Presence of enolase in the M-band of skeletal muscle and possible indirect interaction with the cytosolic muscle isoform of creatine kinase

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Glycerol-skinned skeletal muscle fibres retain the defined sarcomeric structure of the myofibrils. We show here that a small fraction of two enzymes important for energy metabolism, the cytosolic muscle isoform of creatine kinase (EC 2.7.3.2), MM-creatine kinase (MM-CK), and enolase (EC 4.2.1.11), remains bound to skinned fibres. CK is slowly exchangeable, whereas enolase is firmly bound. Two-dimensional gel electrophoresis followed by Western blot analyses demonstrates that both α (ubiquitous) and β (muscle-specific) subunits of enolase are present in these preparations. Enolase and CK were co-localized at the M-band of the sarcomeres, as observed by indirect immunofluorescence and confocal microscopy. Cross-linking experiments were performed on skinned fibres with three bifunctional succinimidyl esters of different lengths and yielded a protein complex of 150 kDa that reacted with antibodies directed against either M-CK or β-enolase. The cross-linking efficiency was greatest for the longest reagent and zero for the shortest one. The length of the cross-linker giving a covalent complex between the two enzymes does not support the notion of a direct interaction between M-CK and enolase. This is the first demonstration of the presence of an enzyme of energy metabolism other than CK at the M-band of myofibres.

Key words: myofibrils, sarcomere, crosslinking, skeletal-muscle enzymes.

INTRODUCTION

In striated muscles, the M-band is located in the middle of the thick filament region and is composed of several elements that seem to stabilize the hexagonal filament lattice of myosin [1]. Some aspects of the M-band structure have been deduced from negatively stained longitudinal sections [2]. Thus the M-band appears as a zone of higher density, approx. 750 Å wide; depending on the fibre type and animal species [3] it consists of three to five cross-striations. The two main components of the M-band, myomesin and protein M, are largely arranged perpendicular to the long axis of myofibrils [4,5] and thus provide a strong connection between thick filaments. The C-terminal phosphorylatable region of the giant protein titin [6] is also located at the M-band [7]. This filament protein extends parallel to myosin and actin filaments towards the Z-line, the anchorage structure of the thin filaments [7,8]. The muscle-specific isoform of creatine kinase (EC 2.7.3.2), MM-CK, is cytosolic but a small fraction of the enzyme is bound specifically to the M-band structure of the sarcomere [9,10], and MM-CK seems to form the M4/M4′ striations [1]. CK has a key role in the energy metabolism of cells with intermittent high-energy requirements such as skeletal muscle fibres [11]. Because many phosphorylatable sites of the M-band proteins are present in this part of the myofibrils [7,12], it is of interest to search for the presence of ATP-regenerating systems, including glycolytic enzymes, at this site.

There is much evidence that the cytosolic enzymes that supply energy for movement and transport are not distributed evenly over the whole cytoplasm but are located in distinct areas of the cell [13,14]. Associations of glycolytic enzymes in multiprotein complexes have been described for various cell types [15,16]. In striated muscles, interactions of many glycolytic enzymes with myofilaments, both *in vivo* and *in vitro*, have been shown by using a variety of techniques [17–20].

Among glycolytic enzymes, enolase (2-phospho-D-glycerate hydro-lyase, EC 4.2.1.11), which presents muscle-specific isoforms, is of particular interest [21]. In striated muscles it is found as homodimers and heterodimers formed from two subunits α and β [21,22]. During ontogenesis, the $\alpha\alpha$ embryonic isoform remains distributed in most adult cell types. A specific transition occurs in striated muscles towards the $\alpha\beta$ and $\beta\beta$ isoforms [22–24]. In fast muscles the β subunit accounts for more than 90 % of total enolase activity [21–23]. Interactions *in vitro* of $\beta\beta$- and $\alpha\alpha$-enolase with several glycolytic enzymes and with MM-CK were recently demonstrated [25]. It was therefore tempting to investigate whether an interaction of enolase with M-CK does occur at the M-band of muscle fibres.

Skinned fibres represent a suitable preparation because they are devoid of freely diffusible proteins and of membranes, yet the functional contractile structure is maintained. In this paper we investigated the localization of enolase isoforms on skinned muscle fibres by using immunological techniques. Cross-linking studies of enolase and CK with bifunctional reagents were used to determine a possible interaction between the two enzymes at the M-band.

EXPERIMENTAL

Reagents

The cross-linking reagents disuccinimidyl tartrate (DST), dithiobis[succinimidyl propionate] (DSP) and ethyleneglycolbis-

Abbreviations used: CK, creatine kinase; DSP, dithiobis[succinimidyl propionate]; DST, disuccinimidyl tartrate; ECL, enhanced chemiluminescence; EGS, ethyleneglycolbis[succinimidyl succinate]; M- (subunit) or MM- (dimeric) CK, cytosolic muscle isoform of creatine kinase.

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[succinimidyl succinate] (EGS) were purchased from Pierce. Other chemicals were of analytical grade. Citifluor was from UKC Chemical Laboratory and the enhanced chemiluminescence (ECL®) kit was from Amersham.

Enzymes

The $\beta\beta$-enolase form, purified from rabbit skeletal muscle, was obtained from Sigma. Enolase activity was measured spectrophotometrically at 340 nm by using the coupled reactions of pyruvate kinase and lactate dehydrogenase [21].

MM-CK was prepared in the laboratory from rabbit muscles by using method B of Kuby et al. [26]. The protein was further purified by chromatography on Biogel P100. CK activity was determined at 340 nm by using the coupled reactions of hexokinase and glucose-6-phosphate dehydrogenase [26].

