Mobility of Creatine Phosphokinase and β-Enolase in Cultured Muscle Cells

Martine Arrio-Dupont,* Georges Foucault,* Monique Vacher,* Aïcha Douhou,* and Sophie Cribier#
*Laboratoire des Gènes et Protéines Musculaires, ERS CNRS 570, F91405 Orsay, and #Laboratoire de Physico-Chimie Moléculaire des Membranes Biologiques, UPR CNRS 9052 et Université Paris 6, Institut de Biologie Physico-Chimique, F75005 Paris

ABSTRACT The diffusion of β-enolase and creatine phosphokinase in muscle cells has been studied by modulated fringe pattern photobleaching. β-Enolase is mobile in the sarcoplasm. At 20°C, the diffusion coefficient is 13.5 ± 2.5 $\mu m^2 s^{-1}$ in the cytosol and 56 $\mu m^2 s^{-1}$ in aqueous media. As in the case of dextrans of the same hydrodynamic radius, its mobility is hindered by both the crowding of the fluid phase of the cytoplasm and the screening effect due to myofilaments. A fraction of creatine phosphokinase is mobile in the sarcoplasm. Its diffusion coefficient in the cytosol, 4.5 ± 1 $\mu m^2 s^{-1}$, is lower than that of the dextran of equivalent size. The other fraction (20 to 50%) is very slightly mobile, with an apparent diffusion coefficient varying from 0.0035 to 0.043 $\mu m^2 s^{-1}$. This low mobility might be attributed to exchange between free and bound creatine phosphokinase. The bound fraction of the endogenous enzyme was localized by immunocytofluorescence on the cultured muscle cells. Our results favor a localization of bound cytosolic creatine phosphokinase on the M-line and a diffuse distribution in all myotubes.

INTRODUCTION

The movement of molecules through the cytoplasm of cells has been the topic of many investigations. It is quite different from diffusion in solution and depends on the structure and viscosity of the cellular components (reviewed by Luby-Phelps, 1994). Striated muscle is one of the most highly ordered of all biological tissues (reviewed by Pollack, 1990). Besides the contractile elements, thick filaments of myosin and thin filaments of actin and associated proteins, whose spacing has been accurately described by electronic microscopy and x-ray diffraction (Sosa et al., 1994), a cytoskeletal lattice maintains the structure of muscle cells. The proteins of the M-line (Obermann et al., 1996) and Z-line serve as structural integrators of the myofilaments and the longitudinal lattice components. Two giant proteins, titin and nebulin, are molecular rulers specifying the length of the contractile filaments (reviewed by Trinick, 1994) and the elastic filament system in skeletal muscle (Labeit and Kolmerer, 1995). Furthermore, a muscle membrane cytoskeleton has been described (reviewed by Small et al., 1992). We recently showed (Arrio-Dupont et al., 1996) that the mobility of inert macromolecules in muscle cells is hindered by both the crowding of the fluid phase of the cytoplasm and the screening effect due to myofilaments.

There is much evidence that the cytosolic enzymes, which supply energy for movement and transport, are not distributed uniformly over the whole cytoplasm, but are located in distinct areas of the cell. Several groups have presented evidence for a close coupling between localization and function of enzymes (Welch, 1977; Srere and Ovadi, 1990). Most but not all of the glycolytic enzymes are compartmentalized in the thin filament region of skeletal muscle (Dölken et al., 1975; Brooks and Storey, 1991). With a variety of techniques, interactions have been observed for phosphoglucose isomerase, phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, aldolase, pyruvate kinase, and F-actin (reviewed by Maughan and Lord, 1988; Maughan and Wegner, 1989; Knull and Walsh, 1992).

