

Translational Diffusion of Globular Proteins in the Cytoplasm of Cultured Muscle Cells

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ABSTRACT Modulated fringe pattern photobleaching (MFPP) was used to measure the translational diffusion of microinjected fluorescein isothiocyanate (FITC)-labeled proteins of different sizes in the cytoplasm of cultured muscle cells. This technique, which is an extension of the classical fluorescence recovery after photobleaching (FRAP) technique, allows the measurement of the translational diffusion of macromolecules over several microns. Proteins used had molecular masses between 21 and 540 kDa. The results clearly indicated that the diffusivity of the various proteins is a decreasing function of their hydrodynamic radius. This decrease is more rapid with globular proteins than with FITC-labeled dextrans (Arrio-Dupont et al., 1996, *Biophys. J.* 70:2327–2332), most likely because, unlike globular proteins, dextrans are randomly coiled macromolecules with a flexible structure. These data do not exclude the possibility of a rapid diffusion over a short distance, unobservable with our experimental set-up, which would take place within the first milliseconds after bleaching and would correspond to the diffusion in restricted domains followed by impeded diffusion provoked by the network of microtubules, microfilaments, and intermediate filaments. Thus our results may complement rather than contradict those of Verkman and collaborators (Seksek et al., 1997, *J. Cell Biol.* 138:1–12). The biological consequence of the size-dependent restriction of the mobility of proteins in the cell cytoplasm is that the formation of intracellular complexes with other proteins considerably reduces their mobility.

INTRODUCTION

Cell cytoplasm is a complex environment comprising a fluid medium, a high concentration of proteins, and a network of cytoskeletal filaments. In this highly organized network, small metabolites, proteins, and mRNAs must move to maintain cell functions and renewal cell constituents. It has been proposed that the crowding might seriously hinder solute diffusion and influence a variety of biochemical processes (see the reviews by Zimmerman and Minton, 1993, and Luby-Phelps, 1994). However, rather contradictory results on solute diffusion in cell cytoplasm have been put forward by experimentalists in recent years.

The studies of small molecule diffusivity found values for the viscosity of the fluid phase close to water viscosity (1–2 cP) (Mastro et al., 1984; Clegg, 1984; Luby-Phelps et al., 1988, 1993; Kao et al., 1993). However, Jacobson and collaborators, using fluorescence recovery after photobleaching (FRAP) to measure the diffusion of proteins in human fibroblasts, found that, from 12 to 440 kDa, the diffusion coefficients were markedly reduced compared to the values in aqueous buffer but exhibited almost no dependence on the molecular weight (Wojcieszyn et al., 1981; Jacobson and Wojcieszyn, 1984). They concluded that the diffusion in the cytosol was dominated not by steric effects

but rather by binding of the diffusing species to elements of the cytosol matrix. To overcome the possibility of either specific or nonspecific interactions of proteins with the intracellular structures and to understand how the intracellular medium hinders diffusion, inert macromolecules such as dextran and Ficoll were later employed (Luby-Phelps et al., 1986, 1987; Peters, 1986). From those investigations, it was inferred that the cytoplasm of Swiss 3T3 fibroblasts could be described as a network of entangled fibers interpenetrated by a fluid phase containing soluble proteins at high concentration, in which self-diffusion of inert tracer particles was hindered in a size-dependent manner (Luby-Phelps et al., 1987, 1988; Luby-Phelps, 1994). But in a recent study, Seksek et al. (1997), by using a microsecond-resolution FRAP apparatus, obtained results that did not support the concept of size-dependent diffusion of dextran or Ficoll macromolecules in the cytoplasm of either 3T3 fibroblasts or Madin-Darby canine kidney epithelial cells. Indeed, from $t_{1/2}$ values of FRAP recovery curves, the latter authors concluded that in the cytoplasm of such cells, the diffusion coefficients of large particles was reduced three- to fourfold relative to the values obtained in water but did not depend on the size of these particles. However, because of technical limitations, Seksek et al. recorded incomplete fluorescence recoveries after photobleaching, and the percentage of recovery appeared as a decreasing function of the size of these molecules (see figure 4D of Seksek et al., 1997).

The diffusion coefficients of soluble molecules have also been measured in the cytoplasm of muscle cells. Striated muscle is one of the most highly ordered of all biological tissues (reviewed by Squire, 1997). Besides the contractile

