MYOSIN FROM CROSS-REINNERVATED CAT MUSCLES. EVIDENCE FOR RECIPROCAL TRANSFORMATION OF HEAVY CHAINS

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1. Introduction

In an earlier paper [1], changes in the physiological properties of cat muscles following cross-reinnervation were correlated with biochemical changes in the constituent myosins. Results showed that cross-reinnervation of soleus (slow-twitch) and flexor hallucis longus (fast-twitch) muscles produced a reciprocal transformation of the maximum shortening velocities of these muscles, and the ATPase activities of the constituent myosins mirrored these changes. Thus the kinetic properties of the myosins were altered according to the type of nerve impulse reaching the muscles. The results were further correlated with the pattern of light chain bands obtained by gel electrophoresis of the myosins in the presence of sodium dodecyl sulphate (SDS). These light chains appear to be essential for the ATPase activity [2,3], and the number of components as well as their apparent molecular weights differ in myosins from fast-twitch and slow-twitch muscles [4,5]. It was concluded that cross-reinnervation of a fast-twitch muscle resulted in the production of a myosin characteristic of slow-twitch muscle and vice versa. This would account for the kinetic and physiological changes observed, and indicates that the myosin phenotype is controlled via the central nervous system.

The techniques used in this study [1] could not resolve changes in the myosin heavy chains, since the molecular weights of these polypeptides from different myosins are very similar. In this paper we wish to report experiments demonstrating that the heavy chains are also reciprocally transformed following cross-reinnervation. These experiments rely on an analysis of the distribution and environment of the cysteine containing peptides in the different myosins. Although differences have been reported in the content of 3-methyl histidine and methylated lysines in cat muscles [6], cysteine residues were chosen for this study because they can be selectively modified using radioactive reagents to facilitate their identification in peptide mapping studies, and can also be cleaved specifically by cyanylation using 2-nitro-5-thiocyanobenzoic acid. Furthermore cysteine residues are among the rarest in myosin so that the number of peptides to be compared is small.

2. Experimental

The myosins used in these experiments were those obtained from May 18 Cat described previously [1]. The transformation of the physiological and biochemical activities was almost complete for this cat in both the cross-reinnervated flexor hallucis longus (X-FHL) and soleus muscles (X-SOL). Indeed the X-SOL muscle from this cat showed a much more marked decrease in the time to peak of the isometric twitch than had been observed for any other cat studied previously. These myosin preparations had been stored at -20 °C in 50% glycerol for about 16 months. After removal of the glycerol by dialysis, the myosins were dissolved in 6M guanidine hydrochloride, 50 mM Tris–Cl pH 8.4 and containing 10 mM EDTA. Dithiothreitol was added to 1.5 mM and the solutions incubated under an atmosphere of nitrogen for one hour to ensure that the protein thiol groups were fully reduced. [^{14}C]iodoacetic

acid was added to 10 mM and the reaction allowed to proceed for 1 hr before adding a large excess of β-mercaptoethanol to eliminate the remaining iodoacetic acid. Following exhaustive dialysis against 0.5% acetic acid, the incorporation of iodoacetic acid was determined by scintillation counting and the protein concentration measured by absorbance at 280 nm (using a value for $E^{1\%}_{280}$ = 6.20 cm^{-1} as described previously [1]. Values obtained for the incorporations varied between 7.8 and 8.8 mol/10^5 g myosin, indicating that the cysteine residues had reacted almost quantitatively. (The cysteine content of cat myosins has not been published, but values obtained for a number of different myosins are all in the range 8.5–9.9 mol/10^5 g [7,8].)