Immunological reagents

Because commercial antibodies against M-CK present cross-reactivity against many proteins of the myofibrils such as myosin and phosphorylase *b*, polyclonal antibodies were obtained in our laboratory by immunization of chicken, as described previously [27]. The antibodies were eventually affinity-purified by chromatography on an M-CK–Sepharose column. No cross-reaction was observed with either the B-cytosolic or the mitochondrial form of CK. The preparation of serum directed against rabbit [28] or mouse β-enolase, and against mouse α-enolase, was as described previously [25,29]. Antibodies directed against chicken M-band proteins (myomesin and protein M) were a gift from T. Wallimann (Zurich, Switzerland).

Biotinylated or peroxidase-labelled antibodies directed against chicken IgY or rabbit IgG, and FITC-labelled anti-(sheep IgG) and anti-(goat IgG), were from Sigma.

Preparation of skinned fibres and myofibrils from rabbit psoas muscle

New Zealand male rabbits (2–2.5 kg) were killed by intravenous injection of a lethal dose of pentobarbital. Psoas muscles were taken out and kept in 0.1 M KCl/1 mM EGTA/5 mM EDTA/1 mM NaN_3 containing 0.1 mM PMSF, 10 μM leupeptin and 10 μM pepstatin (medium A) before dilaceration. Fibre bundles (1–3 mm in diameter, 2–3 cm long) were dissected out at 4 °C in medium A. Cellular membranes were disrupted by using the method of Wallimann et al. [9] and Grove et al. [30] with medium A containing 50 % (v/v) glycerol. This treatment was performed twice. Skinned fibres could be kept at −20 °C for several weeks in medium A containing 50 % (v/v) glycerol. Before use, the skinned fibres were subjected to further washes with medium A.

Myofibrils were prepared from glycerol-treated fibres with the use of a slight modification of the preparation of Kundrat and Pepe [31]. After five washes with medium A, the fibres were homogenized on ice in a Sorvall Omni Mixer with a burst of 10 s at maximal speed, followed by a 2 min cooling period at 4 °C and another burst of 7 s.

FITC labelling of the enzymes

$\beta\beta$-Enolase

A 500 μl portion of suspension of rabbit enolase (5 mg of protein) in 2.8 M $(NH_4)_2SO_4$ was dialysed against buffer B [48 mM K_2HPO_4/14 mM NaH_2PO_4/4.5 mM KH_2PO_4 (pH 7.2)] containing 0.1 mM dithiothreitol and 1 mM $MgSO_4$, then concentrated to 150 μl by ultrafiltration on Microcon 10 (Amicon). The same volume of FITC solution (2.5 mg/ml in 0.2 M K_2HPO_4, pH 8.5), prepared just before the reaction, was added to the enolase solution. After incubation for 2 h at room temperature in the dark, the excess of unreacted dye was eliminated by chromatography on a PD10 column (Pharmacia) equilibrated with buffer B.

CK

A 25 mg/ml solution of CK in buffer B was mixed with the same volume of the FITC solution prepared as above. The incubation time was decreased to 1 h because CK has a very reactive lysine residue in its active site [32]. For both enzymes, the average ratio of dye to protein subunit was evaluated as previously described [27] and found to be close to 1:1.

Incubation of skinned fibres with FITC-labelled MM-CK or $\beta\beta$-enolase

After five washings with medium A, fibres were incubated for 2 h at 4 °C in 5 mM Tris/HCl buffer, pH 8.0, to wash out the CK or $\beta\beta$-enolase firmly bound to the M-band of the sarcomeres. After this incubation, fibres were resuspended in medium A supplemented with 10 mM potassium phosphate, pH 7.5 (medium A plus phosphate). This suspension was incubated at 4 °C overnight with various concentrations of FITC-labelled CK (0.36, 1.3 or 3.6 mg/ml) or of FITC-labelled $\beta\beta$-enolase (3.75 mg/ml) in medium A plus phosphate. The fibres were then washed three to five times with medium A; myofibrils were then prepared as described above and mounted in Citifluor for fluorescence microscopic observations.

Immunolabelling of myofibrils

A 200 μl suspension of myofibrils was incubated with anti-(M-CK) or anti-(M-band protein) antibodies, with anti-(rabbit β-enolase) or anti-(mouse α-enolase) serum. For M-CK, the second antibodies were biotin-labelled anti-(chicken-IgY) produced in rabbit that were then reacted with Texas Red–streptavidin. FITC-labelled anti-sheep antibodies were used to detect β-enolase and FITC-labelled anti-(goat IgG) for anti-(M-band proteins) and anti-(α-enolase). Double-labelling experiments were performed to detect M-CK and β-enolase simultaneously. Preparations were then mounted in Citifluor and observed by confocal microscopy. Control preparations were obtained with the second antibody alone. Special precautions were taken for confocal microscopy observations. The choice of Texas Red as second fluorescent conjugate was important because its excitation spectrum is far from the emission spectrum of fluorescein. The excitation intensity at the two wavelengths was carefully adjusted to avoid artifacts of microscopic observation.