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elements, thick filaments of myosin and thin filaments of actin and associated proteins, the spacing of which has been accurately measured by electron microscopy and x-ray diffraction (Sosa et al., 1994; Xu et al., 1997), a cytoskeletal lattice maintains the structure of muscle cells. The proteins of the M-line and Z-line serve as structural integrators of the myofilaments and of the longitudinal lattice components. Two giant proteins, titin and nebulin, compose the elastic filament system in skeletal muscle and molecular rulers specifying the length of the contractile filaments (reviewed by Trinick, 1994). Furthermore, a cytoskeleton localized under the muscle membrane has been described and reviewed by Small et al. (1992). It has been shown that the diffusion coefficients of small ions, with the exception of Ca^{2+} , and of small molecules were reduced in muscle by a factor of 2 relative to their values in aqueous medium (Kushmerick and Podolsky, 1969; Yoshizaki et al., 1990; Hubley et al., 1995). Previous studies were carried out on the rate of leakage of cytosolic proteins out of skinned skeletal fibers (Maughan and Lord, 1988; Maughan and Wegner, 1989). Measurement of the diffusion by photooxidation and/or microinjection of visible-light-absorbing proteins in a single muscle fiber was recently reported (Jürgens et al., 1994, 1997; Papadopoulos et al., 1995). All investigators found an important reduction of protein mobility in muscle fibers compared to that in water. Furthermore, a strong size-dependent reduction of the diffusion coefficients was detected; the reduction varied from a factor of 10 for small proteins to 60 or more for large proteins. Some of the proteins used in these studies have an affinity for intrasarcolemmal sites, other glycolytic enzymes, and actin. For this reason, in a previous work we have studied the diffusion of fluorescein-dextran (FITC-dextran) in striated muscle cells and found that the ratio $D_{\text{cytoplasm}}/D_{\text{water}}$ decreased monotonously as the hydrodynamic radius R_h of the macromolecules increased (Arrio-Dupont et al., 1996). We concluded that the mobility of inert molecules in muscle cells was hindered by both the crowding of the fluid phase of the cytoplasm and the screening effect due to myofilaments. However, as dextran conformation is that of a randomly coiled hydrated polymer (Luby Phelps et al., 1988; Berk et al., 1993; Arrio-Dupont et al., 1996; Gribbon and Hardingham, 1998), it was interesting to extend our studies to the diffusion of proteins in the cytoplasm of skeletal muscle cells, as globular proteins behave in water like hard spheres (Tanford, 1961).

We have investigated the diffusion of fluorescently labeled proteins of different sizes in the cytoplasm of striated muscle cells. We chose to study the diffusion of proteins known for their absence of interaction with the intracellular constituents of muscle and with molecular masses in the range of 21–540 kDa. The translational diffusion of FITC-labeled proteins was measured with a modulated fringe pattern photobleaching (MFPP) apparatus (Davoust et al., 1982), as in our former investigations (Arrio-Dupont et al.,

1996). This technique is convenient in the case of giant cells such as myotubes. The bleaching pattern is obtained with interference fringes covering the whole cell; the diffusion coefficient is calculated from the evolution of the contrast between bleached and nonbleached regions. The evolution of the fluorescence can be followed over long intervals, allowing one to reach the equilibrium state. In the case of a single diffusion coefficient, the contrast decreases exponentially and gives rise in a semilogarithmic plot to a nonambiguous straight line. In a recent paper Munnelly and collaborators showed the advantage of the fringe photobleaching recovery technique for investigating the entire cell's surface. However, their stem does not take advantage of the fringe modulation (Munnelly et al., 1998).

MATERIALS AND METHODS

Muscle cell cultures

Rabbit satellite cells were cultured from a slow muscle, semimembranosus proprius, as previously described (Arrio-Dupont et al., 1996; Barjot et al., 1998). The satellite cells were grown to confluence in Dulbecco's minimum essential medium containing 20% fetal calf serum, 100 U/ml penicillin, and 1 mg/ml streptomycin; then the medium was changed to Dulbecco's minimum essential medium containing antibiotics, 2% fetal calf serum, 5 $\mu\text{g}/\text{ml}$ insulin, 5 $\mu\text{mol}/\text{ml}$ transferrin, and 5 nmol/ml sodium selenite (ITS medium). In the ITS medium cells fused and differentiated into myotubes. For indirect immunofluorescence assays, muscle cells were cultured on microscopic coverslips. To perform MFPP experiments, cells were grown in Ø 6-cm dishes, the bases of which were replaced by sealed glass coverslips allowing microscopic observations. For microinjections and MFPP experiments, an ITS medium devoid of phenol red and buffered with 20 mM HEPES was used. Most of the photobleaching experiments were performed on myotubes after 7–15 days of differentiation. The cultured myotubes were 10–40 μm wide and up to 1 mm in length.

The differentiation was followed by indirect immunofluorescence, using monoclonal antibodies directed against α -actinin (Sigma), myosin heavy chains (neonatal and fast, clone MY 32; Sigma), or the ryanodine receptor (RYR) (clone 34-C, ABR), and FITC-labeled goat anti-mouse IgG as the secondary antibody or purified polyclonal antibodies produced in rabbit against SERCA2a, SERCA2b, and the biotin-labeled purified anti-rabbit IgG as the secondary antibody, and then Texas red streptavidin. Both the monoclonal antibody (FITC fluorescence) and the polyclonal antibody (Texas red fluorescence) were simultaneously used on the same cell for double labeling.

Proteins

The rabbit muscle forms of myokinase (ATP:AMP phosphotransferase; EC 2.7.4.3), phosphoglucosyltransferase (α -D-glucose-1-phosphate phosphotransferase; EC 5.4.2.2), β -enolase (phosphopyruvate hydratase; EC 4.2.1.11), and β -galactosidase from *Escherichia coli* (β -D-galactoside galactohydrolase; EC 3.2.1.23) were obtained from Sigma. FITC-conjugated anti-mouse IgG developed in goat was from Sigma Immuno Chemicals. The recombinant enhanced green fluorescent protein (EGFP) variant of the *Aequorea victoria* green fluorescent protein was obtained from Clontech; this form has a high absorbency at 488 nm.