The alkylated myosins were digested with trypsin overnight in 0.1 M ammonium acetate at pH 8.1, using an enzyme: substrate ratio of 1:80 (w/w), then freeze dried. Paper electrophoresis at pH 6.5 was carried out as described previously [8], and peptide maps prepared using electrophoresis at pH 2 in the perpendicular dimension and chromatography in butan-1-ol-acetic acid–water–pyridine (15:3:10:12) (BAWP) [8]. To avoid heterogeneity of peptides in the pH 2 dimension due to partial oxidation of the carboxymethyl cysteine residues, the strips of paper were exposed to performic acid vapour following the initial electrophoresis at pH 6.5, to convert all the carboxymethyl cysteine to its sulphone form [8]. Radioactive peptides were detected by radioautography using Kodak Autoprocess film. A quantitative estimate of the labelled peptides was made by cutting out the spots from the peptide maps and scintillation counting.

Chemical cleavage at the cysteine residues was achieved after the myosins had been denatured and fully reduced by incubation for 2 hr at 37 °C in a solution containing 6 M guanidine hydrochloride, 0.2 M Tris–Cl pH 8.0–8.2 and 0.1 mM dithiothreitol. After dialysis for 24 hr against the same solution, the samples were made 10 mM with 2-nitro-5-thiocyanobenzoic acid, using a stock of freshly prepared 0.1 M reagent in 6M guanidine hydrochloride, and maintained at 37 °C for 36 hr. The reaction was terminated by acidification to 50% acetic acid, and the cleaved proteins dialysed against 50% acetic acid exhaustively. This method is based on published techniques [9,10]. After drying under vacuum at room temperature, the fragments were submitted to gel electrophoresis on 8% polyacrylamide gels in 0.1% SDS using a discontinuous buffer system [11]. Gels were stained with 0.05% Coomassie brilliant blue in 50% methanol, 10% acetic acid and destained initially in the same solvent, then in 5% methanol, 10% acetic acid.

3. Results and discussion

Electrophoresis of tryptic digests of the labelled myosins at pH 6.5 showed that with a single exception, all the radioactive peptides were neutral or anionic. The exception occurred in the myosins from normal soleus (N-SOL) and X-FHL muscles, and provided a useful distinction between these myosins and those from N-FHL and X-SOL muscles. (Since the cysteine peptides from the light chains are anionic at pH 6.5, the basic and neutral peptides arise from the heavy chains.) Chromatography of the basic band in BAWP yielded a single spot, suggesting that only one cysteine peptide was present. The acidic and neutral zones from the pH 6.5 electrophoresis were subjected to further electrophoresis at pH 2 to produce the peptide maps shown in fig.1. Radioautographs obtained from digests of N-SOL and X-FHL were indistinguishable as far as the mobilities and numbers of peptides were concerned, and tracings of these spots were superposed to produce the map labelled 'slow-twitch' in fig.1. The same was true for N-FHL and X-SOL myosins, labelled 'fast-twitch' in fig 1. Further peptide maps were obtained from tryptic digests of larger preparations of cat myosins which had been freed of light chains by gel filtration on Biogel A 1.5 M in 0.1% SDS. These controls confirmed that nearly all the radioactive peptides arose from the heavy chains.

Although fig.1 shows clear differences in the neutral peptides obtained from 'fast-twitch' and 'slow-twitch' myosins, the maps of the acidic peptides show striking similarities. This observation suggests that the peptides containing these cysteine residues are similar both in size and net change in the different myosins, which may indicate extensive sequence homologies in these myosins. Strips containing the neutral peptides were submitted to chromatography in BAWP to obtain further fractionation of these peptides, and the radioautographs are shown in fig.2.

