence of SDS–5 M urea pH 7.0 [2] the mobility of tropomyosin was reduced thus separating it from TN-T (fig.1c′). The results indicated that foetal TN-T had a molecular weight of 36100 compared with 34250 for normal adult and dystrophic TN-T (fig.1). No differences were detected in the TN-I component of troponin. TN-I and myosin light chain LC_1 co-electrophoresed in the presence of SDS, pH 7.0, but migrated separately in the presence of SDS–5 M urea, pH 7.0 (fig.1) thus enabling densitometry to be carried out on the separate chains.

Densitometry of fast-green stained gels (table 2) indicated that adult, dystrophic and foetal myofibrils

Fig.1. Polyacrylamide gel electrophoresis of myofibrils. Electrophoresis conditions: (a–c) Sodium dodecyl sulphate (SDS), pH 7.0; (a′–c′). SDS–5 M urea, pH 7.0. Myofibrils were dissolved (1 mg/ml) in 8 M urea, 1% SDS, 1% β-mercaptoethanol, 0.01 M sodium phosphate buffer, pH 7.0, and incubated briefly at 37°C before centrifugation for 10 min at 25°C at 500 *g* to remove the small amount of insoluble material. 50 μg samples were electrophoresed as described previously [9] except that the gels contained 10% acylamide and 0.402% methylene bisacrylamide in 0.075 M sodium phosphate buffer, pH 7.0, 0.1% SDS. Gels were stained in 1% Fast Green in 7% acetic acid for 2 h at 25°C and destained in several changes of 7% acetic acid [28]. Mol. wts. were determined from SDS, pH 7.0, gels as described previously [9]. To enable identification of the various polypeptide chains the following myofibrillar proteins were purified and electrophoresed for comparison: myosin [9], actin [29], tropomyosin [30] and troponin [31]. Myofibrils were prepared from the following mice: (aa′) normal adult; (bb′) dystrophic; (cc′) foetal. HC – myosin heavy chain; LC_1, LC_2 and LC_3 – myosin light chains; α-TM and β-TM – tropomyosin polymorphic forms; TN-T, TN-I and TN-C – the tropomyosin binding, inhibitory and Ca^{2+}-binding components of troponin.

all have approx. 1 mol of TN-T and TN-I per 7 mol of actin suggesting that there were no quantitative differences in troponin. (The molar ratio of TN-C (1.0 mol) could only be determined in normal myofibrils). During development, the relative amount of α-tropomyosin increased from 0.86 to 1.77 mol and, since the β-tropomyosin content did not alter, the total content of tropomyosin polymorphic forms was greater in adult (2.38 mole compared with 1.48 mol). The α-tropomyosin content of dystrophic myofibrils was similar to the adult level.

Adult myofibrils contained an increased amount of the myosin light chain LC_3 compared with foetal and dystrophic (0.60, 0.20, 0.30 mol respectively).

To estimate the weight ratio of myosin heavy chain-to-actin, myofibrils were electrophoresed in gels containing a lower concentration of bis-acrylamide (0.134%) and stained in Coomassie blue [20,21]. The ratios obtained for normal adult, dystrophic and foetal myofibrils were very similar (1.83 ± 0.13, 1.89 ± 0.05, 1.80 ± 0.03 (2 samples) respectively).

4. Discussion

Although mixed skeletal muscle was used in this investigation it is probable that the myofibrillar proteins were predominantly of the fast-twitch type. A previous investigation [9] indicated that myosin from the same musculature of adult, dystrophic and foetal mice was of the fast-twitch type with three light chains. Even at high protein loading slow-twitch myosin light chains were hardly visible in SDS-gels (ref. [9], fig.1). It is therefore assumed in the present investigation that differences detected between the other myofibrillar proteins were not due to changing proportions of fast-twitch and slow-twitch isoenzymatic and polymorphic forms.