Two-dimensional gel electrophoresis and Western blot analysis

The soluble protein fraction for two-dimensional gel electrophoretic analysis was prepared from rabbit muscle as follows: frozen tissue samples were weighed, ground into a powder under liquid nitrogen with a hand homogenizer, and suspended in cold extraction buffer. The extraction buffer was 15 mM sodium phosphate buffer, pH 7.2 (5 ml of buffer per g of tissue), containing 4 mM magnesium acetate and the following cocktail of proteinase inhibitors: 2 mg/ml aprotinin, 2 mM benzamidine, 5 mg/ml leupeptin, 2 mg/ml pepstatin A, 0.5 mM PMSF and 1 mM EGTA. The supernatant obtained with a 60 min centrifugation at 15000 ***g*** was aliquotted and kept frozen at −20 °C

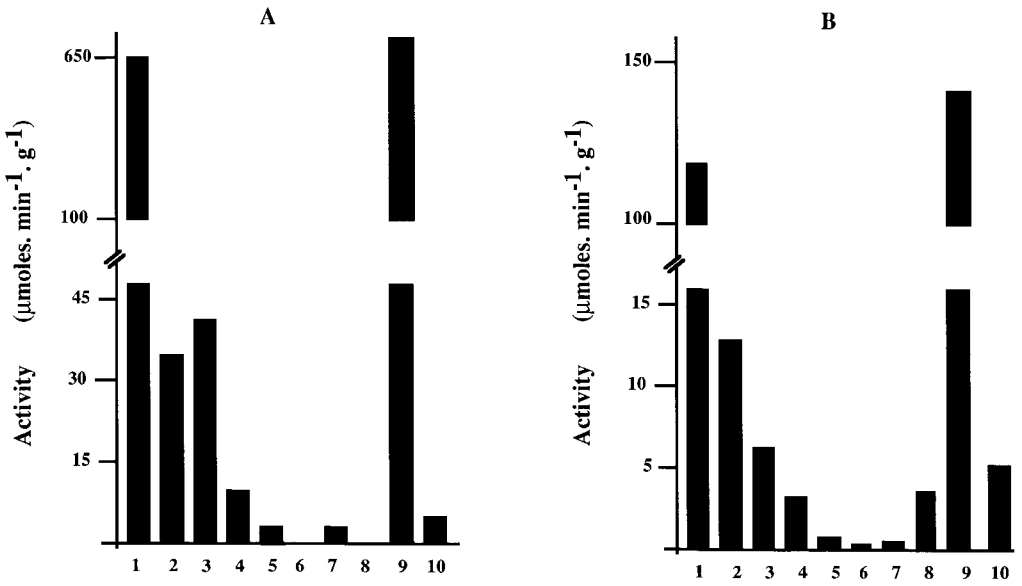

Figure 1 CK activity (A) and enolase activity (B) released during the preparation of skinned fibres and myofibrils

The activities were determined in supernatants obtained at each step of the preparation (columns 1–8). They are expressed relative to the initial mass of muscle. Note the different scales for activities higher than 100 μmol/min per g. Column 1, extraction with medium A; column 2, extraction with medium A plus 50% (v/v) glycerol; columns 3 and 4 represent another extraction cycle similar to that described in columns 1 and 2. At this stage the skinned fibres were kept at −20 °C in medium A plus 50% (v/v) glycerol. Columns 5 and 6, two successive washes of glycerol-skinned fibres with medium A; column 7, supernatant after 2 h of extraction in 5 mM Tris/HCl, pH 8.0; column 8, activities after treatment with 0.6 M KCl/0.1 M potassium phosphate/10 mM potassium pyrophosphate/1 mM $MgCl_2$ of fibres previously treated in 5 mM Tris/HCl; column 9, total CK and enolase activities in homogenates of the starting muscle sample; column 10, total activities in myofibrils prepared from skinned fibres as described in the Experimental section.

until further use. Protein concentration was determined by using the Bio-Rad Bradford protein assay with BSA as a standard.

Two-dimensional gel electrophoresis was performed by the method of O'Farrell, with minor modifications as described previously [25]. The soluble protein extracts were taken into the sample buffer [9.5 M urea/2% (w/v) Nonidet P40/5% (v/v) 2-mercaptoethanol/2% (w/v) ampholines (pH 3.5–10.0)]. Skinned fibres were washed with medium A as above, weighed, then incubated at 37 °C for 30 min in the sample buffer (5 μl/mg of wet weight). Samples were then subjected to basic non-equilibrium pH-gradient electrophoresis in 4% (w/v) polyacrylamide gels containing 2% (w/v) ampholines (pH 3.5–10.0), for 5 h at 500 V. For the second dimension, electrophoresis was run overnight in the presence of 0.1% SDS in 8% (w/v) polyacrylamide gels (acrylamide/bisacrylamide ratio 1:40) at 10 mA per slab gel. The gels were then electrically transferred to Hybond C membranes (Amersham). The membranes thus obtained were kept dry until immunological treatments, which were performed by using the protocol indicated by Amersham for the ECL Western blotting detection system.

Cross-linking of M-CK and β-enolase in glycerol-skinned fibres

To remove any residual soluble MM-CK and $\beta\beta$-enolase, approx. 2 g of glycerol skinned fibres was washed five times with 5 ml of medium A. These fibres were incubated in medium A plus phosphate for 1 h at 4 °C with different concentrations (from 0.5 to 2 mM) of the bifunctional cross-linkers DST, DSP or EGS, which are respectively 6, 12 and 16 Å long. The reaction was stopped by adding 5 mM Tris/HCl, pH 8.0. Fibres were then washed with 0.6 M NaCl to remove myosin; if this step was omitted, electrophoresis patterns yielded a very large protein band in the 200 kDa region that perturbed the observation of the other polypeptide bands. Fibres were finally extracted with 1% (w/v) SDS at 100 °C for 3 min in the presence of 40 mM iodoacetamide to avoid the formation of disulphide bridges.

Treated samples were separated by SDS/PAGE [7% (w/v) gel] with 3% (w/v) stacking gels as described by Laemmli [33]. The polypeptides were transferred electrophoretically to nitrocellulose membranes (reinforced nitrocellulose, 0.45 μm pore size; Schleicher and Schuel), incubated overnight at 4 °C with either anti-(M-CK) or anti-(β-enolase) antibodies and revealed with the ECL detection system as above. After this first reaction, immunoblots were washed with SDS and 2-mercaptoethanol at 60 °C in accordance with the technical notice from Amersham. Immunoblots that had been reacted with anti-(M-CK) in the first step could then be incubated with anti-(β-enolase) in the following step and vice-versa. This procedure allowed us to observe the same membrane decorated with one or the other antibody, thus giving an accurate determination of the polypeptide bands that could react with several specific antibodies.

