DISTRIBUTION OF ISOENZYMES OF THE GLYCOGENOLYTIC CASCADE IN DIFFERENT TYPES OF MUSCLE FIBRE

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1. Introduction

Mammalian skeletal muscle fibres have been classified into at least three major types on the basis of their dynamic and histochemical characteristics, termed (a) fast twitch glycolytic (white), (b) fast twitch oxidative glycolytic (red), and (c) slow twitch oxidative (red) [1,2]. It has been known for some years that certain enzymes of the glycolytic pathway, such as phosphorylase and lactate dehydrogenase, have much higher activities in fast twitch glycolytic fibres, while the content of myoglobin or enzymes of the tricarboxylic acid cycle are much higher in slow twitch oxidative fibres (reflecting a greater number of mitochondria) [2]. However only recently has it been realized that the various muscle fibre types differ considerably in their genotypes, and that such differences might underlie the apparent heterogeneity of enzymes isolated from muscles which are composed of more than one class of fibre. Enzymes which have now been shown to exist in different forms in fast twitch glycolytic and slow twitch oxidative fibres include lactate dehydrogenase [3], AMP deaminase [4], the light chains of myosin [5] and phosphorylase kinase [6].

In this paper we present a survey of the distribution of isoenzymes of the glycogenolytic pathway in the psoas (a muscle comprising > 90% fast twitch glycolytic fibres), the soleus (almost entirely slow twitch oxidative fibres) and cardiac muscle. This study was prompted by the knowledge that two isoenzymes of cyclic AMP dependent protein kinase can be resolved by DEAE-cellulose chromatography of extracts prepared from *mixed* muscles of the hind limb and back [7].

2. Materials and methods

2.1. *Preparation of muscle extracts*

The psoas major, soleus and heart muscles were removed from female New Zealand White rabbits and placed in ice. Care was taken to use only the central fibres of the psoas, since the medial and lateral edges may show appreciable fibre heterogeneity [8]. The muscles were chopped finely and homogenized with 2.5 vols of 4.0 mM EDTA – 14 mM mercaptoethanol, pH 7.0. The suspension was centrifuged at 10 000 *g* for 20 min and the supernatant was decanted through glass wool.

2.2. *Cyclic AMP-dependent protein kinase*

The extracts were acidified to pH 5.5 and the solution centrifuged. After adjusting to pH 7.0, the supernatant was made 50% in ammonium sulphate and recentrifuged. The precipitate was redissolved in 5.0 mM glycerol-2-phosphate-1.0 mM EDTA – 14 mM mercaptoethanol pH 7.0 and dialysed against this buffer. The solution was clarified by centrifugation and applied to a DEAE-cellulose column (8 × 0.8 cm). The column was then eluted successively at pH 7.0 with (a) 5 mM glycerol-2-phosphate (b) 25 mM glycerol-2-phosphate (c) 40 mM glycerol-2-phosphate and (d) 50 mM glycerol-2-phosphate + 0.15 M NaCl. All solutions contained 14 mM mercaptoethanol and 1.0 mM EDTA. Cyclic AMP-dependent protein kinase was eluted exclusively in fractions (b) and (d), corresponding to the two isoenzymes termed Peak-1 and Peak-2 respectively [7]. When either fractions (b) or (d) from any muscle type were rechromatographed, > 95% of the activity emerged in the same fraction as before, demonstrating that

there was no cross contamination of isoenzymes during the initial elution.

2.3. *Phosphorylase kinase*

The muscle extracts were adjusted to pH 7.0, taken to 45% saturation in ammonium sulphate and centrifuged. The precipitate containing phosphorylase kinase was redissolved in 50 mM glycerol-2-phosphate–1.0 mM EDTA-14 mM mercaptoethanol pH 7.0 and dialysed against this buffer. Antigen–antibody precipitation was carrried out in solution by adding to the dialysate a γ-globulin fraction isolated from antiserum raised in hens to purified rabbit muscle phosphorylase kinase [9,10]. The immunoprecipitates formed after two hours at 0°C were collected and analysed by sodium dodecyl sulphate gel electrophoresis [9].