An attempt was made to quantitate these differences by cutting out the radioactive peptides and scintil-

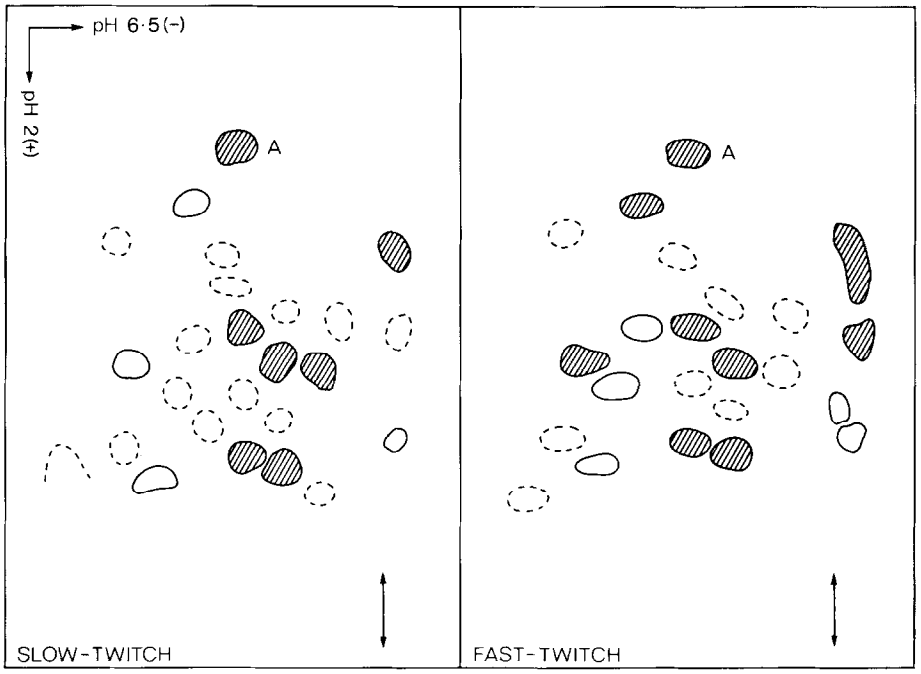

Fig.1. Two-dimensional peptide maps of tryptic digests of slow-twitch and fast-twitch myosins, showing the [^{14}C]carboxymethyl cysteine peptides. These maps show only acidic and neutral peptides separated initially by electrophoresis at pH 6.5 and then at pH 2 in the perpendicular dimension. strong radioactive peptides; moderate peptides; weak peptides. The double-headed arrow marks the position of the origin, and the peptide marked A was used to normalise the counts in the basic and neutral peptides (table 1). Radioactivity at the origin due to insoluble peptide material is not shown.

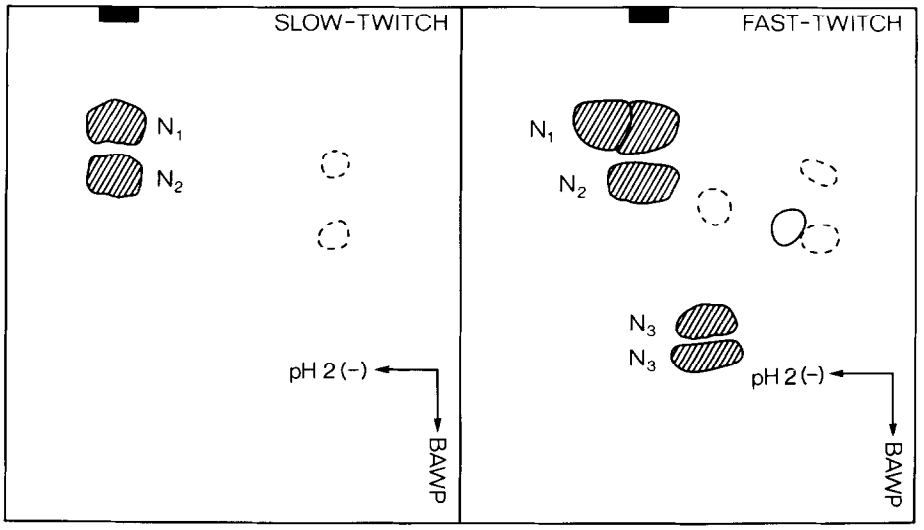

Fig.2. The neutral peptides (lying vertically above the origin in fig.1) were subjected to chromatography in BAWP as indicated. Symbols as in fig.1. The black rectangle marks the position of the serine marker in the pH 2 dimension. Counts in the labelled peptides are shown in table 1.