FITC labeling of the proteins

Protein solutions, ~25 mg/ml in 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 1

mM MgSO_4 and 1 mM of the inhibitor P^1, P^5 -di(adenosine-5')pentaphosphate (AP5A) in the case of myokinase, were incubated in the dark with the same volume of a 2.5 mg/ml FITC solution in 0.2 M K_2HPO_4 (pH 8.5) prepared just before labeling. After 2 h of incubation in the dark at room temperature, the excess unreacted dye and AP5A were eliminated by chromatography on a PD10 column (Pharmacia) equilibrated with buffer A. The dye/protein labeling was evaluated spectrophotometrically at pH 8.5, and the average molar ratio of dye to protein subunit was 0.95:1.

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 $100,000 \times g$ in a Beckman Airfuge. A filled Femtotip was inserted into the needle holder of a Leitz micromanipulator and connected to a pressure microinjector (5242; Eppendorf). For myotubes of length greater than 200 μm , small volumes were injected into several places in the cell. The total volume injected was smaller than 10% of the cell volume. The myotubes were allowed to equilibrate for several hours before the fluorescence experiments were started.

Diffusion measurements

The MFPP technique takes advantage of a spatial and temporal modulation that allows direct recording of the contrast between bleached and non-bleached zones (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 microcomputer for signal analysis. Modulated fringe pattern photobleaching produces a bleaching pattern of interference fringes. Except for a few cases, as mentioned below, the fringes were oriented perpendicular to the muscle fibers. Decay of fluorescence contrast was treated by the Padé-Laplace formalism, which allowed an immediate multiexponential analysis (Yeremian and Claverie, 1987). 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 (Davoust et al., 1982). Anomalous diffusion, if any, can be detected by varying the interfringe spacing (Bouchaud et al., 1991). The experiments were performed at 20°C in a temperature-regulated room. Diffusion coefficients of proteins in aqueous media were measured in buffer A, placed as a thin aqueous layer between a glass slide and a coverslip.

RESULTS

Characterization of the cultured muscle cells

Immunofluorescence assays showed that after 9–17 days of differentiation the myotubes presented the striation of myosin organized in the A-band (Fig. 1 *a*, right). An antibody directed against SERCA2a (sarcoplasmic reticulum Ca^{2+} -ATPase of slow skeletal muscle) indicated a location near the A-band (Fig. 1 *a*, left), whereas anti-SERCA2b (ubiquitous Ca^{2+} -ATPase) reacted with a lower intensity with all cells, including mononucleated ones (not shown). As expected from the preceding observation, the location of α -actinin (Fig. 1 *b*, right) is complementary of that of SERCA2a (Fig. 1 *b*, left), whereas the ryanodine receptor (RYR) responsible for the calcium release from the sarcoplasmic

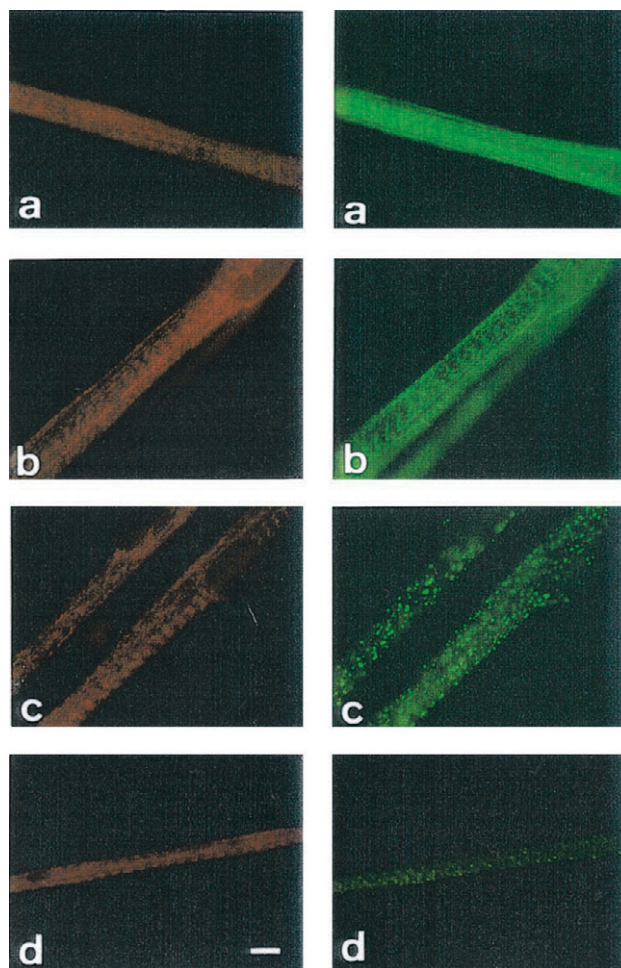


FIGURE 1 Double labeling by immunofluorescence of culture muscle cells after 14 days of differentiation. The left part of the figure (*a–d*) shows the localization of SERCA2a; the right part shows myosin (*a*), α -actinin (*b*), and RYR (*c* and *d*). The bar represents 5 μm .

reticulum is either punctiform or striated (Fig. 1, *c* and *d*, right). In the latter case its location is complementary to that of SERCA2a (Fig. 1 *c* and *d*, left).

These cultured myotubes show the highly organized structure of muscle and thus are a good system for the study of the intracytoplasmic diffusion of proteins. The presence of an organized sarcoplasmic reticulum (Flucher et al., 1993) indicated that they are similar to muscles.