2.4. *Phosphorylase b*

This enzyme was partially purified from the muscle extracts as described for phosphorylase kinase. The dialysate obtained after ammonium sulphate fractionation was subjected to acrylamide gel electrophoresis and stained histochemically for phosphorylase *b* activity as described by Davis et al. [11].

2.5. *Debranching enzyme*

Glycogen debranching enzyme was partially purified from each extract as described for cyclic AMP dependent protein kinase (section 2.2). The debranching enzyme emerging in fraction (b) from DEAE-cellulose was further examined by Ouchterlony double diffusion in 1% agar, buffered with 50 mM glycerol-2-phosphate-2.0 mM EDTA pH 6.8 containing 1.5 M NaCl. Antiserum to a homogeneous preparation of debranching enzyme from mixed rabbit muscle [12] was raised in hens.

The DEAE-cellulose fractions were also subjected to antigen–antibody precipitation in solution using a γ-globulin fraction isolated from the antiserum. The immunoprecipitates formed after 30 min were analysed by sodium dodecyl sulphate gel electrophoresis [9].

2.6. *Enzyme assays*

Cyclic AMP dependent protein kinase was assayed by the incorporation of ^{32}P-radioactivity into histone H-1 from [γ-^{32}P] ATP, using the cellulose phosphate filter paper method of Witt and Roskoski [13]. Phosphorylase kinase [10] and phosphorylase *b* [14] were assayed by standard procedures. Debranching enzyme was measured by the incorporation of [^{14}C] glucose into glycogen [12].

3. Results and discussion

3.1. *Cyclic AMP dependent protein kinase*

The distribution of the Peak-1 and Peak-2 isoenzymes in the different fibre types is shown in table one. The psoas contained predominantly the Peak-1 isoenzyme (Peak-1/Peak-2 = 9.0), whereas soleus and cardiac muscles had almost equal amounts of the two types (Peak-1/Peak-2 = 0.9). The isoenzyme ratio, Peak-1/Peak-2 therefore varies by a factor of ten between fast twitch glycolytic and slow twitch oxidative skeletal muscle fibres. Evidence is now accumulating that each isoenzyme may be composed of an identical catalytic subunit (C) bound to distinct regulatory subunits (R_1 and R_2) [15,16]. If this is the case, then the different ratios arise from the presence of altered proportions of the R-1 and R-2 subunits in different fibre types.

The ratio, Peak-1/Peak-2 in cardiac muscle appears to vary from mammal to mammal. In the present work, rabbit cardiac muscle contained nearly equal amounts of the two types. However in rat cardiac muscle the Peak-1 isoenzyme is predominant [16,17], whereas in bovine cardiac muscle the Peak-2 isoenzyme is predominant [16].

In the rabbit, the soleus is composed almost exclusively of slow twitch oxidative fibres, and it therefore seems clear that both isoenzymes are present in this fibre type. On the other hand, it could be argued that the small amount of the Peak-2 isoenzyme in the psoas derives from contamination with red oxidative fibres. However, such contamination would have to be at least 10% (tables 1 and 2). Since care was taken to use only the central fibres of the psoas, which should be 95–98% fast twitch glycolytic [8] it seems most likely that fast twitch glycolytic fibres do indeed synthesize small amounts of the Peak-2 isoenzyme. The present data cannot however answer the question of whether *single* fibres synthesize both isoenzymes.

Table 1
Elution of the Peak-1 and Peak-2 isoenzymes of cyclic AMP dependent protein kinase from DEAE-cellulose

Fibre type	Peak-1 (%)	Peak-2 (%)	Peak-1/Peak-2
1. Psoas (fast twitch glycolytic)	90 ± 4 (5)	10 ± 4 (5)	9.0
2. Soleus (slow twitch oxidative)	47 ± 2 (3)	53 ± 2 (3)	0.9
3. Cardiac	48 ± 2 (3)	52 ± 2 (3)	0.9

The figures in parentheses refer to the number of preparations examined. The yield of cyclic AMP dependent protein kinase was 80% up to the DEAE-cellulose step, and the yield from DEAE-cellulose (peak-1 + peak-2) averaged 80-90%.

