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Protein Diffusion in Living Skeletal Muscle Fibers: Dependence on Protein Size, Fiber Type, and Contraction

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ABSTRACT Sarcoplasmic protein diffusion was studied under different conditions, using microinjection in combination with microspectrophotometry. Six globular proteins with molecular masses between 12 and 3700 kDa, with diameters from 3 to 30 nm, were used for the experiments. Proteins were injected into single, intact skeletal muscle fibers taken from either soleus or extensor digitorum longus (edl) muscle of adult rats. No correlation was found between sarcomere spacing and the sarcoplasmic diffusion coefficient (*D*) for all proteins studied. *D* of the smaller proteins cytochrome *c* (diameter 3.1 nm), myoglobin (diameter 3.5 nm), and hemoglobin (diameter 5.5 nm) amounted to only ~1/10 of their value in water and was not increased by auxotonic fiber contractions. *D* for cytochrome *c* and myoglobin was significantly higher in fibers from edl (mainly type II fibers) compared to fibers from soleus (mainly type I fibers). Measurements of *D* for myoglobin at 37°C in addition to 22°C led to a Q_{10} of 1.46 for this temperature range. For the larger proteins catalase (diameter 10.5 nm) and ferritin (diameter 12.2 nm), a decrease in *D* to ~1/20 and ~1/50 of that in water was observed, whereas no diffusive flux at all of earthworm hemoglobin (diameter 30 nm) along the fiber axis could be detected. We conclude that 1) sarcoplasmic protein diffusion is strongly impaired by the presence of the myofilamental lattice, which also gives rise to differences in diffusivity between different fiber types; 2) contractions do not cause significant convection in sarcoplasm and do not lead to increased diffusional transport; and 3) in addition to the steric hindrance that slows down the diffusion of smaller proteins, diffusion of large proteins is further hindered when their dimensions approach the interfilament distances. This molecular sieve property progressively reduces intracellular diffusion of proteins when the molecular diameter increases to more than ~10 nm.

INTRODUCTION

Knowledge of the structural and rheological properties of cytoplasm is necessary to develop concepts of intracellular transport processes. Experiments on the intracellular diffusion of molecules turned out to be a valuable tool for this purpose (reviewed by Luby-Phelps et al., 1988). It is known from studies in model systems how the diffusion of molecules is affected by the properties of the diffusion medium. A great deal of information about the motion of molecules in cytoplasm has been obtained from intracellular diffusion measurements of living cells with different techniques (fluorescence recovery after photobleaching (FRAP), electron spin resonance, NMR) (Jacobson and Wojcieszyn, 1984; Gershon et al., 1985; Luby-Phelps et al., 1987; Verkman, 1999; Arrio-Dupont et al., 2000). Most studies were carried out on relatively small, mononucleated cells. These studies showed that cytoplasm is a structurally dynamic, highly complex diffusion medium. The cytoplasmic filamentous and soluble proteins interfere with diffusing molecules in several ways, thereby slowing down intracellular translational diffusion.

The interior of striated muscle fibers exhibits a high degree of both structural order and dynamics due to contractile activity. It was shown in resting fibers that small molecules with a molecular mass of ≤500 Da diffuse about half as fast in skeletal muscle as in dilute aqueous solution (Kushmerick and Podolsky, 1969). There is hardly any information about the diffusional behavior of macromolecules in intact muscle cells. Diffusion experiments on skinned fibers revealed a protein diffusion coefficient that was ~10 times smaller compared with the value in water. Diffusivity decreased with increasing molecular mass in the range of 10–150 kDa (Maughan and Lord, 1988). A low sarcoplasmic protein diffusivity was also observed in living fibers for myoglobin, which has been investigated most frequently because of its involvement in intracellular oxygen transport (Baylor and Pape, 1988; Jürgens et al., 1994; Papadopoulos et al., 1995).

The aim of this study is to characterize protein diffusion in living mammalian muscle fibers for a wide range of sizes of macromolecules (12–3700 kDa). It was investigated whether the structural differences between fast and slow skeletal muscle fibers give rise to appreciable differences in sarcoplasmic diffusion of these proteins. For two of the proteins, we have investigated whether extensive contractile activity enhances diffusive protein transport in sarcoplasm, which would indicate substantial convection of the sarcoplasmic fluid induced by muscle fiber activity. Furthermore, the diffusivity of myoglobin at 37°C was also measured.

Microinjection and microspectrophotometry were employed to measure the diffusive distribution of proteins along the longitudinal axis of intact rat extensor digitorum longus (edl) and soleus fibers. The technique is similar to the one used by Baylor and Pape (1988) to trace myoglobin diffusion in frog muscle fibers, with some modifications

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allowing us to perform fast and repetitive measurements of the diffusion coefficient within the same experiment.

Six globular proteins with molecular diameters ranging from 3 to 30 nm were selected for the measurements. The proteins are well characterized, stable and (except for the biggest, earthworm hemoglobin), widely used as molecular mass markers. The heme and iron content of the molecules made fluorescence labeling unnecessary and allowed us to trace sarcoplasmic protein diffusion photometrically, despite the presence of strongly absorbing intrinsic myoglobin.

MATERIALS AND METHODS

Fiber preparation

Muscle fiber bundles were prepared from the soleus or the edl muscle of female Wistar rats (body mass 230–280 g) as described previously (Papadopoulos et al., 1995). The bundles consisted of 2–20 muscle fibers and had a length of 1.0–1.2 cm. They were mounted in a measuring chamber perfused with carbogen-equilibrated Ringer's solution (pH 7.4; pO_2 300–375 mmHg). The bundles had a thickness less than 200 μm, allowing an adequate light transmittance of the sample. The sarcomere length was noted after the bundles were mounted in the chamber for each experiment and varied randomly between 2.4 and 3.4 μm. The temperature in the chamber was controlled by a thermostat set to either 22 ± 1 or 37 ± 1°C.

Protein solutions

The proteins used for microinjection were prepared as concentrated solutions in phosphate buffer (24.7 mM KH_2PO_4, 57.6 mM K_2HPO_4, 10.0 mM NaCl). NaCl was added to increase the osmolarity to 300 mOsm/liter. The pH of the buffer was 7.17 (22°C), which is within the range of sarcoplasmic pH values found in resting muscles (Geers and Gros, 1990). Cytochrome *c* from horse heart and myoglobin from horse skeletal muscle were purchased as lyophilized powder from Serva (Heidelberg, Germany). Beef liver catalase and horse spleen ferritin were from Pharmacia (Freiburg, Germany). Solutions of each of the lyophilized proteins were prepared by dissolving the protein powder in a surplus of phosphate buffer. Insoluble material was then removed by centrifugation (2000 × *g* for 20 min at 4°C). Human HbA was prepared according to the method of Jürgens et al. (1980) from donated blood received from the local blood bank. Earthworm (*Lumbricus terrestris*) hemoglobin was purified according to the method of Gros (1978).