Creatine phosphokinase (CK) plays a key role in the energy metabolism of cells that have intermittent high-energy requirements, such as skeletal muscle fibers. This enzyme catalyzes the reversible transfer of the phosphoryl group from phosphocreatine to ADP and allows the regeneration of the cellular energy-carrying molecule, ATP (Kenyon and Reed, 1983). Biochemical fractionation and in situ immunolocalization techniques have shown that the different CK isoforms are not distributed evenly in muscle fibers (Otsu et al., 1989; Wegmann et al., 1992; Eppenberger, 1994). The isoform Mi_b-CK is specifically mitochondrial and is bound to the external face of the internal membrane. The three-dimensional structure of the chicken mitochondrial isoform has recently been resolved (Fritzwolf et al., 1996). The MM-CK is cytosolic; a small fraction of this enzyme is bound specifically to the M-line structure of the sarcomeres (Wallimann and Eppenberger, 1985), and another fraction seems to be loosely bound to other sites in the sarcomeres. The exact localization of this loosely bound MM-CK is controversial, as Otsu et al. (1989, 1993) observed a localization in the whole A-band, whereas Wegmann et al. (1992) observed this isoform both localized at the M-line and highly confined to the I-band.

In the so-called creatine shuttle or phosphocreatine circuit (Bessman and Carpenter, 1985; Wallimann et al., 1992), the energy necessary for contraction is transported via the phos-

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Address reprint requests to Dr. Martine Arrio-Dupont, Laboratoire Gènes et Protéines Musculaires, ERS CNRS 570, bâtiment 433, Université Paris-Sud, Centre d'Orsay, F91405 ORSAY. Tel.: 33-1-69157027; Fax: 33-1-69853715; E-mail: martine.arrio@bpc.u-psud.fr.

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phorylated form of creatine from the sites of energy production, mitochondria, to the sites of utilization, myosin ATPases. In this model it may be useful for the contraction to have a cytosolic MM-CK located near the active site of myosin. Nevertheless, Arrio-Dupont et al. (1992) have demonstrated with a model system that direct interaction between CK and myosin was not necessary to observe a functional coupling between the enzymes. Binding of CK and myosin on the same surface produces a channeling of the intermediate ADP in the unstirred layer near the active surface; the thick filaments may represent such an active surface (Arrio-Dupont, 1988). Therefore it was very important to know the mobility of MM-CK in the cytosol of muscle fibers.

To study the mobility of CK in muscle cells, we have injected fluorescently labeled CK into cultured myotubes. The diffusion was observed by a modulated fringe pattern photobleaching technique that has previously been used to study the diffusion of inert tracers in the cytoplasm of myotubes (Arrio-Dupont et al., 1996). β-Enolase, a muscle glycolytic enzyme whose size is near that of CK (Rider and Taylor, 1974) and for which the binding to intracellular structures is low (Baranowski and Wolna, 1975; Pagliaro et al., 1989), was chosen as a reference protein, although a recent report indicates some binding to muscle structures (Merkulova et al., 1997).

MATERIAL AND METHODS

Muscle cell cultures

Rabbit satellite cells were cultured from a slow muscle, *semimembranosus proprius*, as previously described (Arrio-Dupont et al., 1996). The satellite cells were grown to confluence in Dulbecco's minimum essential culture medium containing 20% fetal calf serum, 100 U/ml penicillin, and 1 mg/ml streptomycin, and then the medium was changed to Dulbecco's minimum essential medium containing antibiotics, 2% fetal calf serum, 5 μg/ml insulin, 5 μmol/ml transferrin, and 5 nmol/ml sodium selenite (ITS medium). In the ITS medium cells fused and differentiated to myotubes. For indirect immunofluorescence assays, muscle cells were cultured on microscopic coverslips. To perform fluorescence recovery after photobleaching (FRAP) experiments, cells were grown in Ø 6-cm dishes, the bases of which were replaced with sealed glass coverslips, allowing microscopic observations. For microinjections and FRAP experiments, an ITS medium devoid of phenol red and buffered with 20 mM HEPES was used.

Most of the FRAP experiments were performed on myotubes after 9–17 days of differentiation. These cultured myotubes were similar to young muscle. They were 10–40 μm wide and up to 1 mm in length.

Enzymes

β-Enolase (EC 2.7.3.2), rabbit skeletal muscle form, was obtained from Sigma.