3.2. *Phosphorylase kinase*

Phosphorylase kinase isolated from mixed rabbit and murine muscle of the hind limb and back contains four subunits, termed α (molecular weight 145 000), α' (140 000), β (128 000) and γ (45 000), and the smallest active species of the enzyme has the structure $[(\alpha + \alpha')\beta\gamma]_4$ [9,10]. Jennissen and Heilmeyer [6] reported that phosphorylase kinase isolated from the soleus contained only the α', β and γ subunits, while the psoas contained only the α, β and γ subunits, suggesting that varying proportions of two isoenzymes, $(\alpha\beta\gamma)_4$ and $(\alpha'\beta\gamma)_4$, are present in different mammalian skeletal muscle fibres. However, as slow twitch oxidative fibres are known to contain higher levels of proteinase activities [18] and the isolation of the $(\alpha'\beta\gamma)_4$ isoenzyme from the soleus required several lengthy chromatographic steps [6], it could still be argued that the α'-subunit was derived from the α-subunit during the purification.

Fig.1 shows the sodium dodecyl sulphate gel patterns of immunoprecipitates formed after incubation of muscle extracts with purified antibody to phosphorylase kinase for only two hours. The results confirm that the soleus contains only the α', β and γ subunits, strongly supporting the contention that two isoenzymes exist in mammalian muscles. On the other hand, while the psoas contained predominantly the $(\alpha\beta\gamma)_4$ isoenzyme, small quantities of the $(\alpha'\beta\gamma)_4$ isoenzyme were also present (about

Table 2
Relative specific activities of glycogenolytic enzymes in extracts of different muscle fibres[a]

Fibre type	Cyclic AMP dependent protein kinase[b]	Phosphorylase kinase	Phosphorylase[c]	Debranching enzyme
Psoas (fast twitch glycolytic)	1.0	1.0	1.0	1.0
Soleus (slow twitch oxidative)	2.0 ± 0.2	0.055 ± 0.01	0.11 ± 0.01	0.34 ± 0.2 (4)
Cardiac	1.4 ± 0.2	0.02 ± 0.01	0.15 ± 0.01	0.52 ± 0.25 (4)

[a] Relative to the psoas (1.0).
[b] Peak-1 + Peak-2.
[c] Phosphorylase *b* + phosphorylase *a*.
Each figure represents an average of measurements from four animals.

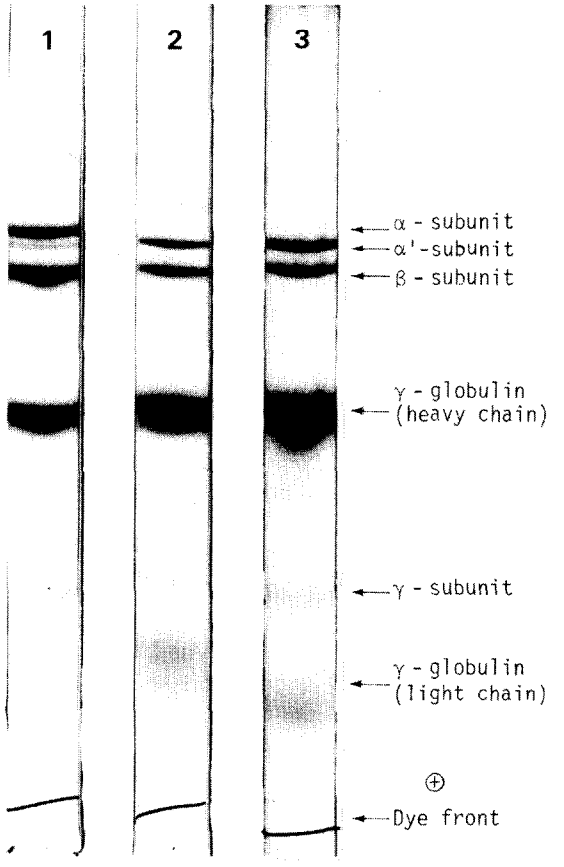

Fig.1. Sodium dodecyl sulphate gel electrophoresis of immunoprecipitates formed by incubation of partially purified phosphorylase kinases with antibody to phosphorylase kinase. (1) psoas major; (2) soleus; (3) cardiac.

