Protein Osmotic Pressure and the State of Water in Frog Myoplasm

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ABSTRACT We measured the osmotic pressure of diffusible myoplasmic proteins in frog (*Rana temporaria*) skeletal muscle fibers by using single Sephadex beads as osmometers and dialysis membranes as protein filters. The state of the myoplasmic water was probed by determining the osmotic coefficient of parvalbumin, a small, abundant diffusible protein distributed throughout the fluid myoplasm. Tiny sections of membrane (3.5- and 12–14-kDa cutoffs) were juxtaposed between the Sephadex beads and skinned semitendinosus muscle fibers under oil. After equilibration, the beads were removed and calibrated by comparing the diameter of each bead to its diameter measured in solutions containing 3–12% Dextran T500 (a long-chain polymer). The method was validated using 4% agarose cylinders loaded with bovine serum albumin (BSA) or parvalbumin. The measured osmotic pressures for 1.5 and 3.0 mM BSA were similar to those calculated by others. The mean osmotic pressure produced by the myoplasmic proteins was 9.7 mOsm (4°C). The osmotic pressure attributable to parvalbumin was estimated to be 3.4 mOsm. The osmotic coefficient of the parvalbumin in fibers is ~3.7 mOsm mM^{-1}, i.e., roughly the same as obtained from parvalbumin-loaded agarose cylinders under comparable conditions, suggesting that the fluid interior of muscle resembles a simple salt solution as in a 4% agarose gel.

INTRODUCTION

As conventionally held, most of the intracellular osmotic pressure in muscle is due to the inorganic ions, phosphate-containing compounds, and free amino acids present in the myoplasm (Maughan and Recchia, 1985; Godt and Maughan, 1988; Wiggins, 1990). Of these, it is widely believed that the most abundant osmotically active constituents are potassium ions and phosphocreatine (Godt and Maughan, 1988, and references therein), although some investigators argue otherwise (e.g., Ling, 1984, and references therein). Intracellular proteins are likely to contribute to the total osmotic pressure, but the extent to which they contribute is unknown. In this study we measured protein osmotic pressures in frog muscle fibers, stripped of their sarcolemmas under oil, using a novel microvolumetric method based on differential dialysis membrane filtration and Sephadex bead osmometry (Edmond et al., 1968). This technique allowed the determination of osmotic pressures and osmotic coefficients of proteins in a cellular environment approximating that in vivo. Osmotic coefficients (the ratio of osmotic pressure to concentration) are routinely used in colloid chemistry to assess the state of a solute with respect to its interaction with a solvent, and vice versa (Tanford, 1961).

In this study we have focused on the osmotic pressure due to parvalbumin, a soluble calcium- and magnesium-binding protein found in high concentrations in fast twitch skeletal muscles (particularly in lower vertebrates). Because of its abundance and relatively small size, parvalbumin is well suited to serve as a probe of the intracellular milieu. Recently we reported estimates of the parvalbumin concentration and diffusion coefficients in frog myoplasm (Maughan and Godt, 1999). From comparison of parvalbumin diffusion in bulk water and myoplasm, we concluded that myoplasmic water (at least that portion through which parvalbumin diffuses) does not differ drastically from pure water. The present study supports this conclusion in that, at roughly equivalent concentrations, the osmotic coefficient of parvalbumin in muscle (3.7 mOsm mM^{-1}) is comparable to that of parvalbumin in 4% agarose (3.4 mOsm mM^{-1}). Other cytosolic proteins (creatine kinase and glycolytic enzymes) appear to have smaller osmotic coefficients (as a group, close to 2), a result that is consistent with some of these other proteins being complexed to one another or to cytomatrix proteins.

MATERIALS AND METHODS

Preparation

A fiber bundle (10–30 fibers) was cut from the isolated semitendinosus muscle of *Rana temporaria* (Charles Sullivan Co., Nashville, TN). Bundles were blotted and placed directly in a glass-bottom dish filled with water-saturated mineral oil (see below). Thus these muscles were never exposed to any artificial solution (such as Ringers) before being immersed in the oil. The temperature of the oil and the bundle was maintained at 4°C with a Peltier device (Cambion, Cambridge, MA). A single fiber segment was isolated by progressively subdividing the bundle. The segment was mechanically stripped of its sarcolemma, straightened, and manually moved to a clear region of the dish, using forceps to delicately pull the skinned fiber through the oil. Segment diameters were ~0.1 mm and lengths were 2–4 mm. The water-saturated mineral oil was produced by shaking one part distilled/deionized water and two parts mineral oil for 2 h, centrifuging the mixture (1000 g for 15 min), and drawing the water-saturated oil off the top.