RESULTS

Determination of CK and enolase activities extracted during the preparation of skinned fibres and myofibrils

CK and enolase activities measured during a typical preparation of skinned fibres and myofibrils are shown in Figures 1(A) and 1(B) respectively. Most of the CK activity was released in the first steps (Figure 1A, columns 1–4) by medium A or medium A plus 50% (v/v) glycerol, yielding the stock of skinned fibres kept at −20 °C (column 4). Before use, the fibres were submitted to further washes (Figure 1A, columns 5 and 6), which released only very little CK activity. The remaining bound CK was removed

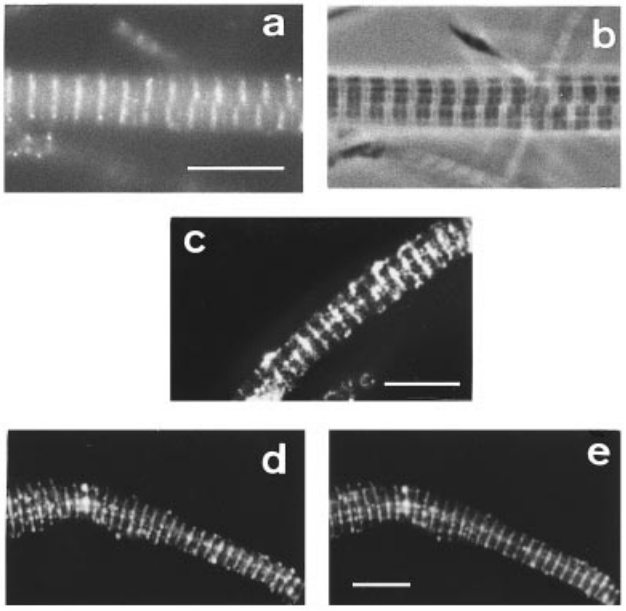

Figure 2 Localization of M-CK and β-enolase in myofibrils

(**a**) Texas Red labelling with anti-(M-CK) primary antibodies; (**b**) the corresponding phase-contrast observation. (**c**) FITC labelling with anti-(β-enolase) primary antiserum (confocal microscopy). (**d**, **e**) Double labelling with anti-(M-CK) and anti-(β-enolase) observed by confocal microscopy: anti-CK (**d**); anti-(β-enolase) (**e**). Scale bar, 10 μm.

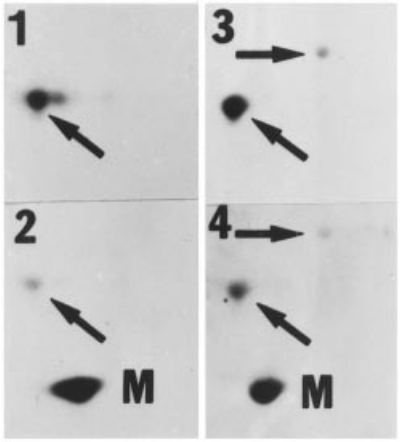

Figure 3 Two-dimensional gel electrophoresis and Western blot identification of enolase isoforms and CK in skinned fibres

Western blots were obtained after two-dimensional gel electrophoresis of 2 μg of rabbit psoas extract (panels 1 and 2) and of 20 mg of skinned fibre extract (panels 3 and 4). In panels 1 and 3, α-enolase and β-enolase subunits have been detected with the ECL detection system (see the Experimental section) after successive incubations of the blots with the corresponding antisera diluted respectively 1:5000 and 1:50000. In panels 2 and 4, the same blots (panels 1 and 3 respectively) were revealed for a third time, after a further incubation with anti-(M-CK) antibodies. Horizontal arrows indicate α-enolase; oblique arrows indicate β-enolase; M indicates M-CK.

from the skinned fibres by extraction at low ionic strength for 2 h in 5 mM Tris/HCl, pH 8.0 (Figure 1A, column 7). The high-ionic-strength medium yielded disorganized skinned fibres but no more CK activity was extracted (Figure 1A, column 8). The CK activity that remained bound to the myofibrils prepared from skinned fibres (Figure 1A, column 10) represented no more than 1% of the total activity measured in homogenates of the starting muscle preparation (column 9).

Figure 1(B) shows the enolase activities measured at the same stages of preparation of skinned fibres and myofibrils. Most of the enolase activity was released by extraction with medium A or medium A plus 50% (v/v) glycerol (Figure 1B, columns 1–6). In contrast with MM-CK, enolase activity was not extracted by 5 mM Tris/HCl (Figure 1B, column 7) but was by high-ionic-strength medium (column 8). Approx. 2–3% of the total enolase activity remained bound to the myofibrils (Figure 1B, columns 9 and 10).

Localization of M-CK and of β-enolase in myofibrils by indirect immunofluorescence labelling

For immunohistochemical observations, myofibrils obtained from skinned fibres were incubated with anti-(M-CK). They exhibited a striated fluorescent pattern (Figure 2a). Comparison with the phase-contrast image indicated that this strong fluorescence signal was located at the M-band of the sarcomere (Figures 2a and 2b). A weak signal was also visible at the Z-band. No signal was visible in controls from which the primary antibody had been omitted.

Similarly, the β-enolase antibody yielded a striated pattern (Figure 2c). Co-localization of M-CK and β-enolase at the M-band of the sarcomeres was observed by double labelling and confocal microscopy (Figures 2d and 2e). A weak labelling of myofibrils at the M-band was sometimes observed with an antiserum specific for α-enolase (results not shown).