Utrafiltration membranes (Amicon, Beverly, MA) were used to concentrate the protein solutions to their final concentrations (Table 1). The protein concentrations were measured photometrically, using the extinction coefficients (ϵ) given in Table 1. Centrifugation of the solutions (15,000 × *g* for 20 min) revealed no formation of protein crystals or precipitates.

The purity of the prepared protein solutions (except for earthworm Hb) was checked by native gel electrophoresis. Cytochrome *c*, myoglobin, hemoglobin, and catalase moved as one distinct band in the gels; ferritin, however, showed a second band. The intensity of the band was measured densitometrically and found to make up maximally 10% of the total amount of the applied protein. The intensity and position of this band indicated the presence of ferritin dimers in the solution (Niitsu and Listowsky, 1973). The possible influence of these dimers on the magnitude of the diffusion coefficients calculated for this protein is considered below.

Absorbance spectra of the proteins were recorded to find the most sensitive wavelength for the microphotometric detection of the proteins injected into the sarcoplasm. For the heme proteins the prominent absorbance maximum (A_{max}) in the Soret band was chosen as the measuring wavelength (Table 1). Horse spleen ferritin has no absorbance maximum in the visible range, but, because of the iron content of the protein, it shows a continuous increment in the light absorption toward shorter wavelengths. Because of the decreasing sensitivity of the microscope photometer in the UV range, an optimal measuring wavelength of 400 nm was used to record ferritin diffusion in the muscle fibers.

After the protein solutions were frozen in liquid nitrogen in 50-μl aliquots, they were stored until use at −80°C.

Micropipettes

Short-shanked pipettes with tip diameters between 0.7 and 2.4 μm were drawn from borosilicate capillaries (1B100F-4; WPI, Sarasota, FL) on a programmable horizontal puller (BB-CH; Mecanex, Switzerland). The pipettes were back-filled (MicroFil; WPI) immediately before use with a small amount of thawed and filtered protein solution and mounted on a Piezo pipette holder that is part of an electronic micromanipulator (PM10; Märzhäuser, Germany). Microinjection was carried out by application of 300–660 kPa of pressure for a defined time interval (PicoPump; WPI).

Setup and measurements

The experimental setup used for the microinjection experiments is based on an assembly described by Jürgens et al. (1994). The sample chamber containing the fiber bundle was mounted on a computer-controlled scanning stage (EK8B; Märzhäuser) of a microscope photometer (UEM; Zeiss, Germany). A halogen bulb provided the light directed to the muscle fibers by a condenser lens. The transmitted light was guided through a water immersion objective lens (40×; Nikon) with a 2-mm working distance. A field diaphragm, variable in height and width, was inserted at a focal plane

TABLE 1 Some properties of the proteins used for microinjection

Protein	Mass (kDa)	Concentration of injection solution (mmol/l), (g/dl)	ε (l/mmol/cm)	Measuring wavelength (nm)
Cytochrome *c*	12.4	13.7; 17.0	106*	410
Myoglobin	17.0	17.0; 29.0	188†	409
Hemoglobin	64.5	3.3; 21.0	125‡	414
Catalase	247.5	0.22; 5.4	140§	405
Ferritin¶	450–900	0.31; 14.3	1,274	400
Earthworm hemoglobin	3,700	0.02; 7.2	27,000‖	415

Extinction coefficients (ε) at the measuring wavelength are from * Margoliash and Frohwirt (1959), † Scheler et al. (1957), ‡ Antonini and Brunori (1966) (ε refers to mmol heme in the oxygenated form), § Keilin and Hartree (1951) (ε refers to mmol heme in the Fe^{3+} state). ¶ Molecular mass and ε of ferritin depend on the iron content of the protein. ε for ferritin was calculated from the amount of lyophilized ferritin that was used to prepare the solution and the final volume after concentration of the solution. ‖ Schlom and Vinogradov (1973) (ε refers to mmol holoprotein).

into the light path to restrict the area of the sample from which the intensity of the transmitted light was measured. In the experiments, the length of the area perpendicular to the longitudinal fiber axis was chosen to be ~20% less than the fiber diameter, which ranged between 40 and 80 μm, and the length parallel to the longitudinal fiber axis was 10–20 μm. After passing through a monochromator (VIS; Zeiss), the transmitted light was received by a photometer (PM3; Zeiss) that was connected to a 12-bit AD converter. The scanning stage, monochromator, and photometer were controlled by a PC via a programmable interface.

To perform a microinjection experiment, an intact muscle fiber with a distinct cross-striation pattern was selected. A segment of the fiber, between 300 and 2000 μm in length, was then defined as the measuring distance. This was done by moving the scanning stage with a joystick and inspecting the fiber along its longitudinal axis. The stage was stopped when the fiber was out of focus and the microscope was focused again to the fiber edges. At each stop the three coordinates of the stage position were transferred to the computer. After the last coordinates were recorded, the computer interpolated between the recorded stage positions and calculated the course of the scanning path so that 100–200 measuring points were accessed along this path. Finally, a microinjection site at the center of the fiber segment was selected, and its coordinates were also transmitted to the computer. Regions near fiber ends were omitted to avoid reflection of the diffusing molecules. During a scan the stage moved stepwise along the scanning path, and after each step the intensity of the transmitted light was measured by the photomultiplier. The duration of a scan depended on the path length, the number of steps, and the averaging frequency set for the light intensity measurements; this amounted to 30–60 s. We calculated that the advancement of the diffusing front during a scan introduced an error of <1% into the results.

A reference scan was performed before microinjection to eliminate disturbances of the measuring signal resulting from extracellular material (connective tissue, capillaries with blood cells). After the reference scan was taken, 20–60 pl of concentrated protein solution was microinjected into the fiber within a few seconds. Up to 15 successive scans were then performed to record the changes in the transmittance profiles caused by axial diffusion of the protein. Scans were performed until peak absorbance fell to values less than 0.05, where signal/noise ratio became unacceptable. Duration of the experiments thus depended mainly on the microinjected volume and on the magnitude of D and ranged from 5 min to 3 h. Fig. 1 shows data from a microinjection experiment, where myoglobin diffusion in a soleus fiber was studied. The first light intensity scan recorded after microinjection (*open circles*, indicated by 0′ in Fig. 1 *A*) shows myoglobin already being distributed over ~100 μm at both sides of the injection point. This relatively large distribution width at the beginning of the diffusion measurements can be attributed to the injection pulse and to the time needed to withdraw the micropipette from the bath and move the stage to the starting point of the scan. At the distal sites of the scanned segment that had not yet been reached by the diffusing front of the protein, light intensity values of the reference scan and those recorded after microinjection exhibited no significant differences. Movement artifacts, occurring in ~10% of the scans, caused drastic changes in the absorbance profiles. Such measurements were discarded.