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Bead osmometry and molecular filtration

Single Sephadex beads (composed of cross-linked dextrans) were used as osmometers for measuring the osmotic pressure of proteins within the skinned fiber. Our method is based on a technique developed by Edmond et al. (1968) and Ogston and Wells (1970), with an added feature of using molecular filters (dialysis membranes) between bead and fiber to differentiate the contributions of specific proteins. A filter with a 3.5-kDa cutoff should block the diffusion of virtually all proteins into the bead, thereby allowing measurement of their summed contributions to osmotic pressure. A 12–14-kDa filter permits parvalbumin (11.6–11.7 kDa (Ogawa and Tanokura, 1986)) to pass to some extent, thereby allowing a differential measurement of osmotic pressure due to parvalbumin.

In the absence of a membrane, a dry bead placed upon the skinned fiber under oil absorbs water and swells until the activity of water in the bead equals the activity of water in the fiber. A fully hydrated bead placed upon the fiber will shrink until the activities of water in bead and fiber are also equal. If the initial dry volumes (diameters) of both beads are equal, their final volumes (diameters) should be equal. By filtering the entry of cytosolic contents into each bead with the prehydrated dialysis membranes, only diffusible molecules below a certain size are permitted to enter the bead. The osmotic pressure difference across the membrane (between bead and fiber) is therefore equal to the osmotic pressure of the myoplasmic proteins that are unable to penetrate the Sephadex bead.

Beads were prepared for osmometry as follows: Sephadex G-200 beads (Pharmacia, Piscataway, NJ), 30–40 μm diameter, were placed in the oil near the skinned fiber, taking care to select only uniformly spherical beads. Bead diameters were measured to within ± 1 μm using a filar micrometer (LaSico, Los Angeles, CA). Beads were paired according to size, and one member of the pair was hydrated in relaxing solution. The relaxing solution contained (in mM) 6 $MgCl_2$, 5 Na_2ATP, 45 K_2CP, 20 imidazole, and 5 EGTA (pH 7). The ionic strength of the relaxing solution was 194 mM, similar to that in the intact frog muscle fiber (Godt and Maughan, 1988, Table 2). Beads were hydrated for a minimum of 1 h in <5 μl of relaxing solution under oil. All chemicals, unless otherwise noted, were obtained from Sigma Chemical Co. (St. Louis, MO).

Membranes used for molecular filtration were prepared as follows: Spectra/Por dialysis membrane tubing (No. 2: 12–14-kDa cutoff; No. 3: 3.5-kDa cutoff; Spectrum Microgon, Laguna Hills, CA) was cut into sections ~0.1 mm square using a scalpel under a dissecting microscope. The membrane sections were hydrated in relaxing solution for a minimum of 24 h before use, yielding a hydrated thickness of ~0.05 mm.

Protocol

Fig. 1 illustrates the experimental approach. Four membrane sections (one pair with 3.5-kDa cutoffs and one pair with 12–14-kDa cutoffs) were placed on the surface of the skinned fiber near its mid-section (Fig. 1, step 1; for simplicity, only two membranes are illustrated). Approximately 15 min was allowed for the hydrated membrane sections to equilibrate with the fiber fluid. A hydrated bead was carefully positioned on one membrane of each pair of membranes (Fig. 1, step 1, protocol A), and a dry bead was placed on the other membrane (Fig. 1, step 1, protocol B). Shrinking of the hydrated beads and swelling of the dry beads were measured at timed intervals using the filar micrometer. After the beads reached a nearly constant size (i.e., after equilibration) the beads were removed and calibrated. The time course of swelling or shrinking was generally fitted by a double exponential to obtain an estimate of the final diameter d_∞ for each bead (usually within a few percent of each other). The two values of d_∞ were averaged to obtain a mean value d*.

Bead calibration

The Sephadex beads were calibrated in calibration solutions containing a high-molecular-weight dextran that cannot penetrate the Sephadex bead

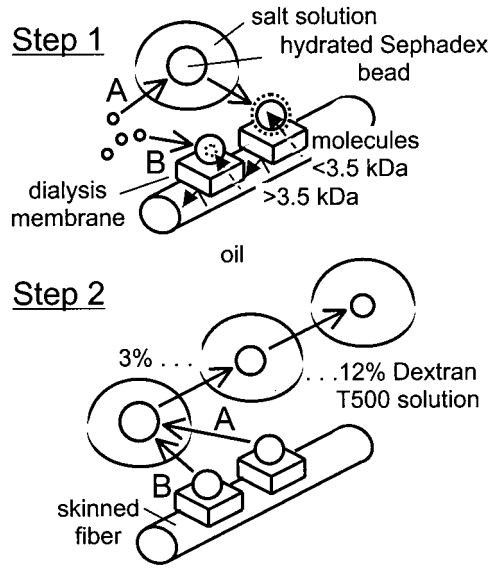

FIGURE 1 Schematic diagram of Sephadex G-200 bead osmometry on a single skinned muscle fiber under oil. Protocol A, equilibration with dry beads; protocol B, equilibration with prehydrated beads. Step 1, placement and equilibration of beads; step 2, calibration. In step 1, the dashed circles represent initial bead diameters and the solid circles represent the final bead diameters. Spectro/Por membranes were used with 3.5-kDa (shown) or 12–14-kDa cutoffs. A portion of fiber is shown. See text for details.