Identification of enolase isoforms in skinned fibres by two-dimensional gel electrophoresis and Western Blotting

Two-dimensional gel electrophoresis allows the clear identification of the three mouse enolase subunits α, β and γ [25]. We present here the first attempt to use our antisera for the recognition of the α- and β-enolase subunits from rabbit. A preliminary analysis of rabbit heart extract, which is richer than other muscles in α-enolase [22,23], showed that the migrations of the α and β subunits were quite similar to those observed in mice (results not shown). Analysis of 2 μg of rabbit psoas extract (Figure 3, panel 1) allowed the identification of the β subunit but not the α subunit, which was visible only when 10-fold more extract was analysed (results not shown). The β-enolase subunit from rabbit presented the same heterogeneity pattern as previously described for the mouse [25]: two major subspecies differing slightly in their pI values but of the same molecular mass. When 20 mg of washed skinned fibres prepared from psoas were analysed, both the α and β subunits were clearly detectable after sequential incubation of Western blots with the anti-(β-enolase) and anti-(α-enolase) serum (Figure 3, panel 3). The α-enolase subunit of rabbit exibits one major spot and seems to be less heterogeneous than that of mouse [25]. In skinned fibres, compared with the soluble fraction, there is an enrichment in the α subunit relative to the β subunit. Indeed, in Figures 3 (panel 1) and 3 (panel 3), the α subunit was detected in skinned fibres (Figure 3, panel 3) but not in the soluble protein fraction (Figure 3, panel 1), although the two blots displayed the same signal intensity for the β subunit. To detect the M-CK subunit, the same blots were incubated with the corresponding specific antibodies (Figure 3, panels 2 and 4). Note that the α and β

subunits were still visible from the previous treatments of the blots.

Cross-linking of M-CK and β-enolase in glycerol-skinned fibres

The immunofluorescence and two-dimensional gel electrophoresis experiments described above clearly indicate that enolase is localized at the M-band of the skinned fibres. As the interactions of muscle enolase *in vitro* with MM-CK have been demonstrated recently [25], we searched for these interactions at the M-band with the use of bifunctional cross-linkers of various lengths. Homobifunctional cross-linkers are commonly used to analyse, *in vitro* and *in vivo*, protein–protein interactions or the protein neighbourhood in protein complexes or membranes [34]. A specially interesting approach consists of using cross-linkers of different lengths but with the same reactive functions. In the present work, experiments were performed with the succinimidyl esters DST, DSP and EGS, which are respectively 6, 12 and 16 Å long. The specificity of the cross-linking reaction is demonstrated by the formation of the same complexes with the two cross-linking reagents (EGS and DSP), showing that the cross-links of MM-CK with other polypeptides are indeed obtained by reaction with specific lysine residues (Figure 4).

The protein complexes were formed by treating the washed skinned fibres with bifunctional reagents. We then detected those reacting with antibodies specific for M-CK and β-enolase. Western blots obtained after one-dimensional gel electrophoresis in denaturing conditions and incubated with antibodies directed against M-CK (Figures 4A and 4C) show the presence of many polypeptide bands. The 43 kDa polypeptide band corresponds to the MM-CK monomer and the 86 kDa polypeptide band probably to the MM-CK dimer. Indeed, only these two peptides were observed when the shortest succinimidyl ester (DST, 6 Å long) was used (results not shown). Minor polypeptide bands representing the cross-linking of M-CK with unknown polypeptides were visible with DSP (Figure 4A); their intensities increased with EGS. Increasing concentrations of cross-linkers yielded increased intensities of labelling (Figures 4A and 4C, lanes 1–3). A weakly labelled 150 kDa polypeptide band was detected at the greatest DSP concentration (Figure 4A, lane 3) that was more intensely labelled in experiments with EGS (Figure 4C). Cross-linking of MM-CK with myomesin, protein M, myosin or titin could not be observed under our conditions of electrophoresis, which did not resolve higher-molecular-mass complexes.

The same blots revealed with the anti-(β-enolase) antiserum (Figures 4B and 4D) showed a polypeptide of approx. 50 kDa, which corresponds to the enolase monomer. This polypeptide was the only band detected with DST as cross-linker (results not shown). A polypeptide band of 150 kDa was clearly visible when using either DSP or EGS as cross-linker (Figures 4B and 4D). This band was the only one recognized by both anti-(M-CK) and anti-(β-enolase) antibodies. It appeared in the presence of high concentrations of the longest reagent and was less detectable when a smaller cross-linker was used. A few polypeptide bands with weak intensities were detected in the 100 kDa region, particularly after cross-linking with EGS (Figure 4D). The β-enolase dimer (100 kDa) was not a major polypeptide in these cross-linking experiments, although it appeared during cross-linking of purified enzyme with the same reagents (results not shown).

Binding of FITC-labelled MM-CK and ββ-enolase to skinned fibres

Glycerol-skinned fibres were washed with medium A, then for 2 h with 5 mM Tris/HCl, pH 8.0, to remove as much unbound

