Interactions between β -enolase and creatine kinase in the cytosol of skeletal muscle cells

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We studied interactions *in vivo* between the cytosolic muscle isoform of creatine kinase (M-CK) and the muscle isoform of 2-phospho-D-glycerate hydrolyase (β -enolase) in muscle sarcoplasm by incubating glycerol-skinned fibres with FITC-labelled β -enolase in the presence or absence of free CK. A small amount of bound β -enolase was observed in the presence of large concentrations of CK. The mobility of enolase was measured in cultured satellite cells by modulated-fringe-pattern photo-

bleaching. FITC-labelled β -enolase was totally mobile in both the presence and the absence of CK but its diffusion coefficient was slightly lower in the presence of CK. This suggests a weak interaction *in vivo* between enolase and CK.

Key words: diffusion, fluorescence recovery, photobleaching, skinned muscle fibres.

INTRODUCTION

Cytosolic enzymes that supply energy for movement and transport are not distributed uniformly throughout the cytoplasm but are located in particular areas of the cell. This distribution is especially important for muscle cells, which require intermittent high-energy outputs. Some glycolytic enzymes are restricted in the thin-filament region of skeletal muscle [1]. Interactions of phosphoglucose isomerase, phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase and aldolase with thin filaments have been shown by using a variety of techniques [2–4]. The association of glycolytic enzymes into multiprotein complexes has been described in various cell types. Furthermore, numerous studies have shown the importance of reversible pH-dependent associations in controlling the efficiency of glycolysis [5–9].

2-Phospho-D-glycerate hydrolyase (EC 4.2.1.11; enolase) is a glycolytic enzyme of particular interest because it has muscle-specific isoforms [10,11]. In adults, subunits of the muscle isoform of enolase (β -enolase) accumulate preferentially in fast-twitch muscle fibres in which the β -isoenzyme accounts for more than 90% of total enolase. The binding of β -enolase to several glycolytic enzymes has been described [12]. The direct interaction of glycolytic enzymes with other processes regenerating ATP suggests the presence in sarcomeres of protein complexes involved in ATP production. This highlights the exceptional energy requirements of muscle. A high-affinity interaction *in vitro* between the cytosolic muscle isoform of creatine kinase (M-CK; EC 2.7.3.2) and enolase has been reported by Merkulova et al. [12]. CK has a key role in the energy metabolism of cells with intermittent high-energy requirements, such as skeletal muscle fibres. M-CK is located in the cytosol, where a small fraction of the enzyme binds specifically to the M-band of the sarcomere [13,14]. A small proportion of enolase is also tightly bound to the M-band of muscle [15]. Unlike CK, this bound enolase is not

exchangeable and there is no direct interaction between M-CK and enolase on the M-band; nevertheless, an indirect interaction is possible [15].

It is therefore of interest to look for interactions *in vivo* between β -enolase and M-CK in the cytosol of muscle cells. The mobility of FITC-labelled CK and FITC-labelled β -enolase was measured in cultured myotubes by using fringe-pattern photobleaching. Previous studies [16] have shown that β -enolase is totally mobile. In contrast, injected M-CK distributes into two populations, one (representing 60–80% of molecules) being mobile and one (20–40% of molecules) having slight mobility. Thus the formation of a complex between FITC-labelled β -enolase and unlabelled CK might be expected to give an immobile fraction of β -enolase and/or a decrease in its intracellular diffusion constant. Endogenous levels of M-CK were very low in our cultured myotubes; the possible formation of complexes was therefore tested by the co-injection of mixtures of FITC-labelled β -enolase and unlabelled CK into cultured muscle cells and by the incubation of these mixtures with skinned muscle fibres.

MATERIALS AND METHODS

Enzymes and proteins

β -Enolase dimeric form, M_r 90 000, 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 [17]. M-CK dimeric form, M_r 86 000, was prepared in the laboratory from rabbit skeletal muscles [18]. The protein was further purified by chromatography on Biogel P100. The proteins were then labelled with FITC as described previously [16]. For both enzymes, the dye-to-protein labelling ratio was approx. 1 dye molecule per protein subunit. FITC-labelled IgG (anti-mouse IgG produced in goat) was purchased from Sigma.

Abbreviations used: CK, creatine kinase (EC 2.7.3.2); M-CK, cytosolic muscle isoform of creatine kinase; enolase, 2-phospho-D-glycerate hydrolyase (EC 4.2.1.11); β -enolase, muscle isoform of enolase; FRAP, fluorescence recovery after photobleaching.

