Fractional synthesis rates *in vivo* of skeletal-muscle myosin isoenzymes

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The synthesis rates of different myosin isoenzymes in a single muscle, and of the same isoenzymes in different muscles (soleus, masseter and plantaris), were measured. The rate of total protein synthesis was significantly higher in the soleus [$> 95\%$ slow myosin (SM)] than in the plantaris [$> 95\%$ fast myosin (FM)]. Two fast isoenzymes, FM2 and FM3, were synthesized at different rates in the masseter, and SM was synthesized at a faster rate than FM. Intermediate myosin had a synthesis rate similar to that of FM. There was a small but significant difference between the synthesis rates of the SM isoenzymes of the soleus and masseter muscles. FM3 was synthesized faster in the masseter than in the plantaris, whereas FM2 was synthesized faster in the plantaris than in the masseter.

INTRODUCTION

Skeletal muscles are broadly classified as fast or slow, according to their contraction speed. The myosin that they contain exists as several different isoenzymes, which can be separated in their native state by electrophoresis under non-denaturing conditions (Hoh *et al.*, 1976; d'Albis *et al.*, 1979). Slow muscles contain primarily slow myosin (SM) isoenzymes, and fast muscles display a pattern of fast myosin (FM) and intermediate myosin (IM) isoenzymes (Fitzsimons & Hoh, 1983; Gregory *et al.*, 1986a). Most muscles are composed of combinations of several different fibre types, and hence contain a mixture of myosin isoenzymes.

The synthesis rates of individual skeletal-muscle myosin isoenzymes have not been reported. It is not known whether a single isoenzyme is synthesized at the same rate in different kinds of muscles, or whether different isoenzymes present in a single muscle are synthesized at the same rate. Such information would provide valuable clues with regard to myosin-heavy-chain heterogeneity, as well as providing the foundation for understanding the adaptive regulation of myosin-heavy-chain synthesis in skeletal muscle.

In the present study, we have measured the synthesis rates *in vivo* of the heavy-chain subunits of individual myosin isoenzymes in the soleus, plantaris and masseter muscles of the rabbit. There were significant differences between the synthesis rates for the same isoenzyme in different muscles, as well as between certain individual isoenzymes within the same muscle.

The results of this study have been reported in a preliminary abstract form (Gregory *et al.*, 1986b).

METHODS

Infusion of label

Six male New Zealand White rabbits weighing 700–900 g (age 5–6 weeks) were allowed access to food and water *ad libitum*. Approx. 15 mCi of L-[4,5- ^3H]leucine

(Amersham) was infused into the animals via an ear-vein catheter (24-gauge Quick-Cath; Travenol) over a period of 100 min, at a rate of 1.55 ml/h via a syringe pump (Sage Instruments, model 341A) fitted with a gas-tight Teflon barrel syringe (Hamilton). Throughout the infusion period, the rabbit was restrained in a towel, with a cloth covering the animal's eyes. Under these conditions anaesthesia was not required to keep the animal calm. Nine blood samples (each 75 μl) were taken from a vein in the opposite ear at regular intervals during the infusion for determination of the specific radioactivity of [^3H]leucine in plasma. The animal was killed by a blow to the head, and the muscles were dissected within 10 min, weighed, frozen in liquid N_2 and then were stored at -85°C until analysed.

Isolation of myosin isoenzymes

Muscles were pulverized at the temperature of liquid N_2 , and a crude extract was prepared by homogenizing a 60 mg portion in 1 ml of extraction buffer (0.1 M-sodium pyrophosphate/5 mM-EDTA/2 mM-2-mercaptoethanol/0.2 mM-dichlorvos, pH 8.8). Myosin isoenzymes were separated under non-dissociating conditions by pyrophosphate/polyacrylamide-gel electrophoresis essentially as described by Hoh *et al.* (1976) and as previously reported by our laboratory (Gregory *et al.*, 1986a). Two electrophoresis chambers were modified to accommodate 38 tubes each by the drilling of additional holes in the rubber gasket. After electrophoresis, the gels were stained for 15 min with Coomassie Brilliant Blue (Cleveland *et al.*, 1977) and then destained in cold distilled water for several hours. Individual isoenzyme bands were carefully isolated by slicing from the gel with a razor blade.