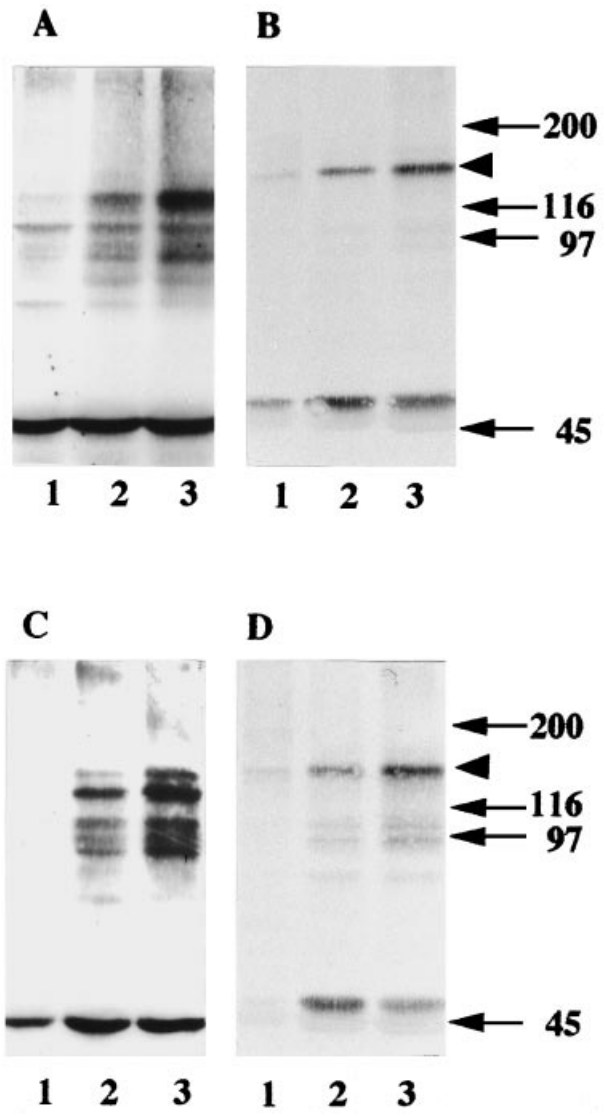

Figure 4 Chemical cross-linking between MM-CK and ββ-enolase in skinned fibres

Western blot analysis of treated extracts after one-dimensional gel electrophoresis. (**A**, **B**) Chemical cross-linking with DSP (length 12 Å). (**C**, **D**) cross-linking with EGS (length 16 Å); lanes 1, 2 and 3 correspond to 0.5, 1 and 2 mM reagent concentration respectively. (**A**, **C**) Incubation with anti-(MM-CK) antibodies; (**B**, **D**) incubation of the same membrane with anti-(β-enolase) serum, after washings with SDS and 2-mercaptoethanol. The arrowhead indicates the 150 kDa band reacting with both anti-(MM-CK) and anti-(β-enolase). The positions of molecular mass size markers migrating in these gels are indicated at the right (in kDa).

CK and enolase as possible. They were then incubated overnight with FITC-labelled MM-CK (0.36–3.6 mg/ml) and washed three to five times with medium A. After these treatments, the bound fluorescent proteins were observed at the M-band of the sarcomere (Figures 5a and 5b). This was confirmed by double immunolabelling of the M-band with antibodies directed against myomesin and protein M. Confocal microscopic observations showed a co-localization of FITC–CK and of M-band proteins (Figures 5c and 5d). A slight binding of fluorescent CK was also observed at the Z-band. Furthermore the binding of fluorescent CK was concentration-dependent. Incubation of the same batch of skinned fibres with FITC-labelled ββ-enolase (3.75 mg/ml)

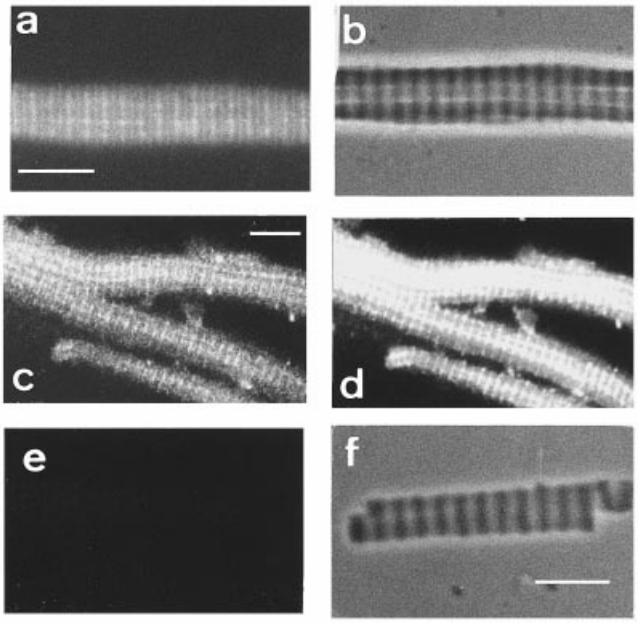

Figure 5 Binding of FITC-labelled MM-CK or β-enolase on skinned fibres after extraction by low-ionic-strength medium

(**a**, **b**) FITC-labelled CK: fluorescence (**a**); phase contrast (**b**). (**c**, **d**) Double labelling with the antibodies specific for M-band proteins (myomesin and protein M) revealed with Texas Red (**c**) and FITC-labelled CK (**d**). (**e**, **f**) FITC-enolase: fluorescence (**e**); phase contrast (**f**). Scale bar, 10 μm.

was performed under the same conditions: no significant binding was observed (Figures 5e and 5f).

DISCUSSION

The M-band in skeletal muscles is a very crowded and dense area of the sarcomere. The few identified protein components of this structure are myosin of the thick filaments, titin, the two structural proteins myomesin and protein M, and M-CK [1,4,7]. Phosphorylation of these M-band proteins has been described and might have an important role in maintaining the dynamic structure of the myofibrils [4,7,12]. The structural organization of the M-band could disturb the free diffusion of molecules so that a discrete localization of some enzymes implicated in ATP production might be required at this site. The present study was undertaken to investigate whether enzymes of energy metabolism other than CK, such as enolase, could be identified at this site.