Evaluation of data

The axial spread of the microinjected proteins in the muscle fibers was regarded as a one-dimensional diffusion process with infinite extension of the diffusion path. The partial differential equation

$$\frac{\partial C}{\partial t} = D \cdot \frac{\partial^2 C}{\partial x^2} \tag{1}$$

describes the proportionality between the change of $\partial C/\partial x$ along the diffusion path x and the change in concentration with time ($\partial C/\partial t$). Assuming Beer's law to hold for the muscle fiber, the observed changes in absorbance can be used instead of C in Eq. 1. D, the translational diffusion coefficient, was calculated using a numerical solution (Crank-Nicolson algorithm) of Eq. 1. The first absorbance profile recorded after injection was always taken as the initial condition (see, for example, Fig. 1 *B*, *open circles*, marked by 0′). The curves recorded afterward (designated by 2.5′, 7.5′, and 17.5′, respectively, in Fig. 1 *B*) were fitted with a least-squares

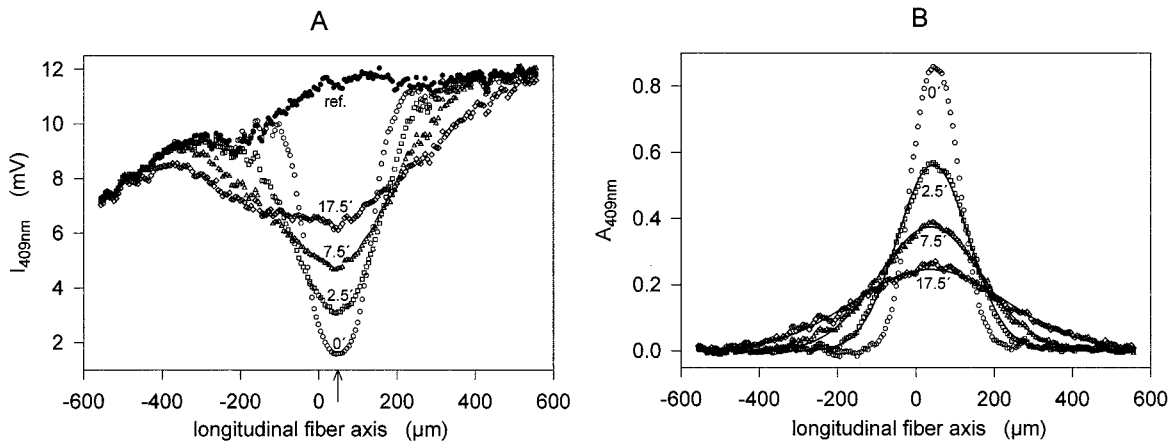

FIGURE 1 (*A*) Light intensity profiles taken before (*filled circles*, reference scan) and at different times after (*empty symbols*) microinjection of 26 pl myoglobin into a rat soleus fiber. The abscissa represents the scanned distance along the longitudinal fiber axis. The injection site was at +50 μm (*arrow*). Ordinate: Intensity of transmitted light (mV) at 409 nm. Each profile consists of 200 measuring points recorded within 50 s. The first intensity profile recorded after microinjection (*empty circles*) is marked 0 min; the second (*empty squares*), third (*empty triangles*), and fourth (*empty diamonds*) intensity profiles were recorded 2.5, 7.5 and 17.5 min later, respectively. (*B*) The abscissa is the same as in Fig. 2 *A*, and on the ordinate the myoglobin related absorbance in the Soret band ($A_{409\ nm}$) is plotted. The absorbance data were calculated by dividing the light intensities of the reference scan and the respective postinjection scan and building the $\log_{10}$ of the result. The data were fitted by a least-square method to the solution of the one-dimensional diffusion equation to calculate sarcoplasmic D. The initial distribution (*empty circles*) is marked 0′. The D found to describe the set of curves most adequately (*solid lines*) in this case is 1.6×10^{-7} cm^2/s (T = 22°C). The sarcomere spacing was 2.8 μm, and the fiber diameter amounted to 73 μm.

method either separately or, as in the example shown in Fig. 1, as an ensemble to the solution of the differential equation by varying D. As Fig. 1 B shows (*solid lines*), the measured profiles are well described by the model of one-dimensional diffusion with a single diffusion coefficient, in this case 1.6×10^{-7} cm^2/s.

Contractions

To investigate the influence of contractions on the intracellular spread of the microinjected proteins, additional experiments were carried out, in which the muscle fibers were electrically stimulated to perform auxotonic contraction series between the scans. For these experiments, either myoglobin or hemoglobin was injected into fibers of the soleus muscle. The experimental protocol for the contraction experiments has been described elsewhere (Papadopoulos et al., 1995). Under the chosen conditions, the fiber bundles contracted to ~60% of their initial length. Each cycle of contractions lasted for 20 min, during which the bundle shortened 1200 times.

RESULTS

Table 2 shows the diffusion coefficients (D) found for the microinjected proteins in single soleus and edl muscle fibers at 22°C and relates the results to the D values of the same proteins in dilute aqueous solution. In general, the D values measured in muscle fibers are relatively low. D of cytochrome c, myoglobin, and hemoglobin in soleus fibers is ~1/10 of the respective value in water. Whereas this is also true for hemoglobin diffusion in edl fibers, D of the smaller proteins cytochrome c and myoglobin is significantly higher ($p < 0.001$) in the fast muscle type. The diffusion coefficient measured for myoglobin at 37°C in soleus fibers ($n = 24$, not shown in Table 2) amounts to $D = (22.0 \pm 1.2) \times 10^{-8}$ cm^2/s.

The relative reduction of D within fibers is higher for catalase and is even more pronounced for ferritin, where sarcoplasmic diffusion is ~20 (catalase) to ~60 (ferritin) times slower than in dilute solution. Like the values for hemoglobin, the respective mean D values in edl and soleus fibers are not significantly different for the two proteins.