The inhibition of Mg^{2+}-ATPase activity due to EGTA reflects the functioning of the troponin–tropomyosin complex; the different degrees of inhibition suggest quantitative or qualitative differences in troponin–tropomyosin. The present results indicated that inhibition of Mg^{2+}-ATPase in foetal myofibrils was significantly less than in adult and dystrophic myofibrils and suggested that this was related to the presence of a foetal form of troponin containing an electrophoretically distinct polymorphic form of TN-T. The different level of inhibition suggested a difference in TN-I but this was not detected by the electrophoresis techniques used. Different polymorphic forms of TN-I with different inhibitory activities have been previously isolated from fast-twitch and slow twitch skeletal muscle [4].

Foetal myofibrils contained 1.48 mol of tropomyosin subunits per 7 mol of actin which is considerably less than in adult and dystrophic myofibrils (2.38 and 2.44 mol respectively). The expected value for the tropomyosin subunit content in myofibrils was 2.0 mol (1.0 mol of native tropomyosin [22]), which suggested that the foetal myofibril preparation may contain some myofibrils without any tropomyosin at all. This is unexpected since it is now accepted that tropomyosin is required with troponin to confer full Ca^{2+} sensitivity to the Mg^{2+}-ATPase of actomyosin.

Eisenberg and Kielley [23] reported that αα and αβ tropomyosin isomers occur in vivo but ββ isomers do not. If this is the case, the data in table 2 may be interpreted in the following way. Of the 0.86 mol of α-tropomyosin in foetal myofibrils, 0.62 mol would be combined with the same amount of β-tropomyosin, and the remaining 0.24 mol would combine to form αα isomers. Adult myofibrils contained 1.77 mol of α-tropomyosin and, since the β-tropomyosin content was similar to foetal (0.61 mol), an increased amount (1.16 mol) would therefore be available for combination into αα isomers. The increase in α-tropomyosin content during development was accompanied by the replacement of foetal TN-T by adult TN-T. This suggests that adult TN-T may bind specifically to the increasing population of αα isomers of tropomyosin.

A previous report from this laboratory [9] indicated that lower specific Ca^{2+}-ATPase activities of myosin from dystrophic and foetal mice correlated with a reduced relative amount of the myosin light chain LC_3. In a further report [24] it was shown that myosin from myotubes in tissue culture contained LC_1 and LC_2 chains only and had a very low specific Ca^{2+}-ATPase activity. It has been suggested that the myosin LC_3 chain is susceptible to removal or degradation during purification and that foetal myosin LC_3 may be more unstable than adult. It has also been suggested that the LC_3 light chain is derived from contaminating connective tissue myosin [25]. In the present investigation, to avoid loss of protein, intact myofibrils were electrophoresed directly and

the previous finding of decreased relative amount of LC_3 in foetal and dystrophic myosin was confirmed. The decreased amount of LC_3 may now also correlate with decreased specific Mg^{2+}-ATPase of myofibrils. Weeds and Taylor [26] have recently separated two myosin subfragment-1 isoenzymes containing either LC_1 or LC_3 chains alone which showed a striking difference in the Mg^{2+}-ATPase in the presence of actin; the V_{max} for S-1 (LC_1) was half that obtained for S-1 (LC_3).

Mg^{2+}-ATPase activities of dystrophic and foetal myofibrils may have been inhibited by the presence of contaminating proteins. Proteins whose subunits have molecular weights similar to LC_2, TN-C and LC_3 were particularly prominent but these were removed when actomyosin was purified from the myofibrils. Actomyosin preparations from adult, dystrophic and foetal myofibrils showed differences in Mg^{2+}-ATPase activity similar to the differences between the original myofibrils.

The evidence of recent cross-reinnervation experiments involving fast- and slow-twitch muscles indicates that the expression of the genes for myosin and troponin subunits is controlled by nerve-mediated factors. The replacement of foetal forms of myosin and troponin by adult forms may occur as the nervous system reaches full differentiation. Dystrophic muscle has a foetal-type myosin light chain composition but adult-type troponin suggesting that there may be different nerve-mediated factors involved in regulating these genes and only myosin synthesis is affected in dystrophy.

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