Diffusion of the labeled proteins in aqueous media

The diffusion of the various proteins was first investigated in buffer A by the MFPP technique. Results are reported in Table 1. The hydrodynamic radii derived from the diffusion coefficients are included in this table. A log-log analysis (not shown) indicated that protein aqueous diffusion coefficients were approximately proportional to the inverse of the cubic root of molecular mass, as expected. This is

TABLE 1 Mobility of proteins in the cytoplasm of cultured muscle cells

Protein	M_r	D_w ($\mu\text{m}^2 \text{s}^{-1}$)	R_h (nm)*	D_{cyt} ($\mu\text{m}^2 \text{s}^{-1}$)	D_{cyt}/D_w
Myokinase	21,000	160 ± 30	1.3 ± 0.2	$46 < D_{\text{cyt}} < 93$	$0.3 < D_{\text{cyt}}/D_w < 0.6$
Phosphoglucumutase	60,000	63 ± 8	3.4 ± 0.4	16.5 ± 3	0.26 ± 0.024
β -Enolase	90,000	56 ± 6	3.8 ± 0.4	10.8 ± 2	0.19 ± 0.024
IgG	160,000	40 ± 5	5.4 ± 0.7	5.5 ± 1	0.14 ± 0.023
β -Galactosidase	540,000	30 ± 3	7.2 ± 0.7	0.004 ± 0.0007	0.00013 ± 0.00004
EGFP	27,000	87 ± 16	2.4 ± 0.4	15.8 ± 3	0.18

*The hydrodynamic radius, R_h , was calculated from the Stokes-Einstein equation: $D = kT/6\pi\eta R_h$.

consistent with the Stokes-Einstein equation for diffusivity, with the assumption that the molecule is a sphere with a volume proportional to its molecular mass (Tanford, 1961).

We had previously shown (Arrio-Dupont et al., 1996) that the dextran diffusion coefficients are proportional to the inverse of the square root of the molecular mass. Similar observations had been made by Berk and his collaborators (Berk et al., 1993).

Note that EGFP was very difficult to photobleach, as previously observed for GFP and some of its mutants (Patterson et al., 1997). Nevertheless, the diffusion coefficient estimated, $87 \mu\text{m}^2 \text{s}^{-1}$, was in agreement with that determined by fluorescence correlation spectroscopy (Terry et al., 1995).

Protein diffusion in the cytoplasm of cultured muscle cells

The diffusion of the various proteins was studied for three different cultures and for cells at 7–15 days of differentiation. For each petri dish, ~10 myotubes were studied. In the case of myokinase, the intracytoplasmic mobility was too

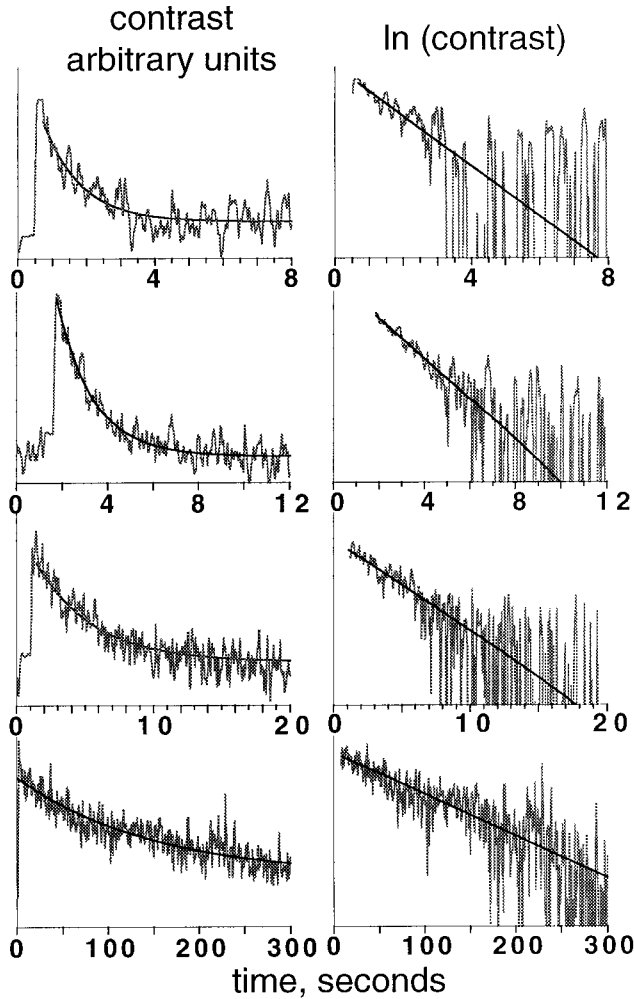


FIGURE 2 Examples of decay of the fluorescence contrast (left) and its semilogarithmic transform (right) for the four proteins studied in this article. The temperature is 20°C in all cases. From top to bottom: phosphoglucumutase, enolase, IgG, and β -galactosidase. PGM: interfringe is $27.0 \mu\text{m}$; decay constant, τ , is 1.09 s. Enolase: interfringe is $26.2 \mu\text{m}$; decay constant, τ , is 1.61 s. IgG: interfringe is $25.1 \mu\text{m}$; decay constant, τ , is 4.27 s. β -Gal: interfringe is $5.28 \mu\text{m}$; decay constant, τ , is 208 s. Note that the apparent noise that appears in the right column is directly related to the use of logarithmic transformation.