An estimate of the degree to which the 10% dimer content in the ferritin solution (see Materials and Methods) may have affected the results obtained for this protein shows that only minor effects can be expected. For the calculation, an arbitrary absorbance profile was created, representing the initial distribution of ferritin at $t = 0$ min. On the basis of this distribution, a second curve was then calculated for $t = 30$ min, assuming as a worst case that 10% of the molecules were immobile during that time and that the remaining 90% diffused with $D = 0.6 \times 10^{-8}$ cm^2/s, the value obtained for ferritin in soleus fibers. The two profiles were then subjected to the usual fitting procedure. The deviation of the calculated D from the inserted D of 0.6×10^{-8} cm^2/s amounted to 8%, which is negligible compared to the scatter in the diffusion data obtained for ferritin (Table 2).

No diffusion along the longitudinal fiber axis in both edl and soleus fibers could be observed for earthworm Hb. Fig. 2 shows nine subsequent absorbance profiles, measured over 35 min after microinjection of the protein. The profiles are almost identical, and no spreading of the molecules along the fiber can be observed (indicated by the *arrows* in Fig. 2). If sarcoplasmic diffusion of that protein takes place at all, the diffusion coefficient should be extremely small. This is illustrated in Fig. 2 by the dotted line profile, which indicates how far the distribution of earthworm Hb would extend after 35 min, if the protein diffuses 50 times more slowly ($D = 2.6 \times 10^{-9}$ cm^2/s) in fibers than in dilute aqueous solution ($D = 1.3 \times 10^{-7}$ cm^2/s; Gros, 1978). For this calculation, a Gaussian-shaped initial distribution, similar to the shape of the first profile measured, was assumed. As the result shows, the distribution of the protein after a 35-min diffusion would extend to ± 200 μm from the injection site, whereas the actually measured profile at 35 min is practically unchanged at ± 100 μm.

TABLE 2 Protein diffusion in muscle and aqueous solution at 22°C

Injected protein	d (nm)	Soleus $D_{cell} \pm$ SE (n)	edl $D_{cell} \pm$ SE (n)	D_{H_2O}	Soleus D_{cell}/D_{H_2O}	edl D_{cell}/D_{H_2O}
Cytochrome c	3.1	13.0 ± 0.6 (27)	16.2 ± 0.6* (22)	120	~1/9	~1/7
Myoglobin	3.5	12.5 ± 1.3 (12)	18.7 ± 0.8* (12)	112	~1/9	~1/6
Hemoglobin	5.5	6.3 ± 0.5 (11)	6.2 ± 0.4 (13)	74	~1/12	~1/12
Catalase	10.2	2.6 ± 0.4 (12)	1.9 ± 0.2 (16)	43	~1/17	~1/23
Ferritin	12.2	0.6 ± 0.1 (10)	0.9 ± 0.1 (26)	38	~1/63	~1/42
Earthworm hemoglobin	30	0	0	13	→ 0	→ 0

d, hydrodynamic diameters; D_{cell}, sarcoplasmic D measured in this study. n, number of experiments, where each fiber was used once for microinjection. D is in 10^{-8} cm^2 s^{-1}. D values in dilute aqueous solution (D_{H_2O}) are from Ehrenberg (1957) for cytochrome c, Riveros-Moreno and Wittenberg (1972) for myoglobin and hemoglobin, Samejima et al. (1962) for catalase, and Gros et al. (1982) for ferritin and earthworm Hb.
* Significant difference ($p < 0.001$, Student's t-test for unpaired samples) between D in soleus and edl fibers.

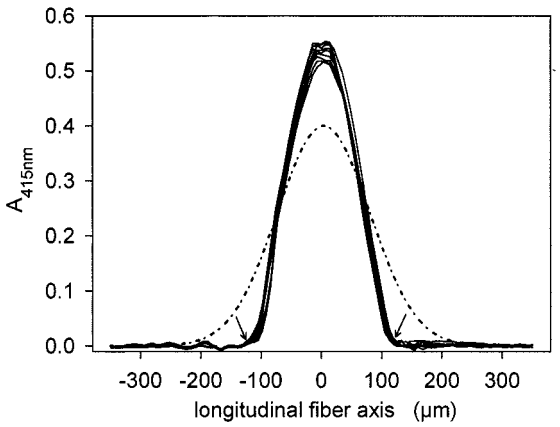

FIGURE 2 Absorbance profiles (continuous lines, λ = 415 nm) measured within 35 min after microinjection of *Lumbricus terrestris* hemoglobin into a soleus muscle fiber. The protein was microinjected at 0 μm on the abscissa; nine profiles are shown. The arrows are pointing at profile regions where a high concentration gradient for earthworm Hb persisted during the experiment, without causing a detectable spread of the molecules. The dotted line illustrates the theoretical distribution of earthworm Hb 35 min after microinjection, if the protein were to diffuse in sarcoplasm 50 times more slowly ($D = 2.6 \times 10^{-9}$ cm^2/s) than in dilute aqueous solution ($D = 1.3 \times 10^{-7}$ cm^2/s).

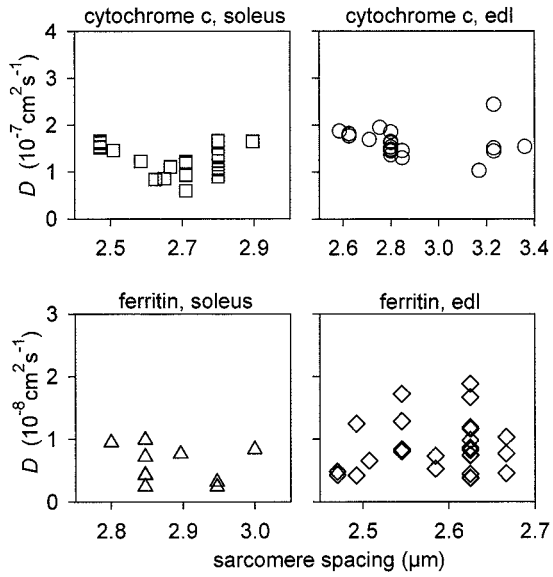

FIGURE 3 Intracellular diffusion coefficients (D) versus sarcomere spacing for cytochrome *c* (*top*) and ferritin (*bottom*) in soleus and edl fibers. The results demonstrate no significant dependency of the values of D on sarcomere length (correlation coefficients between 0.12 and −0.30).

Influence of contractions

Extensive auxotonic contractions at 22°C did not lead to a significantly accelerated spread of microinjected myoglobin and hemoglobin within soleus fibers compared with the mobility of these proteins in resting fibers. For myoglobin, the mean D value found in contraction experiments ($n = 7$) was $(13.3 \pm 0.7) \times 10^{-8}$ cm^2/s. The respective diffusion coefficient for hemoglobin ($n = 10$) amounted to $D = (7.3 \pm 0.6) \times 10^{-8}$ cm^2/s.