M-CK bound to the M-band of rabbit psoas myofibrils is exchangeable

Our results show that the MM-CK activity that is firmly bound to the sarcomere of the rabbit psoas muscle, a fast twitch muscle, represents only 1% of the total MM-CK, an amount smaller than that previously reported by Wallimann [9] for chicken pectoralis muscle. This MM-CK, which remained bound to the sarcomere after washings with 0.1 M KCl, was located on the M-band; however, a weak decoration of the Z-band was also detected by anti-CK immunolabelling. The MM-CK bound to the M-band was removed by incubation at low ionic strength [1,30,31] and could be slowly replaced by external fluorescent CK. These results are in agreement with observations obtained by electron microscopy of beef heart and rabbit muscle [35] and by measuring rebound CK activity on rat heart [36]. The observation of such a slow exchange supports the hypothesis put forward by Arrio-Dupont et al. [27] to explain the presence of a very slightly mobile CK fraction in cultured muscle cells. Binding at the Z-band is also observed but its exact role at this site is not known.

The glycolytic enzyme enolase is firmly bound to the M-band and, in contrast with M-CK, is not exchangeable

We have shown here that 3% of the total enolase activity is firmly bound to the M-band. This is the first demonstration of the presence of a glycolytic enzyme in this sarcomeric structure. Two-dimensional gel electrophoresis followed by Western blot analysis demonstrates that both the muscle-specific β subunit and the ubiquitous α subunit are found in the skinned fibres. Results of the immunocytochemical analysis clearly show a co-localization of β-enolase and M-CK at the M-band of the sarcomere. In contrast with M-CK, enolase is still present in the sarcomeres after incubation with 0.5 mM Tris/HCl and is removed only with the high-ionic-strength treatment that results in the disorganization of sarcomeric structure. The difference in extractability properties of the two proteins might reflect differences in their interactions with other structural components. Our results suggest that enolase is not exchangeable and could explain the lack of fixation of FITC-labelled $\beta\beta$-enolase on skinned fibres: because the binding sites of $\beta\beta$-enolase on the M-band were still occupied, no binding of the newly added molecules was possible.

Enolase and M-CK are not in direct interaction at the M-band

Among the protein complexes formed by treating the skinned fibres with bifunctional reagents, we detected those reacting with the antibodies specific for M-CK and β-enolase. The immunoblots revealed with anti-(M-CK) exhibited several polypeptide bands. It could be postulated that Lys-195 of the CK active site, which is especially reactive [32], is first acylated by one reactive group of the reagent. The second group could modify lysine residues of MM-CK itself (intra-subunit or inter-subunit reaction) or modify the ϵ-amino groups of neighbouring proteins at a maximal distance of 16 Å with DSP or 20 Å with EGS (taking into account the lysine side chain).

The results obtained with the anti-(β-enolase) serum indicate that the $\beta\beta$-enolase dimer is not a major polypeptide in these cross-linking experiments. The major polypeptide detected with the anti-(β-enolase) serum corresponds to a molecular mass of 150 kDa. These results suggest that the neighbouring proteins of the $\beta\beta$-enolase dimer could have reactive lysine residues and yield the major 150 kDa polypeptide directly. This polypeptide band is the only one that also reacted with the anti-CK antibody and it seemed to be more abundant when the longest reagent was used. This result seems to agree with a minimal distance of the order of 16 Å between M-CK and β-enolase in the M-band structure. This value is large compared with the dimensions of MM-CK (92 Å $\times$ 42 Å $\times$ 42 Å) [10,37] and of $\beta\beta$-enolase (a sphere of 34 Å radius [38]) dimers, which does not support the notion of a direct interaction between the two enzymes at the M-band [34]. This 150 kDa polypeptide is larger than the sum of an M-CK monomer with a β-enolase monomer, a result that favours the presence of another protein. It could be accounted for by an MM-CK dimer cross-linked with one β-enolase subunit or one M-CK monomer bound to an enolase dimer. As both α-enolase and β-enolase subunits are clearly present in the skinned myofibres, it could be the homodimer $\beta\beta$ and/or the heterodimer $\alpha\beta$. This polypeptide could be also a complex of M-CK and enolase

monomers with another polypeptide of molecular mass 50 kDa. Furthermore we cannot exclude the possibility that more than one polypeptide of the same 150 kDa apparent size migrate within this electrophoretic band. One of them could be formed from a $\beta\beta$-enolase dimer together with another unknown polypeptide; another could be constituted of an MM-CK dimer and another polypeptide. None of these hypotheses favours a direct association between M-CK and β-enolase.

Metabolic enzymes at the M-band: physiological significance

The MM-CK bound to the M band of the myofibrils is still active ([1], and the present study). Therefore, in addition to its structural role in the organization of the M band, this enzyme could have a role in the regeneration of ATP at a site located far from the actomyosin complex [39,40]. The phosphorylation of components of the M-band, e.g. protein M, myomesin and titin, could have an important role in sarcomere formation and/or maintenance [4,7,12]. The localization of ATP-regenerating MM-CK in the M4/M4′ lines near the phosphorylation site of titin [4] is therefore of physiological significance. The presence of other ATP-generating enzymes such as glycolytic enzymes in the M band could supply the energetic requirements of this particular structure of the sarcomere. For the first time we demonstrate the presence of enolase at the M-band of myofibres. This glycolytic enzyme alone cannot produce ATP. However, the existence of an interaction *in vivo* between $\beta\beta$-enolase and pyruvate kinase has been reported [20], and interactions of phosphoglyceromutase and pyruvate kinase with $\beta\beta$-enolase *in vitro* have been described [25]. Moreover a physical coupling of pyruvate kinase with MM-CK was described by Dillon et al. [41]. Taken together, these observations raise the intriguing possibility that a full ATP-producing segment containing these three glycolytic enzymes and MM-CK might be operating at the M-band of myofibres.