Bands of a single isoenzyme type from 50–200 pyrophosphate gels of one muscle were pooled for subsequent analyses. The myosin light- and heavy-chain subunits contained in the pyrophosphate-gel slices were then separated by denaturing SDS/polyacrylamide-gel electrophoresis, and the heavy-chain band was isolated

Abbreviations used: SM, slow myosin; IM, intermediate myosin; FM, fast myosin; S.A., specific radioactivity; FSR, fractional synthesis rate.

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from the gel with a razor blade as above, and hydrolysed overnight in 6 M-HCl at 110 °C. Hydrolysates were then dried in a Speed-Vac Concentrator (Savant) and redissolved in 100 µl of freshly distilled water. Leucine was separated from acrylamide contaminant by paper chromatography (Whatman no. 3) in butanol/acetic acid/water (2:1:1, by vol.). Before application of the sample, the paper was washed extensively in the chromatographic solvent to remove trace leucine contaminants. After chromatography, leucine was eluted from the top half of the chromatogram and dried by evaporation. The residue was redissolved in 20 µl of 0.05 M-bicarbonate buffer (pH 9), and incubated with diamino[¹⁴C]naphthalene-5-sulphonyl chloride (dansyl chloride; Amersham) of known specific radioactivity. The individual dansyl-amino acid products were separated by two-dimensional chromatography on polyamide plates as described by Airhart *et al.* (1979). Briefly, the dansyl-leucine spot was localized under u.v. light, cut out and placed in a scintillation vial with NCS tissue solubilizer and Econofluor, and counted for radioactivity for 10–100 min in a liquid-scintillation counter (Packard Tri-Carb, model 3320). Specific radioactivity (S.A.) of [³H]leucine in protein was calculated as follows:

$$\text{S.A. of leucine (d.p.m./pmol)} = \frac{{}^3\text{H d.p.m.}}{{}^{14}\text{C d.p.m.}} \times \text{S.A. of } [{}^{14}\text{C}]\text{dansyl chloride (d.p.m./pmol)}$$

For total SDS-soluble protein, a 30–60 mg portion of frozen powdered muscle was homogenized with a Polytron tissue homogenizer in 1.5 ml of 40 mM-sodium pyrophosphate/20 mM-sodium phosphate (NaH₂PO₄/Na₂HPO₄, molar ratio 1:1)/0.5 mM-EDTA/1% SDS, boiled for 3 min and centrifuged (Eppendorf microfuge, 1 min). A 100 µl portion of the resultant supernatant was added to 1 ml of cold 10% (v/v) trichloroacetic acid and left overnight at 4 °C. The precipitate was washed three times with cold 5% trichloroacetic acid, once with 95% (v/v) ethanol/ether (1:1, v/v), and once with acetone. The dried pellet was hydrolysed in 6 M-HCl and taken through the steps described above for determination of S.A. of leucine.

Calculation of fractional synthesis rates (FSR)

FSR values for total protein and for the heavy chains of individual myosin isoenzymes were calculated as follows:

$$\text{FSR (\%/day)} = \frac{\text{S.A. of leucine in protein (d.p.m./pmol)}}{\text{S.A. of leucine in plasma (d.p.m./pmol)} \times \text{time}} \times 100$$

where 'time' refers to the duration of the infusion in days. The S.A. of leucine in the plasma of each individual animal was used to calculate the synthesis rates in muscles from that animal (Kelley *et al.*, 1984).

Statistical analyses

Differences between the FSR of isoenzymes within a single muscle were evaluated by analysis of variance. A paired *t* test was used for comparisons between the synthesis rates of a single isoenzyme synthesized in two different muscles of the same animal, for comparisons between the synthesis rates of FM3 and FM2 within a single muscle, and for evaluating differences between the FSR of total protein in the fast plantaris and slow soleus (Snedecor & Cochran, 1968). *P* < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Myosin isoenzyme composition

The resolution of myosin isoenzymes of rabbit soleus, masseter and plantaris obtained by pyrophosphate/polyacrylamide-gel electrophoresis is shown in Fig. 1. Identification of individual isoenzymes common to different muscles was made by co-electrophoresis of a mixture of two muscles (Fig. 1*d*). Bands were labelled according to current nomenclature (Hoh *et al.*, 1976).

The soleus (Fig. 1*a*) was found to contain a single SM band, as well as a trace of IM, an isoenzyme associated with Type IIa fibres (Fitzsimons & Hoh, 1983). The SM band in the soleus gel may contain more than one isoenzyme, differing in light-chain components (Pinter *et al.*, 1981).