Sarcomere length and calculated diffusion coefficients

The D values listed in Table 2 were calculated from experiments on fiber bundles that differed in sarcomere length. A statistical analysis of sarcomere lengths versus the respective diffusion coefficients revealed no significant correlation for the range of sarcomere lengths between 2.4 and 3.4 μm. This is illustrated in Fig. 3 for the diffusion measurements with the small cytochrome *c* and the relatively large ferritin molecule.

Duration of experiments and calculated diffusion coefficients

As has been mentioned in Materials and Methods, the duration of the experiments varied considerably, ranging from 5 to 180 min. For each set of measurements it was deduced whether there was a correlation between the individually calculated D values and the time at which the corresponding profile was measured. Fig. 4 shows the results obtained for myoglobin diffusion in soleus fibers. It can be seen that there is no systematic variation of the diffusion coefficient with time. Similar results were observed for all other proteins studied. The variation of D seen

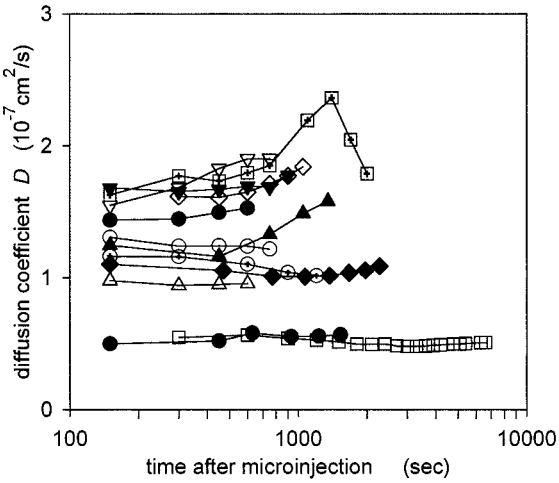

FIGURE 4 Intracellular D versus time at which the corresponding profiles were recorded. Shown are the results obtained for myoglobin diffusion in rat soleus fibers. Different symbols represent measurements carried out in 12 different muscle fibers (mean D value of 12.5×10^{-8} cm^2/s; see Table 2). There is no systematic correlation between the diffusion coefficient and the time at which the profile used to calculate D was recorded. The scatter of D within one series of profile recordings in an individual muscle fiber is smaller than its variation between different muscle fibers.

in subsequently recorded profiles in one fiber was always smaller than the variation of D found between different muscle fibers.

A decrease in D with time would be expected, if the impulse that was used to inject the protein solution into the fiber would only slowly disappear and still affect the first profiles recorded. A continuously decreasing convective component can therefore be excluded as an insignificant factor.

An increase in D during successive scans would be expected if the microinjected solution were to lead to a substantial increase in the protein concentration around the microinjection site. The highest protein concentrations used for the experiments were those of myoglobin (29 g/dl) and hemoglobin (21 g/dl). If such a high concentration (and/or reversible aggregation of proteins) were to affect the present measurements, one would expect D to be smaller at the injection site and to increase when the protein solution is diluted because of the progressing diffusion process. D would thus be a function of profile width and, for successive scans, a function of time. The absence of such an effect in the measurements can be explained by the drastic dilution of the protein solutions already occurring during microinjection. This can be deduced from Fig. 1. From the injected volume and the fiber diameter it can be calculated that the protein solution immediately after microinjection would have filled a fiber segment only 10 μm in length, if no dilution of the injected solution had taken place. But, as the much broader distribution of the protein seen immediately after microinjection (200 μm in Fig. 1 *B*) and the low maximum absorbance value (<1) show, the microinjected solution was diluted by ~20-fold at the injection site. Thus local protein concentrations in the injected fiber are raised only insignificantly above the normal total protein concentration in a fiber of ~25 g/dl.

DISCUSSION

Mainly three factors have been discussed in the literature as being responsible for a low translational diffusivity of macromolecules within cells: a high viscosity of the intracellular diffusion medium due to the soluble cytoplasmic proteins, steric hindrance toward the diffusing molecules exhibited by cytoskeletal structures within cells, and reversible binding of the diffusing proteins to intracellular structures. As to the last point, it cannot be completely excluded that some of the proteins used in the measurements exhibit some affinity for mobile or immobile sarcoplasmic constituents. However, the present study shows a clear dependence of D on protein size in intact skeletal muscle fibers, whereas strong intracellular binding, as has been postulated by Jacobson and Wojcieszyn (1984) for protein diffusion in fibroblasts, would have led more or less to the absence of a dependence of the diffusivities from molecular mass.

Sarcoplasmic viscosity

Because according to the Stokes-Einstein equation D is inversely related to viscosity (η), it is to be expected that a high cytoplasmic viscosity will lead to a low D within fibers, relative to diffusion in dilute solution. If the low D values found here within fibers were attributed solely to sarcoplasmic viscosity, η from diffusion of myoglobin in edl fibers would be 6×10^{-3} Pa s (6 cP), whereas from diffusion of ferritin in soleus fibers it would amount to $\sim 6 \times 10^{-2}$ Pa s (60 cP). Sixty centipoise is the viscosity found in 42 g/dl human hemoglobin solution (Gros, 1978), which is almost twice as concentrated as the total muscle protein concentration. A more appropriate assessment of the influence of soluble sarcoplasmic proteins on diffusion can be made by considering results on the concentration and size dependence of protein self-diffusion in simple protein solutions (Riveros-Moreno and Wittenberg, 1972; Gros, 1978). According to these results, and with the assumption that the soluble protein concentration in sarcoplasm of adult muscle fibers hardly exceeds 10g/dl (Hasselbach and Schneider, 1951; Robinson, 1952; own observations), the sarcoplasmic D of the relatively small microinjected proteins cytochrome *c* and myoglobin should be only slightly decreased by 20–30% compared with dilute solution. The D values of hemoglobin and catalase should be decreased by ~40%, and those of the larger ferritin and earthworm Hb by ~50% within fibers compared with water. Such reductions agree nicely with results presented for small molecules like ATP and PCr (Hubley et al., 1995). But as seen in Fig. 5,