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REFERENCES

1 Wallimann, T. and Eppenberger, H. M. (1985) in Cell and Muscle Motility, vol. 6 (Shay, J. W., ed.), pp. 239–285, Plenum Press, New York
2 Sjöstrom, M. and Squire, J. M. (1977) J. Mol. Biol. **109**, 49–68
3 Thornell, L. E. and Carlsson, E. (1984) in Developmental Process in Normal and Diseased Muscle, vol. 9 (Eppenberger, H. M. and Perriard, J. C., eds.), pp. 141–147, Karger, Basel
4 Obermann, W. M. J., Gautel, M., Steiner, F., Vanderven, P. F. M., Weber, K. and Furst, D. O. (1996) J. Cell Biol. **134**, 1441–1453
5 Vanderven, P. F. M. and Furst, D. O. (1997) Cell Struct. Funct. **22**, 163–171
6 Labeit, S. and Kolmerer, B. (1995) Science **270**, 293–296
7 Obermann, W. M. J., Gautel, M., Weber, K. and Furst, D. O. (1997) EMBO J. **16**, 211–220
8 Trinick, J. (1994) Trends Biochem. Sci. **19**, 405–409
9 Wallimann, T., Turner, D. C. and Eppenberger, H. M. (1977) J. Cell Biol. **75**, 297–317
10 Stolz, M. and Wallimann, T. (1998) J. Cell Sci. **111**, 1207–1216
11 Wallimann, T., Wyss, M., Brdiczka, D., Nicolay, K. and Eppenberger, H. M. (1992) Biochem J. **281**, 21–40
12 Obermann, W. M. J., Vanderven, P. F. M., Steiner, F., Weber, K. and Furst, D. O. (1998) Mol. Biol. Cell. **9**, 829–840
13 Welch, G. R. (1977) Prog. Biophys. Mol. Biol. **32**, 103–191
14 Srere, P. A. and Ovadi, J. (1990) FEBS Lett. **268**, 360–364
15 Brooks, S. P. J. and Storey, K. B. (1991) FEBS Lett. **278**, 135–138
16 Knull, H. R. and Walsh, J. L. (1992) Curr. Top. Cell. Regul. **33**, 15–30
17 Dölken, G., Leisner, E. and Pette, D. (1975) Histochemistry **43**, 113–121
18 Clarke, F. M. and Masters, C. J. (1975) Biochim. Biophys. Acta **381**, 37–46
19 Méjean, C., Pons, F., Benyamin, Y. and Roustan, C. (1989) Biochem. J. **264**, 671–677
20 Maughan, D. and Wegner, E. (1989) Muscle Energetics **315**, 137–147
21 Rider, C. C. and Taylor, C. B. (1974) Biochim. Biophys. Acta **365**, 285–300
22 Kato, K., Shimizu, A., Semba, R. and Satoh, T. (1985) Biochim. Biophys. Acta **481**, 50–58
23 Keller, A., Rouzeau, J. D., Farhadian, F., Wisnewsky, C., Marotte, F., Lamandé, N., Samuel, J. L., Schwartz, K., Lazar, M. and Lucas, M. (1995) Am. J. Physiol. **38**, H1843–H1851
24 Lucas, M., Goblet, C., Keller, A., Lamandé, N., Gros, F., Whalen, R. G. and Lazar, M. (1992) Differentiation **51**, 1–7
25 Merkulova, T., Lucas, M., Jabet, C., Lamandé, N., Rouzeau, J. D., Gros, F., Lazar, M. and Keller, A. (1997) Biochem J. **323**, 791–800
26 Kuby, S. A., Noda, L. and Lardy, H. A. (1954) J. Biol. Chem. **209**, 191–201
27 Arrio-Dupont, M., Foucault, G., Vacher, M., Douhou, A. and Cribier, S. (1997) Biophys J. **73**, 2667–2673
28 Keller, A., Ott, M. O., Lamandé, N., Lucas, M., Gros, F., Buckingham, M. and Lazar, M. (1992) Mech. Dev. **38**, 41–54
29 Keller, A., Scarna, H., Mermet, T. and Pujol, J. F. (1981) J. Neurochem. **36**, 1389–1397
30 Grove, B. K., Kurer, V., Lehner, C., Doetschman, T. C., Perriard, J. C. and Eppenberger, H. M. (1984) J. Cell Biol. **98**, 518–524
31 Kundrat, E. and Pepe, F. A. (1971) J. Cell Biol. **48**, 340–347
32 Kenyon, G. L. and Reed, G. H. (1983) Adv. Enzymol. **54**, 367–426
33 Laemmli, U.K. (1970) Nature (London) **227**, 680–685
34 Bäumert, H. G. and Fasold, H. (1989) Methods Enzymol. **172**, 584–609
35 Herasymowych, O. S., Mani, R. S., Kay, C. M., Bradley, R. D. and Scraba, D. G. (1980) J. Mol. Biol. **136**, 193–198
36 Ventura-Clapier, R., Saks, V. A., Vassort, G., Lauer, C. and Elizarova, G. V. (1987) Am. J. Physiol. **253**, C444–C455
37 Fritzwolf, K., Schnyder, T., Wallimann, T. and Kabsch, W. (1996) Nature (London) **381**, 341–345
38 Duquerroy, S., Camus, C. and Janin, J. (1995) Biochemistry **34**, 12513–12523
39 Arrio-Dupont, M. (1988) FEBS Lett. **240**, 181–185
40 Wallimann, T. (1994) Curr Biol. **4**, 42–46
41 Dillon, P. F., Weberling, M. K., Letarte, S. M., Clark, J. F., Sears, P. R. and Root-Bernstein, R. S. (1995) J. Biol. Phys. **21**, 11–23

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