The MM-CK (EC 4.2.1.11) was prepared in the laboratory, according to method B of Kuby et al. (1954), from rabbit muscles. The protein was further purified by chromatography on Biogel P100. As commercial antibodies against MM-CK presented cross-reactivity against many proteins of the myofibrils such as myosin and phosphorylase b, polyclonal antibodies were obtained in the laboratory by the immunization of chickens. The protein extracted from the CK band after polyacrylamide electrophoresis was injected into chickens, and the antibody was purified from egg yolk by precipitation with polyethyleneglycol. The antibody was eventually purified by affinity chromatogaphy on a CK-Sepharose affinity column. No cross-reaction was observed with either the B cytosolic or mitochondrial forms.

Localization of M creatine phosphokinase in cultured muscle cells

Creatine phosphokinase and M-line proteins (myomesin and M-protein) were localized by indirect immunofluorescence. The primary antiboby was a goat antibody directed against both chicken myomesin and M-protein (generous gift of T. Wallimann, Zurich). A fluorescein isothiocyanate (FITC)-labeled anti-goat antibody was used as the secondary antibody. For the subunit M of CK (M-CK), the primary antibody was the chicken polyclonal antibody prepared in the laboratory. A biotin-labeled anti-chicken IgY and then Texas red-streptavidin was used as the secondary antibody.

FITC labeling of the enzymes

β-Enolase

Five hundred microliters of suspension in 2.8 M ammonium sulfate of purified β-enolase containing 5 mg of protein was dialyzed against the injection buffer (buffer A, 48 mM K_2HPO_4, 14 mM NaH_2PO_4, and 4.5 mM KH_2PO_4, pH 7.2) containing 0.1 mM dithiothreitol and 1 mM $MgSO_4$. The solution was then concentrated by ultrafiltration on Microcon 10 to 150 μl. One hundred fifty microliters of a 2.5 mg/ml FITC solution in 0.2 M K_2HPO_4 (pH 8.5) prepared just before the labeling was added to the β-enolase solution. After 2 h of incubation in the dark at room temperature, the excess of unreacted dye was eliminated by chromatography on a PD10 column (Pharmacia) equilibrated with buffer A.

Creatine phosphokinase

A 25 mg/ml solution of CK in buffer A was mixed with the same volume of 2.5 mg/ml FITC solution in 0.2 M phosphate (pH 8.5). The incubation time was reduced to 1 h because CK has a very reactive lysine in its active center (Kenyon and Reed, 1983; Sheikh et al., 1993).

For both enzymes, the dye/protein labeling was evaluated spectrophotometrically at pH 8.5, using a molar extinction coefficient of 75,000 M^{-1} cm^{-1} at 495 nm for the dye and, respectively, 37,000 M^{-1} cm^{-1} and 35,700 M^{-1} cm^{-1} at 280 nm for the subunit of CK and β-enolase.

Microinjection

Labeled proteins were introduced into myotubes by pressure injection. Sterile Femtotips (Eppendorf) were filled with 2 μl of a solution of protein in buffer A. The concentration of proteins was 5–20 mg/ml, and before use the solutions were centrifuged for 20 min at 100,000 × *g* in a Beckman Airfuge. A filled Femtotip was inserted into the needle holder of a micromanipulator Leitz and connected to a pressure Microinjector 5242 (Eppendorf). The total volume injected was smaller than 10% of the cell volume. For myotubes with a length greater than 200 μm, small volumes were injected into the cell at several places. The myotubes were allowed to equilibrate for several hours before the FRAP experiments were started.

Diffusion measurements

The modulated fringe pattern photobleaching technique takes advantage of a spatial and temporal modulation that allowed direct recording of the contrast modulation data (Davoust et al., 1982). The experiments were performed with an apparatus described earlier (Morrot et al., 1986). This apparatus is built from a fluorescence Zeiss IM-35 inverted microscope, an

argon laser (Coherent, Innova 90–5) tuned to 488 nm as the excitation source, and a Victor computer for signal analysis. The modulated fringe pattern photobleaching produces a bleaching pattern of interference fringes. The fringes were oriented perpendicular to the muscle fibers. The modulated fringes allowed direct recording of decay of fluorescence contrast between bleached and unbleached regions. Decay of fluorescence contrast was treated by the Padé-Laplace formalism, which allowed an 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 is the interfringe spacing. The experiments were performed at 20°C in a temperature-regulated room.