Table 1
Quantitative estimation of peptides characteristic of slow-twitch and fast-twitch myosins

Myosin source	Peptide number			
	Basic	N1	N2	N3
N-FHL	0.19	2.30	1.27	0.93
X-SOL	0.20	2.26	1.28	0.99
N-SOL	1.15	0.87	1.02	0.09
X-FHL	0.85	0.93	0.91	0.18

Counts in the peptides indicated (see fig.2 and text) were normalised to the counts in peptide A (fig.1) for each of the myosins indicated. The counts per minute for peptide A varied between 10 500 and 13 500 for the four samples, due to slight loading differences on the papers. If peptide A represents a unique sequence, values of unity should be obtained for other unique peptides.

lation counting. To facilitate comparison of the different samples, where the initial loadings were not necessarily identical, the acidic peptide (marked A in fig.1) was also counted, since this peptide was common to all myosin digests. The counts in the neutral and basic peptides were then normalised to this peptide A (table 1). Agreement between N-FHL and X-SOL is striking, as is that between N-SOL and X-FHL, indicating the quantitative extent of the reciprocal transformation of the myosin phenotypes following cross-reinnervation. The basic peptide present in slow-twitch myosin is largely absent from fast-twitch myosin, but some residual counts in this area were noted. Fast-twitch myosin contains a pair of neutral peptides (N3) whose combined radioactivity equals that of the reference acidic peptide. (The splitting of this band in BAWP may indicate partial deamidation of the peptide during exposure to performic acid, since maps of unoxidised peptides gave only a single spot of high Rf in BAWP.) Once again a low level of radioactivity was found in the area of the N3 peptides in slow-twitch myosins (table 1). Counts in N1 of fast-twitch myosins are significantly larger than for slow-twitch myosins suggesting additional neutral peptides in these myosins. Since all these peptides arise form the heavy chains, the results confirm that different myosin heavy chains are produced following cross-reinnervation of these muscles.

Differences in the mobility of peptides may arise through deamidation or incomplete cleavage by the digesting enzyme, and they do not always reflect sequence differences. Whilst it is impossible that all the additional neutral peptides in the fast-twitch myosins could have arisen from deamidation of the single basic peptide in the slow-twitch myosin, a further approach was used to demonstrate differences in these heavy chains. Chemical cleavage at the cysteine residues was achieved after reaction with 2-nitro-5-