The masseter, on the other hand, contained a mixture of SM, IM and FM isoenzymes in approximately equal proportions (Fig. 1*b*). The mobility of masseter SM was markedly different from that of soleus SM, in agreement with Mabuchi *et al.* (1984), who showed that these two SM isoenzymes differ in both light-chain and heavy-chain composition. Occasionally a trace amount of SM with

the same mobility as soleus SM was apparent in masseter gels, but the amount was insufficient for measurement of its synthesis rate. The two FM isoenzymes of the masseter had electrophoretic mobilities similar to FM3 and FM2, and co-migrated with FM3 and FM2 of the plantaris when extracts of both muscles were co-electrophoresed (Fig. 1*d*).

The plantaris was found to contain all three fast isoenzymes (FM3, FM2, FM1), together with small amounts of IM and SM (Fig. 1*c*). The amounts of these two minor isoenzyme components were insufficient for assessment of their synthesis rate. The SM in the plantaris could be distinguished as two faint bands which co-migrated with the single wide band of soleus SM when extracts of these two muscles were co-electrophoresed. These two faint SM bands probably represent the SM1 and SM2 isoenzymes found in mammalian muscles (Hoh, 1978; Lyons *et al.*, 1983; Maréchal *et al.*, 1984).

Specific radioactivity of plasma [³H]leucine

The time course of equilibration of plasma and infused [³H]leucine is shown in Fig. 2. There was a rapid rise in plasma leucine specific radioactivity, to about 80% of the mean plateau value of 55–60 d.p.m./pmol, within the first 5 min of infusion. The average plasma leucine S.A. for each animal over the entire infusion period was used for calculation of FSR to correct for the initial time to plateau.

Accurate determination of protein FSR values requires knowledge of the specific radioactivity of the amino acid precursor. In the present study, it was necessary to use the method of constant tracer infusion in order to allow a sufficient period of time for incorporation of labelled

Fig. 1. Myosin isoenzymes of rabbit skeletal muscles

Myosin isoenzymes of (a) soleus, (b) masseter and (c) plantaris were separated by pyrophosphate/polyacrylamide gel electrophoresis. Gel (d) represents co-electrophoresis of myosin from masseter and plantaris. Bands are identified on the schematic at the left: SM, slow myosin; IM, intermediate myosin; FM, fast myosin.

Fig. 2. Specific radioactivity of plasma $[^3\text{H}]$ leucine during infusion

Time course of the rise in plasma leucine S.A. during continuous infusion of $[^3\text{H}]$ leucine. Each point represents the mean $\pm$ S.E.M. for six animals.

leucine to measurable extents in myosin. This methodology, however, is subject to the following limitations.

Since our calculations of synthesis rates are based on the specific radioactivity of free leucine in plasma, the first concern is that equilibration between plasma free leucine and the leucyl-tRNA pool utilized for protein synthesis may not have occurred (see Rannels *et al.*, 1972). The second potential problem is that the relationship between plasma leucine and leucyl-tRNA may not be the same in each of the different muscles studied, even after allowing a sufficient period for equilibration of precursor pools, which may equilibrate at different rates. Finally, it is possible that, even within a single muscle, the plasma leucine/leucyl-tRNA re-

lationship may differ among individual muscle fibres, thereby affecting the calculated synthesis rates for proteins that may be synthesized in different fibres.

If our first assumption is incorrect, i.e. if the specific radioactivity of leucyl-tRNA is less than that of plasma leucine, the absolute values obtained for synthesis rates would be underestimated, although the relationship between the FSR of various myosin isoenzymes would still be correct. Lack of precursor-pool equilibration has proved troublesome in some circumstances *in vitro* (see Rannels *et al.*, 1982), including muscle (Schneible *et al.*, 1981; Stirewalt & Low, 1983). The problem does not appear to occur in perfused hearts (McKee *et al.*, 1978), or in heart (Martin *et al.*, 1977) and lung (Kelley *et al.*, 1984) *in vivo*, based on comparisons of the specific radioactivity of tRNA-leucine and plasma free leucine. Similar measurements for skeletal muscle *in vivo* have yet to be reported. Regarding the second potential pitfall, if the relationship between plasma leucine and leucyl-tRNA was different for different muscles, comparisons between the synthesis rates measured in different muscles would be subject to error, but the relative synthesis rates of different myosin isoenzymes within a single muscle would not be affected. Although it is conceivable that the precursor-pool relationships may differ even among individual muscle fibres within a single muscle, it is technically impossible to measure the specific radioactivity of leucyl-tRNA in each muscle fibre. However, we have observed differences even between two myosin isoenzymes (FM2 and FM3), which have been found to be present together in a single fibre (Mabuchi *et al.*, 1984).