Diffusion coefficients of proteins in aqueous media were measured for solutions in buffer A, placed as a thin layer between a glass slide and a coverslip.

RESULTS

Labeling of β-enolase and creatine phosphokinase with FITC

The absorption spectra of both labeled enzymes at pH 8.5 are shown in Fig. 1. The average ratio of dye to protein subunit was 0.95. In the case of CK, the reactive lysine of the active center was modified, so the enzymatic activity was lost. Many experiments have shown that CK does not bind to cellular sites via its active center; the C-terminal tail of MM-CK is implicated in this binding (Schäfer and Perriard, 1988), and bound CK is fully active (Krause and Jacobus, 1992). Despite the lost enzymatic activity, the binding capacity of CK is maintained after FITC labeling. In the case of β-enolase, no very reactive lysines have been described, and the enzymatic activity is maintained (results not shown and Pagliaro et al., 1989).

Diffusion of proteins in aqueous medium

The diffusion coefficient of the proteins diluted in injection buffer was determined by FRAP. The protein solutions were placed as a thin layer between a glass slide and a coverslip. The values were similar to those obtained by classical methods: 65 $\mu m^2\ s^{-1}$ for CK (Yue et al., 1967) and 56 $\mu m^2\ s^{-1}$ for β-enolase (Baranowski and Wolna, 1975). In another experiment, the diffusion rate was decreased by adding various sucrose concentrations to increase the viscosity of the medium. The diffusion coefficients were inversely proportional to viscosity, as shown in Fig. 2.

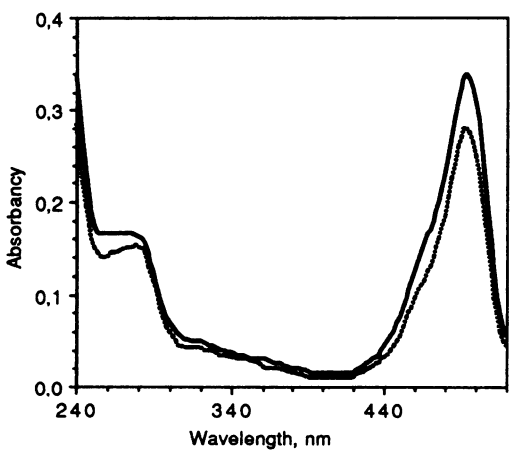

FIGURE 1 Absorption spectra of β-enolase (——) and CK (·····) after labeling by FITC. For both enzymes, an average value of 0.95 lysine per subunit was modified by the fluorescent dye. The spectrum of β-enolase was obtained after a 1:41 dilution of the injected protein in 0.2 M K_2HPO_4, pH 8.5, and that of CK after it was diluted 101 times.

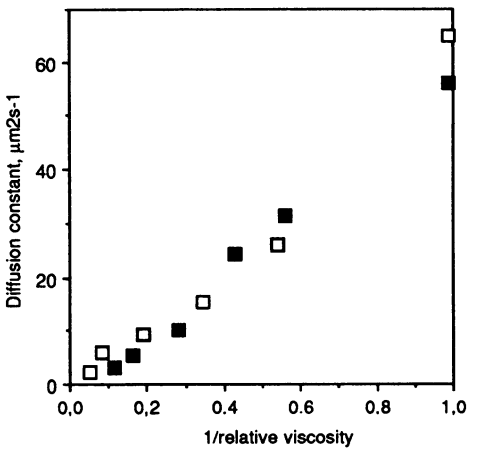

FIGURE 2 Effect of viscosity on the diffusion coefficient of β-enolase and CK in aqueous media. The diffusion coefficients of β-enolase (■) and CK (□) in sucrose solutions were plotted versus the reciprocal viscosity of sucrose relative to water.