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Preparation of skinned fibres and myofibrils

Skinned fibres were prepared from rabbit psoas muscles as described previously [15]. Skinned fibres were kept for up to several weeks at -20°C in medium A (0.1 M KCl/1 mM EGTA/5 mM EDTA/1 mM NaN_3) plus 0.1 mM PMSF, 10 μM leupeptin and 10 μM pepstatin in 50% (v/v) glycerol. Before use, the skinned fibres were washed again with medium A. Myofibrils were prepared from glycerol-treated fibres [19]: the fibres were washed five times with medium A and 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.

Incubation of skinned fibre bundles with labelled β -enolase in the presence or absence of CK

Skinned fibres were washed five times with medium A. Fibres were then incubated for 2 h at 4°C in 5 mM Tris/HCl buffer, pH 8.0, to wash out CK firmly bound to the M-line of the sarcomere. Fibres were resuspended in medium A supplemented with 10 mM phosphate buffer, pH 7.5, and incubated at 4°C overnight with various concentrations of FITC-labelled CK (0.36, 1.36 or 3.60 mg/ml), with FITC-labelled β -enolase (1.44 mg/ml) or with an equimolar mixture of 1.36 mg/ml unlabelled CK and 1.44 mg/ml FITC-labelled β -enolase (16 μM). The fibres were then washed five times with medium A. Myofibrils were prepared as described above and mounted in Citifluor for fluorescence microscopy.

Muscle cell culture

Rabbit satellite cells were cultured as described previously [20] from semimembranosus proprius, a slow-twitch muscle [21]. Satellite cells were isolated by digestion of muscle fibres with pronase [20,21]. The isolated cells were grown in dishes 6 cm in diameter, the bases of which had been replaced with sealed glass coverslips to allow microscopic observation. Cells were grown to confluence in Dulbecco's modified Eagle's medium containing 20% (v/v) foetal calf serum, 100 i.u./ml penicillin and 1 mg/ml streptomycin. The medium was replaced with ITS medium [Dulbecco's modified Eagle's medium containing antibiotics, 2% (v/v) foetal calf serum, 5 $\mu\text{g}/\text{ml}$ insulin, 5 $\mu\text{mol}/\text{ml}$ transferrin and 5 nmol/ml sodium selenite]. In ITS medium the cells fused and differentiated into myotubes. For microinjections and experiments with fluorescence recovery after photobleaching (FRAP), an ITS medium devoid of Phenol Red and buffered with 20 mM Hepes was used. As described previously [20], these cultured myotubes had characteristics similar to those of a young muscle. They were 10–40 μm wide and up to 1 mm in length.

Diffusion measurements

Modulated-fringe-pattern photobleaching is a technique based on spatial and temporal modulation to provide direct recordings of contrast modulation data [22]. The experiments were performed with an apparatus that has been described elsewhere [23]. Modulated-fringe-pattern photobleaching produces a bleaching pattern of interference fringes. The fringes were oriented perpendicular to the muscle fibres. The modulated fringes allowed the direct recording of the decay of fluorescence contrast between bleached and unbleached regions. The decay of fluorescence contrast was treated by Padé–Laplace formalism, allowing immediate multiexponential analysis. Translational diffusion coefficients (D) were deduced from the relation $D = i^2/4\pi^2\tau$, where τ is the time constant of the exponential decay and i the

interfringe spacing. The experiments were performed at 20°C in a temperature-regulated room.

For globular proteins, diffusion constants are proportional to $M_r^{1/3}$ [24]. Protein interactions are reflected in changes in these diffusion constants. A strong complex formed by the interaction between CK (M_r 86000) and β -enolase (M_r 90000) might have a diffusion constant close to that of an IgG (M_r 160000), assuming that the proteins are globular. For weaker interactions, diffusion constants between that of the complex and those of individual proteins might be recorded if the equilibrium is rapid, whereas two constants respectively equal to that of the individual proteins and to that of the complex might be observed in the case of a slow equilibrium.

Labelled proteins were introduced into myotubes by pressure injection. Sterile Femtotips (Eppendorf) were filled with 2 μl of protein solution in 48 mM K_2HPO_4 /14 mM NaH_2PO_4 /4.5 mM KH_2PO_4 (pH 7.2) plus 1 mM MgSO_4 . The total volume injected was less than 10% of the cell volume. The myotubes were allowed to equilibrate for several hours before starting the FRAP experiments. Two mixtures of FITC-labelled enolase and free CK were prepared, containing respectively 9.8 mg/ml labelled enolase and 5 mg/ml CK, or 3.5 mg/ml labelled enolase and 10 mg/ml CK. These mixtures were incubated for 2 h before injection. Two or three Petri dishes were injected with each mixture or with FITC-labelled β -enolase alone (5–20 mg/ml) and two different batches of cells were studied on day 15 or day 34 of differentiation. A control experiment was also performed with FITC-labelled IgG.