Fractional synthesis rates of total protein and myosin isoenzymes

FSR values of total SDS-soluble protein and of the heavy chains of individual myosin isoenzymes are presented in Table 1. The synthesis rate for total protein was significantly higher in the slow soleus than in the fast plantaris ($P < 0.05$), in agreement with Goldspink *et al.* (1983). Although the masseter contains a mixture of fast and slow fibres, the synthesis rate for total protein in the masseter was found to be the same as in the soleus.

The synthesis rates of individual myosin isoenzymes present in different muscles were compared first. The FSR of masseter SM was found to be slightly faster than that of soleus SM ($P < 0.05$). As stated above, these two forms of SM appear to be structurally distinct, and may therefore be products of separate genes. Two of the fast isoenzymes, FM3 and FM2, were found to have different FSR values, which varied in opposite directions depending on the muscle in which they were synthesized. Thus the FSR of FM3 was higher in the masseter than in the plantaris ($P < 0.05$), whereas that of FM2 was higher in the plantaris than in the masseter ($P < 0.05$). Although differences in measured synthesis rates between muscles would be affected by a variable relationship between plasma leucine and the precursor, another possibility is that there is a spectrum of FSR values among individual fast (or individual slow) fibres. In other words, some fast fibres may synthesize and degrade protein faster than others, and the relative abundance of fibres with more rapid turnover may differ in masseter and plantaris. Such differences between overall synthesis rates in different fibres would explain the different FSR values obtained for FM2 and FM3 if they were present

Table 1. Fractional synthesis rates *in vivo* of total protein and of heavy chains of individual myosin isoenzymes in three skeletal muscles

Values are expressed as means $\pm$ S.E.M.; numbers in parentheses indicate numbers of animals studied. Abbreviations: n.p., isoenzyme not present in muscle; n.p.s., isoenzyme not present in sufficient quantity for measurement of synthesis rate. The value of the *t* ratio is included in the footnotes for data evaluated by a paired *t* test.

	FSR (%/day)		
	Soleus	Masseter	Plantaris
Total protein	6.6 $\pm$ 0.2 ¹ (6)	6.7 $\pm$ 0.8 (6)	4.9 $\pm$ 0.7 (6)
Myosin isoenzymes			
SM	4.3 $\pm$ 0.2 ² (6)	5.0 $\pm$ 0.2 (6)	n.p.s.
IM	n.p.s.	4.1 $\pm$ 0.3 (6)	n.p.s.
FM3	n.p.	4.5 $\pm$ 0.3 (5) ^{3,5}	3.3 $\pm$ 0.5 (5)
FM2	n.p.	3.2 $\pm$ 0.4 (5) ⁴	4.5 $\pm$ 0.9 (5)
FM1	n.p.	n.p.	3.9 $\pm$ 0.9 (5)

¹*P* < 0.05 versus plantaris total protein (*t* = 3.29)

²*P* < 0.05 versus masseter SM (*t* = 2.78).

³*P* < 0.05 versus FM3 of plantaris (*t* = 2.98).

⁴*P* < 0.05 versus FM2 of plantaris (*t* = 4.02).

⁵*P* < 0.01 versus FM2 of masseter (*t* = 7.00).

in different fibre types in the masseter and in the plantaris. Understanding the meaning of findings such as these will require analyses of the synthesis rates of individual isoenzymes and precursor-pool relationships in single muscle fibres.

The FSR values of individual isoenzymes in the same muscle were compared next. Although the synthesis rates varied from animal to animal, the relative synthesis rates of constituent myosin isoenzymes within a given muscle were consistent. Within the masseter, the synthesis rate of SM was higher than that of FM2 and FM3 (*P* < 0.01), whereas IM was synthesized at a rate similar to that of FM3. The synthesis rate of FM2 was significantly lower than that of FM3 (*P* < 0.01). This was not the case in the plantaris, for which the FSR of FM2 tended to be higher than FM3, although the difference was not statistically significant. Although the differences between the FSR of FM2 and FM3 in the masseter are not striking when the group means are compared (Table 1), the differences within each muscle were remarkably consistent. A paired *t* test was therefore used to determine the statistical significance of these findings. These opposite relationships for FM in plantaris and in masseter might be explained in terms of differential synthesis rates in different fibre types, particularly if the synthesis rate for a given fibre type is not the same in different muscle types. Different precursor-pool relationships also might be a factor.