Mobility of β-enolase in cultured muscle cells

FRAP experiments were performed at least 3 h after FITC–β-enolase injection. After this incubation time, the protein was equally distributed along the myotubes, but was excluded from the nuclei. The measurements were performed on two different cultures of satellite cells at 10 and 18 days of differentiation. For each dish, 5–10 myotubes were observed. As the interfringes were set in the range 10–20 µm, the mobilities from several sarcomeres were averaged. Fig. 3 gives an example of the decay of the fluorescence con-

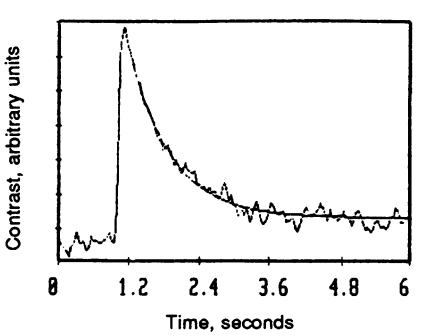

FIGURE 3 Decay of the fluorescence contrast for FITC-β-enolase in muscle cell at 20°C. The curve corresponds to the direct recording. No immobile fraction is observed. The interfringe was 19.4 µm. The time constant, τ, is 0.72 s. According to the relation $D = i^2/4\pi^2\tau$, this gives a diffusion coefficient of $D_{cyt} = 13.3\ \mu m^2\ s^{-1}$.

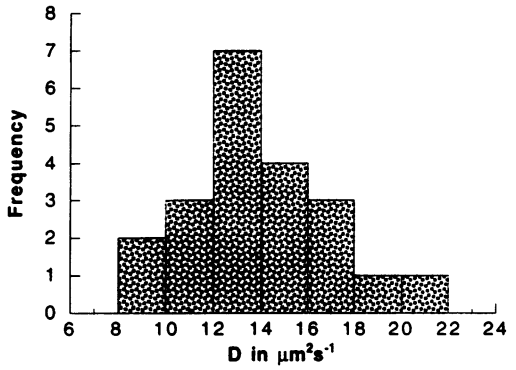

FIGURE 4 Distribution of the diffusion coefficients of β-enolase in cultured muscle cells. An average value of 13.5 ± 2.5 $\mu m^2 s^{-1}$ is obtained.

trast. No immobile fraction is observed. The distribution of the diffusion coefficients of β-enolase in cultured muscle cells is shown in Fig. 4. The results are summarized in Table 1. An average value of 13.5 ± 2.5 $\mu m^2 s^{-1}$ is obtained. The mobility of β-enolase in the cytosol of cultured skeletal muscle cells is four times lower than in aqueous solution, but is near that of a dextran of equivalent hydrodynamic radius, previously determined in these myotubes (Arrio-Dupont et al., 1996). As in the case of inert molecules, the mobility of β-enolase in muscle cells is hindered by both the crowding of the fluid phase of the cytoplasm and the screening effect due to myofilaments.

Mobility of creatine phosphokinase in cultured muscle cells

Fluorescently labeled CK was injected into the myotubes at least 3 h before modulated fringe pattern photobleaching experiments. The measurements were performed on six different cultures of satellite cells at 9–22 days of differentiation. For each dish, 4–10 myotubes were observed. The behavior of CK in the cytosol of myotubes is very different from that of β-enolase. As shown in Fig. 5, at the time scale of the experiment, an important fraction of immobile enzyme is observed, representing 20–50% of the observed signal. The cells injected with fluorescent CK clearly indicated an inhomogeneous distribution (Fig. 6). At these stages of differentiation, there is no correlation between the age of the preparation and the percentage of immobile fraction. The distribution of the diffusion coefficients of the mobile fraction of CK in cultured muscle cells is shown in Fig. 7, and the results are summarized in Table 1. An average value of 4.5 ± 1.0 $\mu m^2 s^{-1}$ is obtained for the diffusion constant.

A contracture was induced on the myotubes by an increase of the external concentration of potassium ion. This contracture is linked to an increase of a few percent of the immobile fraction and a significant increase of the diffusion coefficient of the mobile fraction to 10 ± 2 $\mu m^2 s^{-1}$.