10% as judged by densitometric tracings of the gels). If it is assumed that the two isoenzymes have the same specific activity, this cannot be due to contamination with oxidative fibres in view of the 20-fold lower activity of phosphorylase kinase in such fibres (table 2). Indeed the activity measurements (table 2) indicate that the concentration of the minor $(\alpha'\beta\gamma)_4$ isoenzyme in fast twitch glycolytic fibres is at least as high as in slow twitch oxidative or cardiac fibres.

The results also demonstrate that cardiac muscle contains exclusively the $(\alpha'\beta\gamma)_4$ isoenzyme (fig.1).

All four subunits α, α', β and γ are absent in ICR/IAn mice which completely lack phosphorylase kinase in skeletal and cardiac muscle fibres [9].

3.3. *Phosphorylase*

Cardiac muscle of the rabbit and certain other mammals contains three active forms of phosphorylase *b* which can be resolved by gel electrophoresis or DEAE-cellulose chromatography, termed I_b, II_b and III_b [11]. Type III_b has properties identical to phosphorylase *b* isolated from mixed skeletal muscle, while type II_b is a hybrid formed from one subunit of type I_b and one of type III_b [11]. Fig.2 shows that phosphorylase *b* in psoas or soleus muscle is exclusively type III_b. Types II_b and I_b are found only in cardiac muscle.

3.4. *Debranching enzyme*

Partially purified preparations of debranching enzyme from each fibre type showed cross reactions of identity in Ouchterlony double diffusion

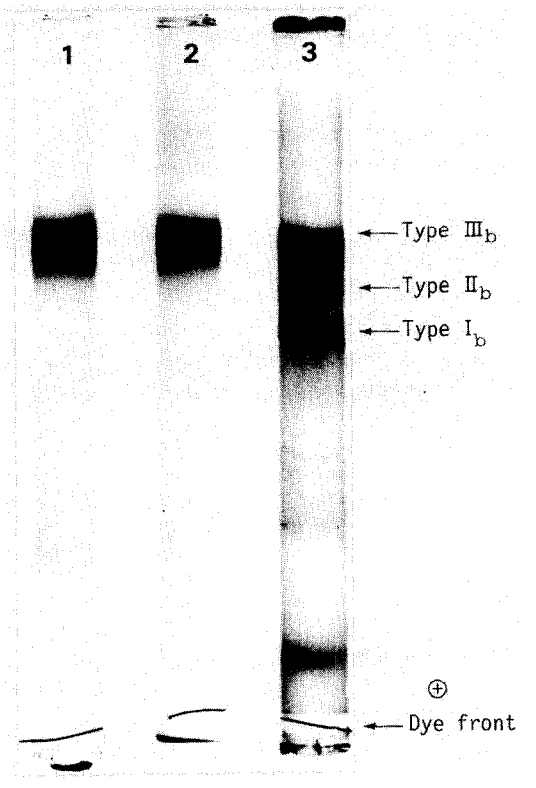

Fig.2. Separation of isoenzymes of phosphorylase *b* by acrylamide gel electrophoresis. Partially purified preparations were electrophoresed at pH 7.9 and stained for phosphorylase *b* activity [11]. (1) psoas major; (2) soleus; (3) cardiac.