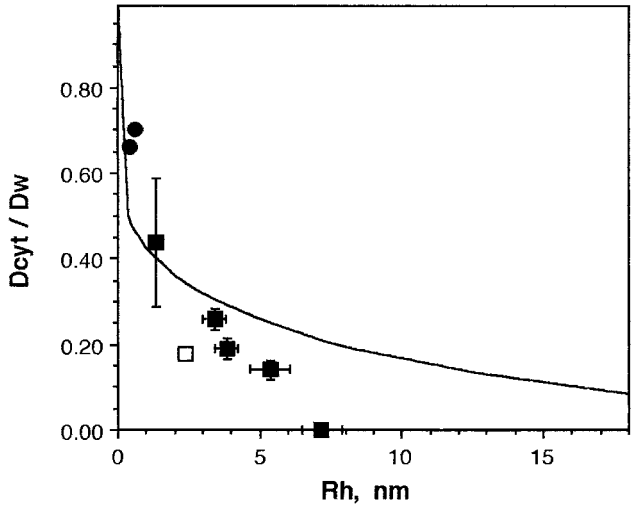


FIGURE 3 Variation of the relative diffusion (D_{cyt}/D_w) coefficient of various globular proteins in the cytoplasm of muscle cells as a function of protein hydrodynamic radius. The closed squares correspond to the various proteins; EGFP is indicated as an open square. The closed circles are the values observed for the diffusion of ATP and creatine-phosphate in muscle fibers (Hubley et al., 1995). The line corresponds to the fit of our previous observations (Arrio-Dupont et al., 1996) about the diffusion of FITC-dextran in the cytoplasm of our cultured muscle cells.

fast to be determined with accuracy. It was only possible to estimate that $46 \mu\text{m}^2 \text{s}^{-1} < D_{\text{cyt}} < 93 \mu\text{m}^2 \text{s}^{-1}$. For phosphoglucosomutase, β -enolase, IgG, and β -galactosidase, typical decays of the fluorescence contrast are shown in Fig. 2. In Table 1, the values of the diffusion constants determined by the Padé-Laplace formalism are indicated. The diffusion constant was always found to be independent of the interfringe spacing (a minimum of two different values were used for each measurement). As in the case of dextran (Arrio-Dupont et al., 1996), no significant difference was observed when fringes were oriented oblique to the myofilaments. Note that the cell size and shape (Fig. 1) did not allow us to work in a parallel orientation; in such a configuration the number of fringes covering the cell would lead to insignificant diffusion values.

As shown in Fig. 3, the diffusion coefficients of the various proteins decreased with their hydrodynamic radius R_h . Note that $R_h = 0.665R_g$, where R_h is the experimental value (Tanford, 1961). To compare with the mobility of small molecules, we have included in Fig. 3 the values obtained from ^{31}P NMR spectroscopy by Hubley and collaborators (Hubley et al., 1995) for ATP and creatine phosphate in isolated skeletal muscle. The line in the same graph is the fit corresponding to our former studies on the diffusion of inert macromolecules in these cultured muscle cells. This fit was obtained by taking into account both crowding due to free soluble proteins (concentration c) and screening due to myofilaments (constant κ_L): $D_{\text{cyt}}/D_w = \exp(-0.035c^{0.635}R_h^{0.16}) \times \exp(-\kappa_L R_h)$, where $c = 135 \text{ mg/ml}$ and $\kappa_L = 0.066 \text{ nm}^{-1}$. Obviously, the curve, which was well adapted to dextran diffusion, does not fit protein diffusion in the cytoplasm of muscle cells.

DISCUSSION

We have determined the mobility of globular proteins in the cytoplasm of cultured muscle cells. Proteins of different sizes were selected for their absence of interaction with the components of muscle cytoplasm, with the exception of myokinase, for which an interaction is possible. The results clearly indicate that the relative diffusion coefficient, D_{cyt}/D_w , of the various proteins is a decreasing function of their hydrodynamic radius. This decrease is more rapid for the globular proteins than for the series of dextran molecules previously studied in the same cultured muscle cells (Arrio-Dupont et al., 1996), as shown in Fig. 3. The difference is not due to electrostatic interactions between the charge surface of the various proteins and intracellular elements of muscle cells, because some of them are positively charged and others are negatively charged at pH 7. It is more likely that the difference between dextrans and proteins is due to the randomly coiled structure of dextran macromolecules, with a high hydration and a flexible structure. We had already pointed out that an FITC-dextran of molecular mass

148,000 Da has an R_h two times higher than that of a protein of equal molecular mass (Arrio-Dupont et al., 1996).

Particular attention has to be paid to the intracellular mobility of EGFP. First, this protein, which has an intrinsic fluorescent chromophore due to the posttranslational modification of the internal Ser-Tyr-Gly sequence, is very difficult to bleach. This resistance to bleaching, already pointed out by Patterson and collaborators (Patterson et al., 1997), is very likely due to the structure of the protein and the manner of formation of the chromophore by cyclization and then oxidation (Phillips, 1997). The second observation is that, in muscle cells, its relative diffusivity is low compared to that of the other proteins studied. The intracellular diffusion coefficient ($15.7 \mu\text{m}^2 \text{s}^{-1}$) is near that of phosphoglucosomutase (PGM), a 60-kDa protein. As it is known that GFP easily assumes dimeric forms, a plausible explanation for this behavior is that after injection into the cells (initial concentration of the protein in injection buffer 6–7 mg/ml), EGFP is present as a dimer in the intracellular medium.