The other fraction of CK seemed immobile at the time scale of the experiment described in Fig. 5. As indicated in Fig. 8, when the decrease in the fluorescence contrast was followed for a longer time, a slight mobility of the enzyme was detected. In another set of experiments, a quantitative study of the rate of diffusion of the slightly mobile CK in relaxed myotubes was performed with narrower fringes than those previously used. Very dispersed values were obtained, as indicated in the table. These values varied from 0.0035 to 0.043 $\mu m^2 s^{-1}$, i.e., 1000 to 100 times lower than the value of the mobile fraction. There is no correlation between the stage of differentiation of the myotubes (from 7 to 22 days after changing the growth medium to the differentiation medium), the percentage of immobile fraction, and the diffusion constants. Furthermore, on the same petri dish, the values obtained for the slightly mobile fraction are much more dispersed than the values obtained for the mobile fraction. A possible explanation for the slow movement of this fraction is that it corresponds to an exchange between mobile and bound enzyme.

Localization of endogenous M creatine phosphokinase in cultured muscle cells

In our cultured muscle cells, the amount of endogeneous CK is ~10 times lower than that of adult muscles. This has already been observed on cultured human satellite cells (Benders et al., 1991).

As shown in Fig. 9 *a*, after 7 days of differentiation, the M-line is well organized in our cultured muscle cells. Myotubes, but not mononuclear cells, are positive for CK M (not shown). The organization of M-CK is observed after 14 days (at least) of differentiation (Fig. 9 *b*). The observation required a high amplification consisting of biotinylated second antibody and Texas red-streptavidin. Double immunofluorescent labelings were performed with an antiboby against M-line proteins 10 times more diluted than those used for Fig. 9 *a*. As indicated in Fig. 9 *c*, a colocalization of endogenous M-CK and M-line proteins is observed.

TABLE 1 Mobility of β-enolase and creatine kinase in the cytosol of cultured muscle cells

Protein	MW	D_{aq} ($\mu m^2 s^{-1}$)	Hydrodynamic radius (nm)	% mobile fraction in the cytosol	D_{cyt} ($\mu m^2 s^{-1}$)	D_{cyt} of a dextran of same size ($\mu m^2 s^{-1}$)*	D_{cyt}/D_{aq}	D_{cyt}, slightly mobile fraction ($\mu m^2 s^{-1}$)
β-Enolase	90,000	56	3.8	100	13.5 ± 2.5	14	0.24	
Creatine kinase	83,000	65	3.3	80 to 50	4.5 ± 1	16	0.07	0.0035 to 0.043

*According to Arrio-Dupont et al. (1996).

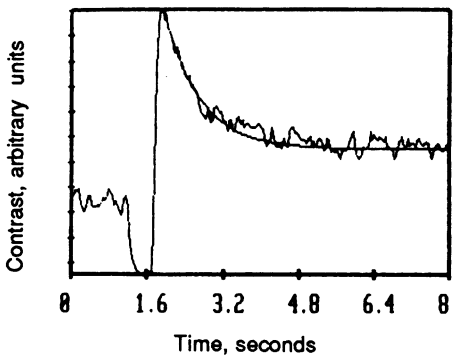

FIGURE 5 Decay of the fluorescence contrast for FITC-CK in muscle cell at 20°C. The curve corresponds to the direct recording. An immobile fraction is observed representing 38% of the signal. The interfringe was 23.7 μm. The time constant, τ, is 3.1 s for the mobile fraction. This gives a diffusion coefficient of $D_{cyt} = 4.6\ \mu m^2\ s^{-1}$.

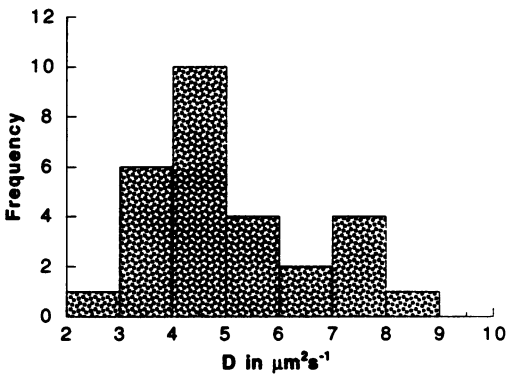

FIGURE 7 Distribution of the diffusion coefficients of the mobile fraction of CK in cultured muscle cells. An average value of $4.5 \pm 1.3\ \mu m^2\ s^{-1}$ is obtained.