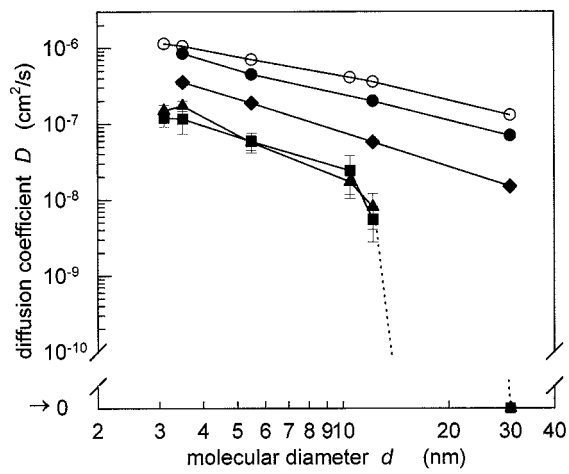

FIGURE 5 Relation between molecular size (d) and diffusion coefficient (D) for the six microinjected proteins in different diffusion media (20°C). ○, D values for diffusion in dilute aqueous solution. ●, ♦, D measured in a 10 g/dl and 24 g/dl protein solution, respectively (no D values for these protein concentrations are available for cytochrome *c* and catalase). The results obtained for intracellular D are indicated by ■ (soleus fibers) and ▲ (edl fibers). Bars indicate SD. The dotted line indicates the drop in the diffusion coefficient of earthworm Hb (d = 30 nm) to a nonmeasurable low value.

these diffusivities are much higher than the measured intracellular ones. In addition to the *D* values measured for the injected proteins at 22°C in soleus and edl fibers, the figure shows the diffusion coefficients in dilute aqueous solution as well as in 10 g/dl and 24 g/dl protein solution. The *D* values shown for 24 g/dl indicate that even if the total muscle protein concentration (structural plus soluble proteins) of ~24 g/dl (Moll, 1968) is assumed to determine the intracellular viscosity, the expected *D* values are still 2–10 times higher than the protein diffusivities actually found in intact fibers. It is clear from these results that viscosity effects alone cannot account for the low intracellular protein diffusivities observed in skeletal muscle fibers.

Effects due to the presence of structural barriers

It is known from theoretical (Wang, 1954; Blum et al., 1989) and experimental (Hou et al., 1990) studies that dimensions and arrangement of filamental structures exert a strong influence on the translational movement of molecules. The diffusional spread of proteins is probably hindered in several ways because of the presence of intracellular structures. First, the sarcoplasmic constituents occupy space that otherwise would be free for molecular movements (Blum et al., 1989). Second, the filaments (including cross-bridges) act as obstacles and make the diffusion paths longer because they have to be bypassed by the diffusing molecules. This tortuosity effect contributes to the impaired diffusion of the microinjected proteins along the fiber. Third, it is expected that relatively rigid globular particles like the proteins used are excluded from sarcoplasmic regions where the interobstacle distances are smaller than the dimensions of the diffusing molecule. Thus the intracellular structures may act like molecular sieves. About 80% of the volume of adult rat skeletal muscle fibers contains myofibrils (Table 3). The remaining volume is largely filled with organelles (nuclei, sarcoplasmic reticulum, mitochondria) that probably represent impermeable obstacles to the diffusion of proteins. It can thus be expected that it should be mainly the structural properties of the myofibrillar space that determine protein diffusion. Because the mobility of proteins was measured in a direction parallel to the fiber axis, we have to consider the cross-sectional pattern of the different sarcomeric elements. In the overlap zone of the A-band, where the myofilaments show the typical hexagonal arrangement, the surface-to-surface distance of the filaments is only between 8 and 15 nm. The variation is due to the dependence of filament spacing on sarcomere length (Elliott et al., 1963). In this region, protruding cross-bridges might additionally obstruct the diffusional spread in longitudinal direction. In the H-zone, where thin filaments are absent, the surface-to-surface distance between thick filaments is 25–30 nm, and at the M-line region filaments that are oriented either parallel or perpendicular to the longitudinal fiber axis form a lattice with spaces ~20 nm wide (calculated from Knappeis and Carlsen, 1968). In the I-band region, where thick filaments are lacking, the regular arrangement of filaments is absent and the dimensions of the interfilament spaces vary strongly. In immediate vicinity to the Z-disc, the thin filaments are oriented tetragonally with interfilamental surface distances of ~20 nm. Finally, in the complex Z-disc structure (Kelly and Cahill, 1972) there seem to be two lattice patterns that are typical for contracted and relaxed muscle, respectively (Goldstein et al., 1991). In this region, surface-to-surface filament distances in the range of 15–20 nm are likely to exist. In addition to the filaments that functionally belong to the contractile apparatus, muscle fibers also contain a cytoskeletal system comprising microtubules and intermediate filaments (reviews of Lazarides (1982) and Waterman-Storer (1991); see also Table 3).

As has been stated (Table 2), diffusion of the three smallest proteins (3–6-nm diameter) is hindered to about the same relative extent. This may be so because the sizes of these molecules are all below the listed intermyofibrillar and lattice space distances, and thus no significant exclusion from the myofibrillar meshwork occurs. Thus the main diffusion hindrance for these molecules should be the tortuosity of the diffusion path (e.g., due to myofilaments, cross-bridges, filaments of the Z-disc and M-lines, organelles). For molecules of the size of catalase (diameter ~10 nm) and, to a greater extent, of ferritin (diameter of 12.2 nm), additional diffusion barriers within the sarcoplasm may become critical. Because the sizes of these bigger proteins are very similar to the mesh width of the myofibrillar lattice at certain locations, the molecules should experience serious hindrance from these structures, although there is evidence that there is no absolute exclusion of molecules the size of catalase and ferritin from the myofibrillar space (Franzini-Armstrong, 1970). On the basis of the filament distances listed above, the critical regions are likely to be the zone of actin-myosin overlap and perhaps the Z-disc. These large proteins may have to circumvent these regions by diffusion through the limited extramyofi-

TABLE 3 Morphological data from adult rat edl and soleus

Parameter	edl	Soleus
Type I fibers (SO) (%)*	3	84
Type IIa fibers (FOG) (%)*	59	16
Type IIb fibers (FG) (%)*	38	0
Mitochondria (vol%)†	4.9	8.3
Sarcoplasmic reticulum (vol%)†	9.3	5.3
Z-disc width (nm)*	64	112
M-line‡	Narrow, three M-bridges	Wide, five M-bridges
Relative content of microtubuli§	1.0	1.7
Nuclei (vol%)†	1.0	1.7
Myofibrils (vol%)¶	81.0	80.2

* Shah and Sahgal (1991), † Davey and Wong (1980), ‡ Sjöström et al. (1982), § Stromer (1995), ¶ van Ekeren et al. (1992).

brillar space or may be delayed until local structural fluctuations allow passage of the bottleneck. These proteins will thus be retarded to a greater extent while traversing a sarcomere in the longitudinal direction than the smaller proteins. The fact that no diffusional movement of earthworm Hb (diameter 30 nm) along the longitudinal fiber axis could be observed indicates that this particle is trapped in both myofibrillar (if it has access to the myofibrillar space at all) and extramyofibrillar regions. It seems, therefore, that even the extramyofibrillar space cannot serve as an effective diffusion path for the diffusion of molecules the size of earthworm hemoglobin.