Our results were obtained with cultured muscle cells. Previous studies were carried out on skeletal muscle fibers (Maughan and Lord, 1988; Maughan and Wegner, 1989; Jürgens et al., 1994, 1997; Papadopoulos et al., 1995). All investigators found an important reduction of protein mobility in muscle fibers compared to that in water. Furthermore, a strong size dependence of the diffusion coefficients was detected. The comparison of these previous observations with ours is shown in Fig. 4, where the intracellular diffusion constants are expressed as a function of the molecular mass of the various proteins. This difference cannot

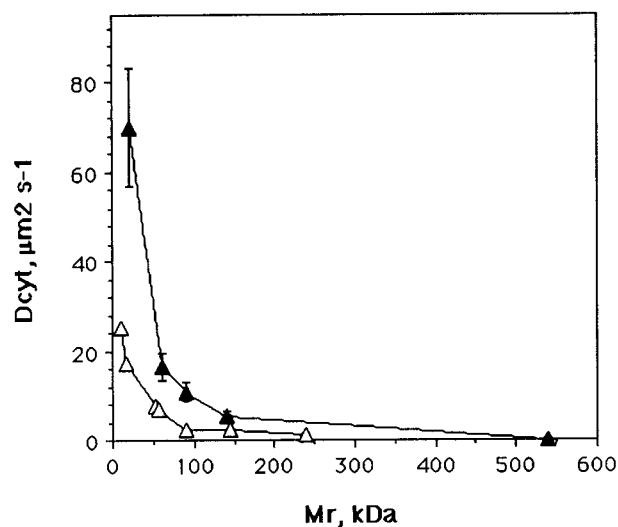


FIGURE 4 Variation of the diffusion coefficient of various globular proteins in the cytoplasm of cultured muscle cells as a function of their molecular mass (▲). In the same graph, the values deduced from the leakage of proteins out of skinned muscle fibers by Maughan and Lord (1988) and for the diffusivity of myoglobin in muscle fibers (Papadopoulos et al., 1995) are reported (△).

be attributed to our observations parallel to the myofilament, as Maughan and his collaborators measure radial diffusion in muscle fiber (Maughan and Lord, 1988; Maughan and Wegner, 1989), and Jürgens and his collaborators measure the lateral diffusion after protein microinjection into muscle fiber (Jürgens et al., 1997) and obtain similar results. Therefore it appears that the diffusivity of proteins is higher in cultured muscle cells than in native muscle fibers. This observation confirms that despite the high organization of the cultured cells, with a sarcoplasmic reticulum in place, they are not as well organized as a true muscle. The cell characteristics are those of a young muscle rather than of an adult one.

Several theoretical models were developed to describe the diffusion of particles in the presence of obstacles in two-dimensional systems (Qian et al., 1991; Saxton, 1993, 1994). These models are suitable for diffusion in membranes (2D) but are not applicable to intracytoplasmic diffusion (3D). Han and Herzfeld (1993) predicted that, under conditions in which proteins can be approximated by hard particles, the hindrance of globular proteins by other proteins at a given volume fraction is reduced when the background proteins are aggregated, and the hindrance is further reduced if rodlike aggregates are aligned. A more elaborate model for obstructed diffusion in three dimensions was proposed recently by Olveczky and Verkman (1998). The latter model considered small objects in a well-organized space; it was applied to molecular transport in the aqueous lumen of organelles, and the application to mitochondria might be transposed to muscle cells. The authors reached the following conclusions: 1) for short times, the diffusion hindrance imposed by immobile obstacles is negligible; 2) for long times, on the other hand, the presence of multiple barriers impedes the diffusion of large particles. The latter phenomenon can be detected only if photobleaching experiments are carried over sufficiently long periods, i.e., if fluorescent molecules can diffuse over long distances. This is likely because Seksek et al. (1997) observed fluorescence recoveries for short periods without following fluorescence intensities until full recovery, and because they obtained relatively free and rapid diffusion of macromolecule-sized solutes up to at least 500 kDa. Hence they could conclude that the cytoplasm is not so crowded that solute motion is seriously impeded. In fact in the recent article of Olveczky and Verkman (1998), the authors concluded, as a recommendation, that measurements of solute diffusion in organelles by photobleaching methods should be carried out over long time intervals to reveal anomalous diffusion associated with spatial heterogeneity.

In the present study, as well as in our former investigation (Arrio-Dupont et al., 1996), sample illumination by several 10–17- μm fringes represented an observation involving many sarcomeres of 2.5 μm . With the exception of nuclei regions, where large macromolecules do not penetrate, fluorescence distribution after microinjection and equilibration

(i.e., before bleaching) was homogeneous. The fluorescence contrast between bleached and unbleached regions in the cytoplasm of muscle cells was studied until at least 90% of the contrast had disappeared. Typically, with the larger protein used, β -galactosidase, the contrast was 10% of its initial value after 300 s. Thus diffusion constants were calculated on the basis of a theoretical 100% fluorescence recovery. The Padé-Laplace analysis as well as the semi-logarithmic plots of the fluorescence contrast decline (Fig. 2) indicated unambiguously a single exponential coefficient. It should be pointed out that our instrumental set-up allows us to record long periods of contrast variations and, hence, to measure with accuracy diffusion over a long distance. Typically the $t_{1/2}$ of the fluorescence contrast extinction in our experiments was several seconds, and the bleaching time varied from 50 to 500 ms. On the other hand, our apparatus is not adapted for the measurement of events taking place within tens of milliseconds, as reported by Seksek et al. (1997). The possibility of a size-independent diffusion taking place during the first fraction of a second cannot be ruled out. In conclusion, the two sets of experiments may be complementary rather than contradictory.