DISCUSSION

We recently showed that the mobility of inert macromolecules in muscle cells is hindered by both the crowding of the fluid phase of the cytoplasm and the screening effect due to myofilaments (Arrio-Dupont et al., 1996). The relative diffusion coefficient ($D_{cytosol}/D_{aqueous\ medium}$) of FITC-dextrans in muscle cells decreased with the hydrodynamic radius, R_h, of the dextrans. A free cytosolic protein concentration of 135 mg/ml, a solvent viscosity of cytoplasm near that of bulk water, and a calculated screening constant of 0.066 nm^{-1}, which took into account the sarcomeric organization of filaments, accurately represented our data.

Our study suggests that β-enolase is 100% mobile in the cytoplasm of cultured muscle cells, as previously observed in 3T3 cells by Pagliaro et al. (1989). The mobility of β-enolase in the cytosol of cultured muscle cells is hindered in the same way as a dextran of the same hydrodynamic radius (Table 1). This hindering has been accurately described by both a screening due to the organized myofilaments and an effect of crowding linked to the high protein concentration in the cytosolic compartment of the myotubes.

Our study clearly demonstrates that, despite the similar sizes of these proteins, the behavior of CK injected into the myotubes is very different from that of β-enolase. First, the mobile fraction represents only 50–80% of the fluorescent CK, and the mobility of this fraction is lower than that of a dextran of same size in the cytosol of rabbit skeletal myotubes ($4.5\ \mu m^2\ s^{-1}$ instead of $16\ \mu m^2\ s^{-1}$). A possible explanation is that a charged surface effect may slow down the movements of CK in the cytosol of muscle cells. One may note that upon induction of a contraction, the mobility of this fraction of CK is increased to a value near that of β-enolase. This observation is comparable to that of Otsu et al. (1993), who noticed that in heart, CK is localized in the A-band, whereas after ischemia this enzyme is more dispersed in the sarcomeres.

Another important observation is on the very slightly mobile fraction corresponding to 20–50% of the fluorescent CK. This fraction is from 100 to more than 1000 times slower than the mobile CK. A possible explanation for this observation is that it corresponds to an exchange between

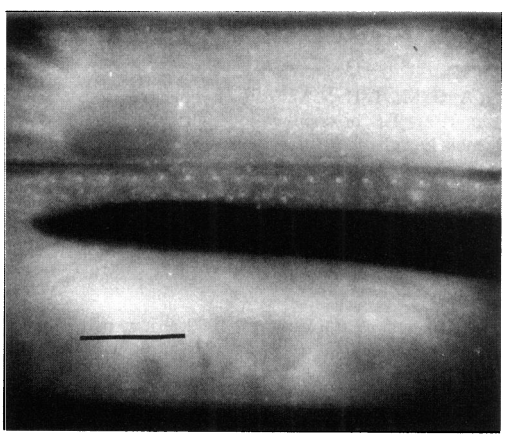

FIGURE 6 Myotubes injected with FITC-CK. These myotubes were injected with the fluorescent protein after 9 days of differentiation and were used 15 h after injection. The bar represents 10 μm.

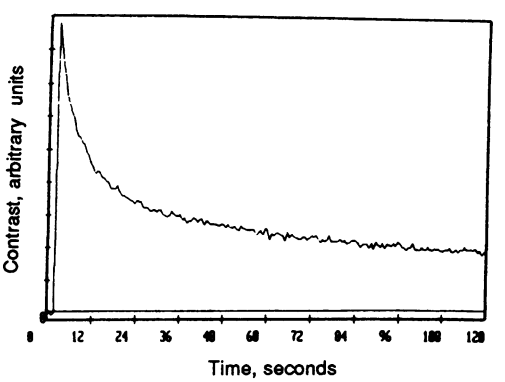

FIGURE 8 Decay of the fluorescence contrast for FITC-CK in muscle cell at 20°C: study of the slightly mobile fraction in relaxed myotubes. The interfringe was 13.6 μm. The time constant, τ, is 110 s. This gives a diffusion coefficient of $D_{cyt} = 0.043\ \mu m^2\ s^{-1}$.