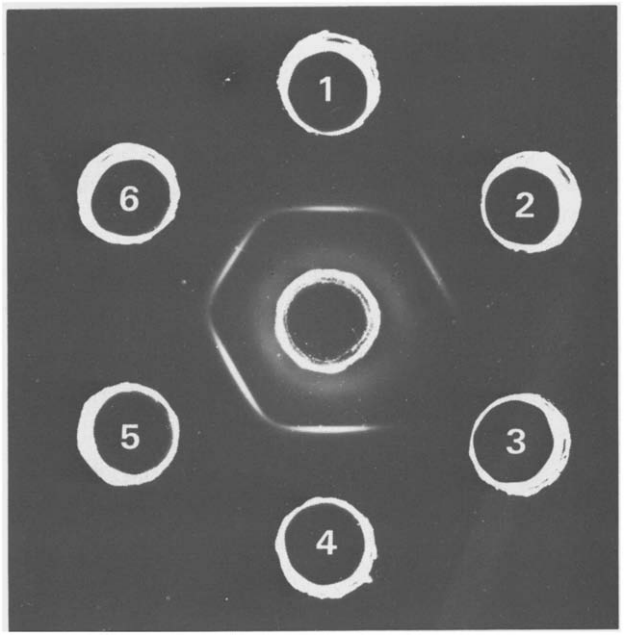

Fig.3a

Fig.3b

experiments (fig.3a) and the gel electrophoretic patterns of immunoprecipitates formed in solution were the same (fig.3b). The results show that the psoas, the soleus and cardiac muscles contain a single type of debranching enzyme which has a subunit mol. wt. of 166 000 [12,19].

3.5. *Activities of the glycogenolytic enzymes*

The activities of the glycogenolytic enzymes (table 2) are much higher in fast twitch glycolytic fibres as would be expected from the metabolism of this fibre type. In contrast, cyclic AMP dependent protein kinase activity is relatively constant between fibres, supporting the idea of a wider role for this enzyme [20]. These results lead however to interesting observations regarding the molar proportions of the various enzymes of the glycogenolytic cascade. If the molecular weight of the smallest unit of cyclic AMP dependent protein kinase (R_1C or R_2C) is taken as 95 000 [16,21] of phosphorylase kinase ($\alpha\beta\gamma$ or $\alpha'\beta\gamma$) as 315 000 [10], and of phosphorylase as 100 000 [22], then the relative molar proportions of these three enzymes, in terms of active centres, is approximately 1 : 10 : 240 respectively in the psoas [23]. This is consistent with the classical view, that this sequence of three enzymes acts as an amplification system for the action of adrenalin on glycogenolysis. However, if it is assumed that the different isoenzymes have very similar specific activities, which is well established for two of the three enzymes [6,24], then the molar ratios, cyclic AMP dependent protein kinase: phosphorylase kinase: phosphorylase become 1 : 0.25 : 14 in the soleus, or 1 : 0.15 : 25 in cardiac muscle. The second component of the cascade (phosphorylase kinase) is therefore present at a much lower molar concentration than the first (cyclic AMP dependent

Fig.3. Immunological characterisation of partially purified debranching enzymes. (a) Ouchterlony double diffusion in agar. Centre well, antiserum to debranching enzyme; (1) and (5) psoas major; (2) and (4) soleus; (6) cardiac, (3) purified phosphorylase *b*. (b) Sodium dodecyl sulphate gel electrophoresis of immunoprecipitates formed by incubation of fraction (b) from DEAE-cellulose with antibody to debranching enzyme. (1) psoas major; (2) soleus; (3) cardiac; (D) purified debranching enzyme from rabbit skeletal muscle [12].

protein kinase). The potential for amplification is therefore much decreased in the soleus and the heart, and amplification should be most significant in these muscle fibres when the degree of activation of cyclic AMP dependent protein kinase is relatively low. There are of course many other possible advantages of linking two or more regulatory enzymes in a sequence than merely the amplification of an initial stimulus [25].

4. Concluding remarks

Distinct isoenzyme patterns of the glycogenolytic enzymes exist in different fibre types. Fast twitch glycolytic and slow twitch oxidative fibres differ in the proportion of the two isoenzymes of cyclic AMP dependent protein kinase and in the type of phosphorylase kinase that is present. Slow twitch oxidative fibres and cardiac fibres resemble one another in these two respects, but differ in that the type I phosphorylase of cardiac muscle is absent in slow twitch oxidative fibres.

In all examples, the functional differences between the isoenzymes seem to be related to the regulatory rather than the catalytic behaviour of the molecules. In the case of cyclic AMP dependent protein kinase and phosphorylase kinase, it is a regulatory subunit that appears to be affected [16.23], while in the case of phosphorylase, the type I isoenzyme is known to have a five to eight-fold K_a for the allosteric activator 5'AMP [6]. However, the precise physiological significance of these differences remains to be elucidated.

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