Finally we would like to make a remark about the biological consequence of the size-dependent restriction of mobility of proteins in the cell cytoplasm. If proteins in the intracellular medium form complexes with other proteins, their mobility should be considerably reduced. A complex of ~500 kDa would be almost immobile. Under such conditions, an ensemble of enzymes implicated in a chain of metabolic reactions may be compared to in vitro immobilized enzymes, for which it has been shown that the functional coupling of activities is channeled because the intermediate metabolites are transformed by the neighboring enzyme before diffusing into the bulk medium (Mosbach and Mattiasson, 1976; Arrio-Dupont, 1988; Arrio-Dupont et al., 1992; Srere et al., 1990).

REFERENCES

- Arrio-Dupont, M. 1988. An example of substrate channeling between co-immobilized enzymes. Coupled activity of myosin ATPase and creatine kinase bound to frog heart myofilaments. *FEBS Lett.* 240:181–185.
- Arrio-Dupont, M., J. J. Bechet, and A. D'Albis. 1992. A model system of coupled activity of co-immobilized creatine kinase and myosin. *Eur. J. Biochem.* 207:951–955.
- Arrio-Dupont, M., S. Cribier, G. Foucault, P. F. Devaux, and A. D'Albis. 1996. Diffusion of fluorescently labeled macromolecules in cultured muscle cells. *Biophys. J.* 70:2327–2332.
- Barjot, C., P. Rouanet, P. Vigneron, C. Janmot, A. Dalbis, and F. Bacou. 1998. Transformation of slow- or fast-twitch rabbit muscles after cross-reinnervation or low frequency stimulation does not alter the in vitro properties of their satellite cells. *J. Muscle Res. Cell Motil.* 19:25–32.
- Berk, D. A., F. Yuan, M. Leunig, and R. K. Jain. 1993. Fluorescence photobleaching with spatial Fourier analysis: measurement of diffusion in light-scattering media. *Biophys. J.* 65:2428–2436.
- Bouchaud, J.-Ph., A. Ott, D. Langevin, and W. Urbach. 1991. Anomalous diffusion in elongated micelles and its Levy flight interpretation. *J. Phys. II France.* 1:1465–1482.