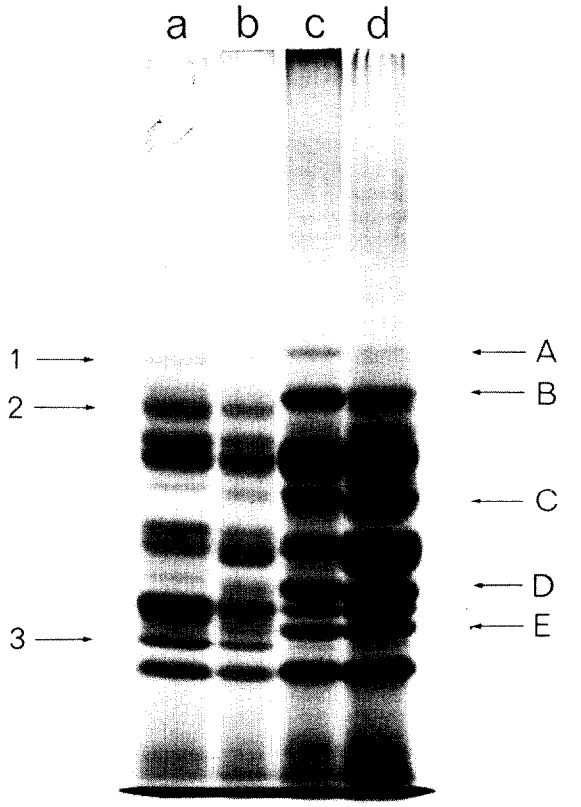

Fig.3. 8% polyacrylamide gel electrophoresis in the presence of SDS of fragments formed by cyanylation cleavage. (a), N-SOL myosin; (b), X-FHL myosin; (c), N-FHL myosin; (d), X-SOL myosin. The arrows 1–3 mark fragments prominent only in the N-SOL and X-FHL cleavage products, whereas arrows A–E mark those prominent only in the N-FHL and X-SOL cleavage products. As a guide to the approximate molecular weights of the fragments, bovine serum albumin (68 000) migrated just in front of fragment 2, while actin (42 000) appeared to co-migrate with fragment D. Fragments with a molecular weight of 27 000 or less migrated to the bottom of the gel.

thiocyanobenzoic acid, and the fragments separated according to size on polyacrylamide gels (fig.3). Although the loading of the X-SOL sample is much greater than the others, there is clear matching of the electrophoretic bands in X-FHL with N-SOL and also in X-SOL with N-FHL. The fragmentation shows a number of bands which are distinctive for each class of myosin as indicated in the figure. Thus while there are bands common to both classes of myosin indicating fragments of equivalent sizes produced in both, other bands of different mobility on polyacrylamide gels indicate that the distribution of cysteine residues is not identically maintained in both fast-twitch and slow-twitch myosins.

The results of these experiments support the conclusion that following cross-reinnervation, myosin genes are expressed which are specified by the newly innervating nerve. Thus in X-FHL muscle, both heavy and light chains characteristic of N-SOL are produced and the kinetic properties of the muscle are consequently changed. Peptide mapping studies, while confirming differences in the heavy chains of fast-twitch and slow-twitch myosins, show a pronounced similarity in the mobilities of many of the labelled cysteine peptides, which may be indicative of the extent of sequence homology to be found in these myosins. If the positions of these cysteine residues in the chains are accurately maintained, then cleavage should produce fragments of identical sizes in both classes of myosin. Whilst this is true for some fragments in fig.3, it is not generally observed, since cyanylation produced a distinctive pattern of bands in both fast-twitch and slow-twitch myosins. These fragments might arise from additional cysteine residues in one or other protein, or because the distribution of cysteine residues is different.

One puzzling feature arising from fig.3 is that the sum of the molecular weights of all the fragments exceeds that for the heavy chain of myosin (about 200 000). The number of bands observed also exceeds that found for rabbit myosin or chicken breast muscle myosin, each of which gives about 6–7 components in this molecular weight range [12]. Whilst this excess apparent molecular weight may be due to incomplete cleavage, it may also reflect the presence of isoenzyme in the individual muscle types. The presence of isoenzymes of myosin has been demonstrated in the case of the light chains in histochemically identified fibres of rabbit fast-twitch muscle [13], and both rabbit and cat slow-twitch muscles contain heterogeneous myosin light chains which are chemically related but contain amino acid substitutions (A.G. Weeds, unpublished results). Thus the heterogeneity of light chains is present in both fast-twitch and slow-twitch myosins. Furthermore, there is limited evidence for heterogeneity of the heavy chains in rabbit fast-twitch myosin [14,15].

Recently other evidence has been reported to show a change in the myosin phenotype following a change in the pattern of stimulation. These results were based on the staining of light meromyosin paracrystals [16], and on gel electrophoresis of the native myosins [17] and also of their tryptic digestion products [18]. Thus there is now a considerable body of evidence to support the hypothesis that the myosin phenotype is controlled by the central nervous system, and that both light and heavy chains are changed in response to cross-reinnervation. The suggestion that the enzymatic character of myosin is determined by the light chains alone is cast into considerable doubt by these results. Other techniques will be required to demonstrate the function of the different light chains in muscle activity.

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