Affinity chromatography

M-CK (120 mg in 10 mM Mes buffer, pH 6.5) was coupled to 10 ml of Affigel 15[®] from Bio-Rad. The support was washed, after which 100 mg of the initial protein remained bound to the gel. The modified gel was poured into two 10 ml syringes that were used as chromatography columns and equilibrated with PBS, pH 7.0, with or without 4 mM magnesium acetate. β -Enolase was dialysed against PBS with or without 4 mM magnesium acetate; 0.5 ml of 3.2 mg/ml β -enolase was then applied to the column and incubated for 2 h at room temperature. β -Enolase was eluted with PBS, pH 7.0, with or without Mg^{2+} , followed by 0.6 M NaCl in PBS. The elution of β -enolase was followed by measuring A_{280} and its enzymic activity. A control experiment was performed under the same conditions, with 1.5 mg of M-CK instead of β -enolase.

RESULTS

Binding of labelled CK or β -enolase to skinned fibres

The skinned fibres were incubated with various concentrations of either labelled CK or labelled β -enolase or an equimolar mixture of native CK plus labelled β -enolase. Myofibrils were then prepared and mounted for fluorescence microscopy (Figure 1).

After incubation with the two higher concentrations (1.36 or 3.6 mg/ml) of FITC-labelled CK, the fluorescent protein was observed both on the M-line and near the Z-line (Figures 1a and 1b). Low levels of binding were observed on the M-line after incubation with a very low concentration (0.36 mg/ml) of FITC-labelled CK.

After five washes with medium A, no binding of FITC-labelled β -enolase was observed (Figures 1c and 1d). After incubation of the fibres with FITC-labelled β -enolase in the presence of unlabelled CK, at a concentration at which binding has been observed *in vitro* [12], little or no binding of FITC-labelled β -enolase to the bound CK was observed (Figures 1e and 1f).

Figure 1 Binding of FITC-labelled CK and FITC-labelled enolase, with or without saturating levels of native CK on skinned fibres

The following conditions were used: (a, b) 1.36 mg/ml FITC-labelled CK; (c, d) 1.44 mg/ml FITC-labelled enolase; (e, f) 1.44 mg/ml FITC-labelled enolase plus 1.36 mg/ml unlabelled CK. Photography was by phase contrast (a, c, e) or by fluorescence (b, d, f). Note that the exposure times in (d) and (f) were longer than that in (b), to improve detection of the weak binding. Scale bar, 10 μ m.

Mobility of β -enolase in muscle cells in the presence of CK

The diffusion constant of FITC-labelled β -enolase in the muscle cells was assessed by modulated-fringe-pattern photobleaching. As indicated in Table 1, β -enolase was completely mobile, regardless of the amount of CK, showing that there was no interaction with immobile CK. However, there was evidence for a slight slowing of enolase diffusion in the presence of an excess of CK. An IgG of M_r 160 000 had a diffusion coefficient of $5.5 \mu\text{m}^2 \cdot \text{s}^{-1}$ in the cytoplasm of cultured muscle cells. This M_r corresponds to a complex between β -enolase and CK, suggesting that there is not a strong complex between these proteins in the cytosol of muscle cells.

Affinity chromatography

Thus mobility determinations and binding assays with fluorescently labelled enolase and skinned fibres in the presence of CK provided no evidence of a strong interaction between these enzymes *in vivo*. We checked whether the apparent high affinity previously observed by ELISA was overestimated owing to surface-bound ligand, by using another method to determine

the affinity of β -enolase for M-CK. An affinity column with covalently bound CK was prepared and β -enolase was applied to the column. Too little β -enolase was retained on the CK column to be detected (Figure 2). The presence of magnesium acetate in the medium had no effect. Elution with 0.6 M NaCl in PBS did not result in the detection of retained β -enolase. CK, used as a control, was eluted with the same volume of buffer as enolase and peaked in fractions 2 and 3, corresponding to non-retained material. These results are consistent with a very low level of binding of enolase to the CK column. The retarded material might have been a contaminant of the enolase preparation with a M_r of 45 000, as observed by SDS/PAGE (results not shown).

DISCUSSION

The existence of specific complexes between glycolytic enzymes and CK near the subcellular sites at which large amounts of ATP are consumed for muscle contraction seemed a very plausible suggestion. The existence of complexes between CK and pyruvate kinase *in vitro* [25] and interactions *in vitro* between enolase and

Table 1 Mobility of β -enolase in the cytoplasm of cultured muscle cells in the presence of CK

The mobility of FITC-labelled β -enolase in muscle cells was studied by modulated-fringe-pattern photobleaching in the presence or absence of unlabelled CK (see the Materials and methods section). The values reported are means $\pm$ S.D. for three different cell cultures, two or three dishes per culture and five to ten myotubes per dish. Under the conditions shown in rows 2 and 3, we have observed previously that 20–50% of the injected CK was immobile [16]. An IgG of M_r 160 000 has a diffusion coefficient (D) of $5.5 \mu\text{m}^2 \cdot \text{s}^{-1}$ in the cytoplasm of these cultured muscle cells (M. Arrio-Dupont, G. Foucault, M. Vacher, P. F. Devaux and S. Cribier, unpublished work).