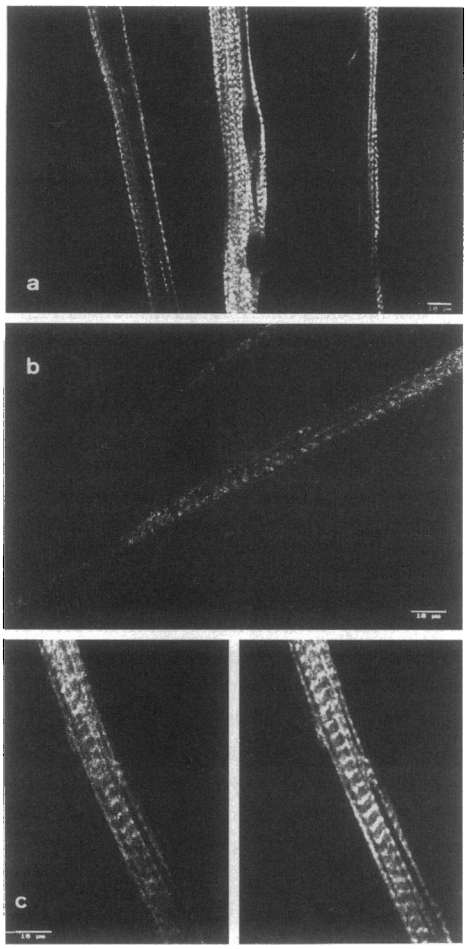

FIGURE 9 Immunolocalization of M-CK and M-line proteins (myomesin and M-protein) observed by confocal microscopy. These myotubes were studied after 7 days of differentiation for the *a*, showing the localization of M-line proteins. For *b* and *c*, the myotubes were studied after 14 days of differentiation. (*b*) Single labeling against CK. (*c*) Double labeling against CK and M-line proteins observed in the same focal plane. *Left*: CK; *right*: M-line proteins. In *c*, the antibody against M-line proteins was 10 times more diluted than in *a*. The bar represents 10 μm.

mobile and bound enzyme. The picture of myotubes injected with FITC-CK clearly indicated a striated repartition of the enzyme in the cells, making an exchange between partially free and bound CK a plausible hypothesis.

It had previously been shown that the extra-M-line cytosolic CK was not free in the cytosol. According to Wegmann et al. (1992), it is localized in the I-band, but it has been viewed near myosin heads by Otsu et al. (1989). In parallel with these contradictory observations, axial diffusion experiments of several proteins (Kraft et al., 1995), among them MM CK, in skinned skeletal myofibrils showed an inhomogeneous repartition of fluorescent CK, attributed to an interaction with the M-line. In our cultured muscle cells the appearance of organized M-CK is very late, as it is scarcely viewed before 14 days of differentiation. Furthermore, the global cellular CK activity is about one-tenth of CK activity in the muscle from which the satellite cells were isolated. Benders et al. (1991) had already observed this difference between cultured human muscle cells and adult muscle. Our cultured myotubes are similar to young muscle (Arrio-Dupont et al., 1996), and this very likely explains the differences between cultured cells (Fig. 9) and localization of CK in adult muscle (Wegmann et al., 1992).

Although the modulated fringe pattern photobleaching technique is particularly suited for the analysis of molecular association processes (Davoust et al., 1982; Lanni and Ware, 1984), we are not able to correlate the percentage of immobile component to the exact bound fraction. In fact, we do not know the exact amount of CK injected into each myotube, or the amount of endogenous MM-CK. Endogenous MM-CK is observed in the myotubes, but the exact amount per myotube is difficult to quantify, because total extracts give only an average value. That also explains the dispersion of the percentage of immobilized fraction, as the ratio of injected to endogenous CK may vary from one cell to the other and from one experiment to the other. The injected CK partly restablishes a cellular concentration comparable to that of the initial muscle.

Based on our data, we demonstrate that there two distinct fractions of CK, a mobile fraction that is cytosolic and a very slightly mobile fraction, probably due to an exchange between mobile CK and CK bound to the M-line.

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