The different FSR values for FM2 and FM3 within the same muscle must be considered against four possibilities regarding their heavy-chain structure and cellular distribution. These two isoforms may be the product of separate genes, and may occur either within the same or within different cell (fibre) types. The second pair of alternatives is that they are the product of a single gene and occur within the same or different cells.

Regarding the first possibility, it is clear that there is more than one isoform of fast myosin heavy chain (Starr & Offer, 1973; Gauthier & Lowey, 1979; Dalla Libera *et al.*, 1980; Zweig, 1981; Billeter *et al.*, 1981; Mabuchi *et al.*, 1984; Izumo *et al.*, 1986; Gregory *et al.*, 1986a). One isoform appears to be associated with fibres that

stain histochemically as Type IIa, and the other with Type IIb fibres. However, it is not known with which native myosin isoenzyme (FM1, FM2, FM3, IM) each fast heavy chain is associated. The most likely candidate is IM, which has been found to be associated with Type IIa fibres (Fitzsimons & Hoh, 1983; Hoh & Chow, 1983). Indeed, we have previously shown that the IM in rat plantaris has a different heavy-chain structure from that of FM1 and FM2, on the basis of peptide-mapping analysis, and therefore may represent the isoenzyme that contains the fast IIa heavy-chain isoform (Gregory *et al.*, 1986a). However, it is not clear whether IM is the only isoenzyme containing the fast IIa heavy chain. Type IIa fibres contain three isoenzymes (Mabuchi *et al.*, 1982, 1984), but it is not known whether all three contain the fast IIa heavy chain. Taken together with our data indicating differential FSR values for FM2 and FM3, this suggests that these two isoenzymes are the result of translations of separate gene products. The potential caveat for this conclusion is that our analyses were of whole muscle containing a mixture of fibres. A spectrum of synthesis rates of individual fibres combined with a spectrum of proportions of FM2 and FM3 in different fibres conceivably could lead to our result, even though the heavy chains were derived from the same gene product. The data of Mabuchi *et al.* (1984) indicate that the proportion of FM isoenzymes is different between Type IIa and IIb fibres of the masseter. However, it is not known whether the FSR values of total protein for these two fibres are different. Again, resolution would require analyses of the synthesis rates of each isoenzyme on an individual fibre basis.

Interpretation of our results requires knowledge as to whether or not the proportions of myosin isoenzymes in the muscles that we have examined are in steady state. Differences in the relative proportions of the various isoenzymes in different muscles or in different fibres would not account for differences in their synthesis rates if we assume a uniform, random, degradation rate and a steady-state isoenzyme composition. Although the rabbits used in the present study were young, they are beyond the early developmental period in which myosin

isoenzymes change dramatically. Similarly, it is unlikely that the isoenzymes are in a period of rapid change such as occurs with thyroid-hormone treatment (Everett *et al.*, 1983) or pressure-overload cardiac hypertrophy (Litten *et al.*, 1982), which would give rise to the kinds of differences in synthesis rates that we have observed in the present study.

In summary, we have found that there are differences between the synthesis rates of individual myosin isoenzymes in the same and in different muscles. The results suggest that the heavy-chain subunits of isomyosins with different synthesis rates are derived from different genes, and/or that there are differences in the synthesis rates characteristic of the fibres in which they are synthesized. The complexities revealed in this study indicate that measurements of the synthesis rates of individual myosin isoenzymes in single muscle fibres, as well as accurate assessment of precursor-pool relationships, may be necessary in order to distinguish between the several alternative explanations of different rates of isoenzyme synthesis. An appreciation of the separate but co-ordinated synthesis of the different myosin isoenzymes is an important element of changes that occur under physiological and pathological conditions which alter the relative amounts of these skeletal-muscle isoenzymes, such as exercise, muscle hypertrophy (Gregory *et al.*, 1986a), diabetes (Rutschmann *et al.*, 1984), muscular dystrophy (Fitzsimons & Hoh, 1983) and hormonal treatment (Izumo *et al.*, 1986; Lyons *et al.*, 1986).

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