Bound water?

It has been postulated that structural proteins within cells are surrounded by relatively thick layers of bound water with increased viscosity. Morel (1985) suggested that the water shell of myofilaments could have a thickness between 3 and 9 nm. The existence of such layers in muscle fibers could impair the diffusion of molecules with dimensions well below 10 nm. Wegmann et al. (1992) favored this effect as a mechanism by which enzymes of average size like creatine kinase (83 kDa; diameter ~6 nm) are largely excluded from the actomyosin overlap zone. However, the presence of significant amounts of bound water within cells has been questioned (Fushimi and Verkman, 1991). Fushimi and Verkman, employing picosecond polarization microfluorimetry of small molecules, measured the fluid phase viscosity within fibroblasts to be not very different (η = 1.2–1.4 cP) from that of free water. They concluded that most of the water within cells has no altered physical properties that would be expected if a significant fraction of water would be in a bound state. Similar results were obtained by Luby-Phelps et al. (1993). A comparison of rotational and translational protein diffusion in muscle argues also against a marked amount of bound water (see below).

Protein diffusion in different fiber types

The edl and the soleus muscle of rats differ markedly in their fiber type composition and their ultrastructures. To see if these differences have an impact on diffusion, measurements were made in fibers from both muscles. As shown in Table 2, the diffusivities of cytochrome *c* and myoglobin were found to be significantly ($p < 0.001$) higher in fibers of the edl compared to soleus. Table 3 lists some morphological parameters of adult rat edl and soleus muscles. The most striking structural difference between the fiber types appears to be represented by the thickness of the Z-disk and the number of M-bridges that connect the filaments of the M-line. As has been stated above, these structures could represent effective barriers to longitudinal protein diffusion, so that the differences in these structures could explain the different diffusivities observed in the two muscles for both cytochrome *c* and myoglobin. However, it is not clear why the intracellular *D* of the larger proteins was not significantly different in fibers of the two muscles.

Influence of contraction

We find that extensive contractile activity does not increase sarcoplasmic *D* of myoglobin. Baylor and Pape (1988) also could not observe such an effect in frog skeletal muscle fibers that were either electrically stimulated or passively stretched and shortened. To determine whether this behavior is unique to myoglobin, we also conducted contraction experiments with hemoglobin, which has a fourfold higher molecular mass. Moreover, no significant difference was observed for this protein between resting and contracting fibers. These results show that contractions do not lead to appreciable convection within fibers, thereby enhancing the diffusional spread of molecules. The hypothesis of convectional transport of myoglobin (Mb) in sarcoplasm during contractions was proposed by Gayeski and Honig (1983) to explain the flat intracellular [MbO_2] gradients that the authors observed in heavily working muscles in the direction perpendicular to the fiber axis.

Temperature dependence

The diffusion coefficient of myoglobin, which was of special interest to us because of its potential role in intracellular O_2 transport, was determined at 37°C in addition to 22°C. The figure obtained at 37°C is 1.8 times higher than that determined at 22°C. From the two *D* values a Q_{10} of 1.46 is calculated. This value is somewhat higher than the Q_{10} of 1.3 derived using the Einstein equation.

Comparison of diffusivities of small molecules versus proteins in muscle cells

Fig. 6 gives a compilation of diffusion data obtained for low-molecular-mass molecules and for proteins in muscle preparations. In many studies (Kushmerick and Podolsky, 1969; Caille and Hinke, 1974; Cleveland et al., 1976; Yoshizaki et al., 1982; Bentley et al., 1993), the mobilities of small molecules (ions, ATP, P_i, phosphocreatine, O_2) were measured. Relative to their *D* values in water, intracellular diffusivities of small molecules were reduced by a factor of ~2. Furthermore, an anisotropy of the diffusion coefficient was observed for Ca^{2+} (Engel et al., 1994), O_2 (Bentley et al., 1993), and H_2O (Cleveland et al., 1976), where diffusion in radial direction was slower than along the longitudinal fiber axis. An anisotropy of sarcoplasmic *D* is also very likely for ATP and phosphocreatine (Hubley et al., 1995). This phenomenon was attributed to the higher tortu-

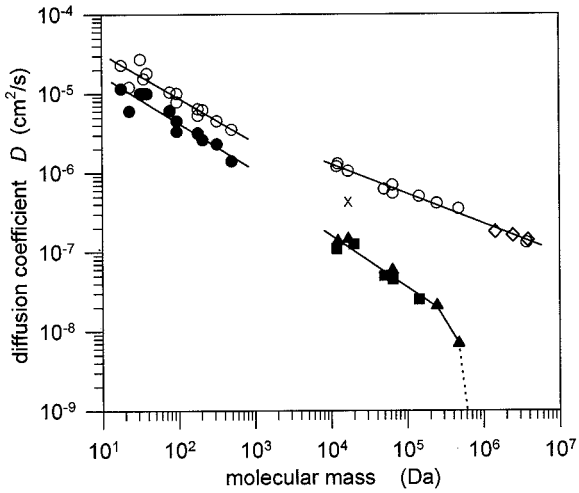

FIGURE 6 Dependency of the diffusion coefficient (D) on molecular mass for diffusion in aqueous solution (*empty symbols*) and in different muscle fiber preparations (*filled symbols*) at 20°C. In the low molecular mass range (<1 kDa) the results obtained for the diffusion of ions, metabolites, and small inert molecules are plotted (*filled circles*), whereas on the right side of the figure (>10 kDa), the D values found for proteins are shown: *filled triangles*, this work; *filled squares*, Ashley et al. (1974) and Maughan and Lord (1988). For earthworm Hb (3.7×10^6 kDa), only the D for diffusion in water is shown, as it is for eukaryotic ribosomal particles (*empty squares*, subunits and complete ribosome). ×, translational D value for sarcoplasmic diffusion of myoglobin as derived from the results obtained by Livingston et al. (1983) for rotational myoglobin diffusion in bovine heart muscle.

osity effect that is exerted by the myofilamental lattice in the radial direction.