Protein	Mobile fraction (%)	$D_{\text{cytoplasm}}$ ($\mu\text{m}^2 \cdot \text{s}^{-1}$)
β -Enolase alone (3.5–20 mg/ml)	100	10.8 ± 3.5
β -Enolase (9.8 mg/ml) + CK (5 mg/ml)	100	10.3 ± 2.1
β -Enolase (3.5 mg/ml) + CK (10 mg/ml)	100	9.9 ± 3.2

Figure 2 Affinity chromatography for enolase on a CK-Affigel column

The fraction volume was 1 ml. Fractions 1–16 were recovered by elution with PBS; fractions 16–25 were obtained by elution with 0.6 M NaCl in PBS.

various glycolytic enzymes or CK as observed by ELISA [12] were also consistent with this possibility.

Previous experiments had shown that FITC-labelled enolase injected into cultured muscle cells was totally mobile in the cytoplasm. Nevertheless, the amount of endogenous M-CK in these cultured cells was still very small after 15 days of differentiation [16], unlike the situation in adult skeletal muscle [13,15]. Thus it was possible that a complex would form between β -enolase and M-CK if both enzymes were present in cells. We performed two series of experiments to test this possibility.

First, chemically skinned fibres were incubated with FITC-labelled enolase in the presence or absence of native M-CK. These experiments enabled us to investigate strong interactions between proteins and myofilaments. We have previously observed that M-CK binds to the M-line of skinned fibres, whereas enolase alone does not [15]. Here we found that, even in the presence of CK, β -enolase did not bind significantly to myofilaments. These experiments show that enolase cannot interact directly with M-CK of the M-line even if the amount of bound CK is increased by exogenous M-CK. These observations are consistent with previous experiments with cross-linking reagents, which showed an absence of direct interactions between these enzymes on the M-line [15].

However, these observations do not preclude the existence of a complex between these enzymes in the cytosol of muscle cells. The second series of experiments involved searching for such complexes. Two observations were expected after the injection of FITC-labelled enolase in the presence of native M-CK: a fraction of immobile enolase indicating a binding to immobile CK, and/or a decrease in the diffusion constant of enolase in the presence of CK indicating the presence of a soluble complex between the proteins (expected M_r 180 000). In our cultured muscle cells, the diffusion constant of an IgG (M_r 160 000) is approximately one-half that of enolase. No immobile fraction of FITC-labelled enolase was detected. This observation is consistent with the absence of binding of FITC-labelled enolase to skinned fibres if no CK is added and a very minor binding in the presence of added CK.

The slight decrease in the diffusion coefficient of β -enolase in the presence of an excess of CK might indicate that no more than 20% of enolase is complexed with CK in the cytosol of the cultured cells. Previous ELISA experiments [12] have shown that β -enolase binds to the adjacent enzymes in the glycolytic pathway (pyruvate kinase and phosphoglycerate mutase) and M-CK with high affinity. A dissociation constant of 5–7 nM has been reported for the complex between enolase and CK [12]. Assuming a dilution of between 1:10 and 1:50 of the protein solutions after injection into myotubes, and with initial concentrations of 30 μM enolase and 100 μM CK (Table 1, row 3), the intracellular concentrations would be respectively 0.6–3 μM and 2–10 μM ; in these conditions, assuming a K_d of 5–7 nM, enolase would be entirely bound to CK. Presumably, ELISA, as used previously, is not suitable for the quantitative evaluation of affinity constants [26,27] and the K_d values were greatly overestimated. A more physiologically appropriate value for K_d would be 1–10 μM , as has been obtained by using other approaches for the interactions between pyruvate kinase or phosphoglycerate mutase and enolase [4,28] or between pyruvate kinase and CK [25]. This value is consistent with our findings by FRAP and affinity chromatography. Whatever the real value of K_d , the previous observations indicated an affinity of enolase for the adjacent enzymes in the glycolytic pathway and for M-CK. From a functional point of view, the weak binding of enolase to CK and to the adjacent enzymes in the glycolytic pathway is of greater interest than strong binding. Several models and experimental studies have demonstrated the importance of associations of the components of a metabolic pathway for channelling the intermediates [5,6,8,9]. Strong interactions would therefore be too rigid and would be impossible to regulate. In contrast, changing enzyme associations by changing intracellular pH or metabolite concentrations facilitates the regulation of metabolic pathways.

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