- Clegg, J. S. 1984. Properties and metabolism of the aqueous cytoplasm and its boundaries. *Am. J. Physiol.* 246:R133–R151.
- Davoust, J., P. F. Devaux, and L. Léger. 1982. Fringe pattern photobleaching, a new method for the measurement of transport coefficients of biological macromolecules. *EMBO J.* 1:1233–1238.
- Flucher, B. E., S. B. Andrews, S. Fleischer, A. R. Marks, A. Caswell, and J. A. Powell. 1993. Triad formation: organization and function of the sarcoplasmic reticulum calcium release channel and triadin in normal and dysgenic muscle in vitro. *J. Cell Biol.* 123:1161–1174.
- Gibbon, P., and T. E. Hardingham. 1998. Macromolecular diffusion of biological polymers measured by confocal fluorescence recovery after photobleaching. *Biophys. J.* 75:1032–1039.
- Han, J., and J. Herzfeld. 1993. Macromolecular diffusion in crowded solutions. *Biophys. J.* 65:1155–1161.
- Hubley, M. J., R. C. Rosanske, and T. S. Moerland. 1995. Diffusion coefficients of ATP and creatine phosphate in isolated muscle: pulsed gradient P-31 NMR of small biological samples. *NMR Biomed.* 8:72–78.
- Jacobson, K., and J. Wojcieszyn. 1984. The translational mobility of substances within the cytoplasmic matrix. *Proc. Natl. Acad. Sci. USA.* 81:6747–6751.
- Jürgens, K. D., S. Papadopoulos, T. Peters, and G. Gros. 1997. Determination of the diffusion coefficient of myoglobin in muscle cells. Photo-oxidation and microinjection method. *Adv. Exp. Med. Biol.* 428:293–298.
- Jürgens, K. D., T. Peters, and G. Gros. 1994. Diffusivity of myoglobin in intact skeletal muscle cells. *Proc. Natl. Acad. Sci. USA.* 91:3829–3833.
- Kao, H. P., J. R. Abney, and A. S. Verkman. 1993. Determinants of the translational mobility of a small solute in cell cytoplasm. *J. Cell Biol.* 120:175–184.
- Kushmerick, M. J., and R. J. Podolsky. 1969. Ionic mobility in muscle cells. *Science.* 166:1297–1298.
- Luby-Phelps, K. 1994. Physical properties of cytoplasm. *Curr. Opin. Cell Biol.* 6:3–9.
- Luby-Phelps, K., P. E. Castle, D. L. Taylor, and F. Lanni. 1987. Hindered diffusion of inert tracer particles in the cytoplasm of mouse 3T3 cells. *Proc. Natl. Acad. Sci. USA.* 84:4910–4913.
- Luby-Phelps, K., F. Lanni, and D. L. Taylor. 1988. The submicroscopic properties of cytoplasm as a determinant of cellular function. *Annu. Rev. Biophys. Biophys. Chem.* 17:369–396.
- Luby-Phelps, K., S. Mujumbar, R. B. Mujumbar, L. A. Ernst, W. Galbraith, and A. S. Waggoner. 1993. A novel fluorescence ratiometric method confirms the low viscosity of the cytoplasm. *Biophys. J.* 65:236–242.
- Luby-Phelps, K., D. L. Taylor, and F. Lanni. 1986. Probing the structure of the cytosol. *J. Cell Biol.* 102:2015–2022.
- Mastro, A. M., M. A. Babich, W. D. Taylor, and A. D. Keith. 1984. Diffusion of a small molecule in the cytoplasm of mammalian cells. *Proc. Natl. Acad. Sci. USA.* 81:3414–3418.
- Maughan, D., and C. Lord. 1988. Protein diffusivities in skinned frog skeletal muscle fibers. *Adv. Exp. Med. Biol.* 226:75–84.
- Maughan, D., and E. Wegner. 1989. On the organization and diffusion of glycolytic enzymes in skeletal muscle. *Muscle Energetics.* 315:137–147.
- Morrot, G., S. Cribier, P. F. Devaux, D. Geldwerth, J. Davoust, J. F. Bureau, P. Fellmann, P. Hervé, and B. Friley. 1986. Asymmetric lateral mobility of phospholipids in the human erythrocyte membrane. *Proc. Natl. Acad. Sci. USA.* 83:6863–6867.
- Mosbach, K., and B. Mattiasson. 1976. Multistep enzyme systems. *Methods Enzymol.* 44:453–478.
- Munnely, H. M., D. A. Roess, W. F. Wade, and B. G. Barisas. 1998. Interferometric fringe fluorescence photobleaching recovery interrogates entire cell surfaces. *Biophys. J.* 75:1131–1138.
- Olviczky, B. P., and A. S. Verkman. 1998. Monte Carlo analysis of obstructed diffusion in three dimensions: application to molecular diffusion in organelles. *Biophys. J.* 74:2722–2730.
- Papadopoulos, S., K. D. Jurgens, and G. Gros. 1995. Diffusion of myoglobin in skeletal muscle cells—dependence on fibre type, contraction and temperature. *Pflugers Arch. Eur. J. Physiol.* 430:519–525.
- Patterson, G. H., S. M. Knobel, W. D. Sharif, S. R. Kain, and D. W. Piston. 1997. Use of the green fluorescent protein and its mutants in quantitative fluorescence microscopy. *Biophys. J.* 73:2782–2790.
- Peters, R. 1986. Fluorescence microphotolysis to measure nucleocytoplasmic transport and intracellular mobility. *Biochim. Biophys. Acta.* 864:305–359.
- Phillips, G. N., Jr. 1997. Structure and dynamics of green fluorescent protein. *Curr. Opin. Struct. Biol.* 7:821–827.
- Qian, H., M. P. Sheetz, and E. L. Elson. 1991. Single particle tracking. Analysis of diffusion and flow in two-dimensional systems. *Biophys. J.* 60:910–921.
- Saxton, M. J. 1993. Lateral diffusion in an archipelago. Dependence on tracer size. *Biophys. J.* 64:1053–1062.
- Saxton, M. J. 1994. Anomalous diffusion due to obstacles: a Monte Carlo study. *Biophys. J.* 66:394–401.
- Seksek, O., J. Biwersi, and A. S. Verkman. 1997. Translational diffusion of macromolecule-sized solutes in cytoplasm and nucleus. *J. Cell Biol.* 138:131–142.
- Small, J. V., D. O. Fürst, and L. E. Thornell. 1992. The cytoskeletal lattice of muscle cells. *Eur. J. Biochem.* 208:559–572.
- Sosa, H., D. Popp, G. Ouyang, and H. E. Huxley. 1994. Ultrastructure of skeletal muscle fibers studied by a plunge quick freezing method: myofilament lengths. *Biophys. J.* 67:283–292.
- Squire, J. M. 1997. Architecture and function in the muscle sarcomere. *Curr. Opin. Struct. Biol.* 7:247–257.
- Srere, P. A., M. E. Jones, and C. K. Mathews, editors. 1990. Structural and Organizational Aspects of Metabolic Regulation. Wiley-Liss, New York.
- Tanford, C. 1961. Physical Chemistry of Macromolecules. Wiley and Sons, New York.
- Terry, B. R., E. K. Matthews, and J. Haseloff. 1995. Molecular characterization of recombinant green fluorescent protein by fluorescence correlation microscopy. *Biochem. Biophys. Res. Commun.* 217:21–27.
- Trinick, J. 1994. Titin and nebulin: Protein rulers in muscle? *Trends Biochem. Sci.* 19:405–409.
- Wojcieszyn, J., R. A. Schlegel, and K. A. Jacobson. 1981. Measurements of the diffusion of macromolecules injected into the cytoplasm of living cells. *Cold Spring Harb. Symp. Quant. Biol.* 46:39–44.
- Xu, S., S. Malinchik, D. Gilroy, T. Kraft, B. Brenner, and L. C. Yu. 1997. X-ray diffraction studies of cross-bridges weakly bound to actin in relaxed skinned fibers of rabbit psoas muscle. *Biophys. J.* 73:2292–2303.
- Yeremian, E., and P. Claverie. 1987. Analysis of multiexponential functions without a hypothesis as to the number of components. *Nature.* 326:169–174.
- Yoshizaki, K., H. Watari, and G. K. Radda. 1990. Role of phosphocreatine in energy transport in skeletal muscle of bullfrog studied by ³¹P-NMR. *Biochim. Biophys. Acta.* 1051:144–150.
- Zimmerman, S. B., and A. P. Minton. 1993. Macromolecular crowding—biochemical, biophysical, and physiological consequences. *Annu. Rev. Biophys. Biomol. Struct.* 22:27–65.