Sarcoplasmic diffusion of proteins seems to be different from the diffusion of smaller molecules in at least two respects. First, translational protein diffusivities (*right-hand part* of Fig. 6) are considerably more reduced in muscle fibers (~10-fold for a molecular mass between 10 and 100 kDa). Second, there seems to be no significant anisotropy in the sarcoplasmic diffusion of proteins. The D values obtained in skinned muscle fibers by Maughan and Lord (1988) for radial diffusion of intrinsic muscle proteins in the molecular mass range between 12 and 144 kDa are very similar to the D values presented in this study for diffusion in the longitudinal direction in the same range of molecular mass. Ashley et al. (1974) found the radial diffusion coefficient of aequorin (~20 kDa) to be $1–1.5 \times 10^{-7}$ cm²/s in bundles of barnacle myofibrils at 20°C, a result that agrees nicely with the longitudinal D for the similarly sized myoglobin presented here and in previous studies (Baylor and Pape, 1988; Jürgens et al., 1994). However, the longitudinal D value for aequorin found by Blinks et al. (1978) in amphibian skeletal muscle fibers is two times lower than that of Ashley et al. (1974) when corrected to 22°C, using a Q_{10} of 1.46.

Recently, Arrio-Dupont et al. (2000) measured the diffusion coefficients of proteins (between 21 and 540 kDa) in the cytoplasm of cultured muscle cells. As in this study, the authors found a negative correlation between protein size and intracellular D, but their values (except for the 540-kDa protein) were considerably higher than the diffusion coefficients reported here. This may indicate that protein diffusion is less hindered in myotubes compared to adult muscle fibers. The lower actomyosin content of immature fibers compared to mature adult skeletal muscle fibers (Robinson, 1952) may account for this observation.

An abrupt decrease of sarcoplasmic D with increasing molecular size is obvious for a molecular mass of >100 kDa (Fig. 6). Proteins like glycerine-aldehyde-3-P-dehydrogenase (144 kDa), catalase (248 kDa), ferritin (450 kDa), and β-galactosidase (540 kDa) diffuse remarkably slowly and earthworm Hb (3700 kDa) probably does not diffuse at all in muscle cells. Fig. 6 also shows the diffusion coefficients measured in dilute aqueous solution for both the subunits (38S and 59S) and the complete particle (81S) of eukaryotic ribosomes (Nieuwenhuysen and Clauwart, 1981). The dimensions of the complete mammalian ribosome are 26 × 32 nm (Verschoor and Frank, 1990), which are very similar to those of earthworm Hb. There are more particles or macromolecules within fibers that have similar dimensions. Glycogen granules (20–30-nm diameter), multienzyme complexes with a molecular mass of ~2600 kDa (20–30-nm diameter; Kurganov et al., 1985), and mRNAs (diameter of a 1-kb transcript = 20.3 nm; Luby-Phelps et al., 1993) should hardly be mobile within fibers. The results obtained by Pavlath et al. (1989) and Ono et al. (1994) on the distribution of gene products from individual nuclei within muscle cells support our assumption. Pavlath et al. produced myotube heterokaryons by fusing human and murine myoblasts and, using species-specific antibodies, could show that the gene products were localized mainly in the vicinity of the nuclei responsible for their synthesis. Ono et al. (1994) transfected mouse muscle fibers with β-galactosidase genes and found a regionally restricted distribution of the enzymes after gene expression; the gene products were most abundant near the transfected nuclei. It can be assumed that in both cases the limited sarcoplasmic diffusivity of macromolecules plays a role in the formation of nucleus-associated domains in muscle fibers.

Rotational versus translational protein diffusion in muscle

Measurements of the rotational diffusion coefficient of proteins within muscle cells were also employed to obtain information about the physical properties of sarcoplasm. Wang et al. (1997) found a Mb rotational correlation time in muscle that is only 1.4 times longer than in solution. Furthermore, Livingston et al. (1983), applying proton NMR to bovine heart muscle cells, derived a rotational diffusion coefficient that is one-half to one-third that in water. From this relatively small decrease in the rotational D in muscle

cells the authors concluded that myoglobin possesses a high intracellular mobility. By analogy, this would mean that the sarcoplasmic D for myoglobin translational diffusion amounts to at least 4.2×10^{-7} cm^2/s, the value measured in 18 g/dl protein solution. But, as can be seen in Fig. 6, this value is far higher compared with the myoglobin diffusivities found in muscle by direct measurement. This illustrates that it is not possible to extrapolate the results obtained for the highly localized molecular movements like rotational diffusion, which may be unaffected by tortuosity and excluded volume effects, to long-range translational movements, especially in complex diffusion media such as the interior of a muscle cell. Moreover, the relatively high rotational mobility of proteins in muscle also argues against a significant fraction of bound water. Rotational diffusion of proteins occurring within "bound water" would be expected to be markedly slowed down. The fact that this does not appear to be so constitutes another argument against the presence of significant amounts of bound water in muscle. Thus our observations are not likely to be influenced by anomalous solvent properties of intracellular water.

Macromolecular diffusion in muscle cells versus nonmuscle cells

According to the results presented here, a critical pore size may exist within skeletal muscle fibers that prevents diffusional movement of particles with a diameter of ≥20–30 nm. Smaller proteins that are mobile in the sarcoplasm diffuse considerably more slowly (≥10-fold) than in dilute aqueous solution. Comparable results were obtained from measurements in nonmuscle cells, although hindrance to diffusion in these systems seems to be less pronounced compared with muscle fibers. Popov and Poo (1992) measured the diffusion of fluorescently labeled lactalbumin (15 kDa), ovalbumin (45 kDa), and bovine serum albumin (68 kDa) in neurites of growing *Xenopus* neurons after protein microinjection into the soma. They observed a decrease in D with molecular mass; the intracellular diffusion coefficients were five times smaller than the values in dilute solution. Swaminathan et al. (1997) used a noninvasive FRAP method to study intracellular protein diffusion by expressing green fluorescent protein (a 30-kDa green fluorescent protein) in the cytoplasm of Chinese hamster ovary cells. Translational cytoplasmic diffusion of green fluorescent protein was 3.2 times slower compared to the value in aqueous saline. Luby-Phelps et al. (1987) investigated the size dependence of the cytoplasmic diffusion of dextrans with the FRAP technique. Although D was found to decrease with increasing dextran size, no fall of diffusivity to zero even for very big dextrans (hydrodynamic diameters up to 60 nm) could be observed. The authors calculated a mean pore size of the cytoskeletal network in cultured nonmuscle cells (e.g., Swiss 3T3 cells) of ~52 nm. In addition, compartments with a smaller cutoff size for diffusion (28–36 nm) were found to exist in cytoplasm. It should be noted that dextrans are long-chain polysaccharides that do not have a fixed three-dimensional structure. Therefore, molecules with fairly well-defined dimensions like globular proteins present an advantage for the investigation of the molecular sieve properties of intracellular structures compared with flexible random coil polymers like dextrans.

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