(Fig. 1, step 2). Drops of calibration solution, pipetted into the bottom of the oil-filled dish, were made by adding known amounts of Dextran T-500 (Pharmacia) to relaxing solution to obtain concentrations of 3, 5, 7, 10, and 12% w/v, i.e., 3–12 g added to 100 ml of relaxing solution.

The beads were placed sequentially into dextran solutions of increasing concentration. Beads remained in each solution long enough to allow equilibration with the dextran (5–10 min), indicated by no further diameter change. The diameter of each bead was plotted as a function of dextran concentration to produce a calibration curve linking osmotic pressure to bead diameter (Fig. 2). From the curve, a dextran concentration c* was obtained that corresponded to bead diameter d*. From c*, an osmotic pressure P* was calculated using the following relationship between os-

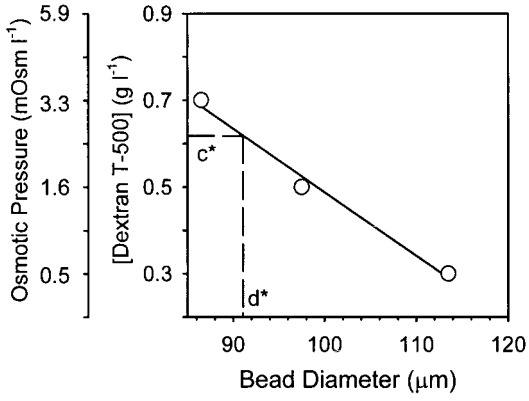

FIGURE 2 Typical calibration curve of Sephadex bead diameter versus Dextran T500 concentration. The diameter d* corresponds to that shown in Fig. 3. Experiment 013087.

motic pressure P (in atm) and concentration c (in g cm^{-3}):

$$P = A_1c + A_2c^2 + A_3c^3, \quad (1)$$

where $A_1 = 0.0655$ atm cm^3 g^{-1}, $A_2 = 10.38$ atm cm^6 g^{-2}, and $A_3 = 75.3$ atm cm^9 g^{-3} for Dextran T-500 (Vink, 1971).

Agarose cylinder preparation

To validate the approach, cylindrical rods consisting of 4% w/v agarose loaded with known amounts of bovine serum albumin (BSA) or parvalbumin in relaxing solution were used to simulate frog muscle experiments. BSA was used because previous independent determinations of its osmotic pressure have been reported over this concentration range (Scatchard et al., 1946). A 4% concentration of agarose was used because the porosity of the gel provides an aqueous environment approximating bulk water (the cylinders are ~96% water). Agarose is an extract of red algae consisting of neutral polysaccharides and sulfated agaropectins.

The 4% concentrated agarose mixture was made by adding 0.2 g of agarose (Sigma) to 5 ml of distilled water. The mixture was heated slowly in a beaker until it melted. The warm agarose mixture was quickly pumped via a syringe into tubing (0.015 inch inner diameter) that served as a mold. After cooling, the agarose cylinders were ejected into distilled water and stored in the refrigerator for up to a month before use.

The agarose cylinders were transferred to the oil bath and equilibrated with 20 μl of relaxing solution containing measured amounts of BSA or parvalbumin. After 24 h, all excess fluid was removed by suction from each protein-loaded cylinder. The agarose cylinders were moved to a clear area in the oil-filled dish, and prehydrated 3.5- and 12–14-kDa membrane sections were placed on the surface of each cylinder. Sephadex bead osmometry was performed on the protein-loaded cylinders following procedures identical to those used for the skinned muscle fiber experiments.

Determination of reflection coefficients for the dialysis membranes

From the data sheets for the dialysis membranes provided by Spectrum Microgon, we assumed that all myoplasmic proteins, including low-molecular-weight species like parvalbumin, are excluded by the dialysis membrane with the 3.5-kDa cutoff. However, for molecular masses above 6 kDa, Spectrum Microgon used various dextrans to calibrate membrane retention of protein. Because the hydrated radius of dextran is considerably larger than that of a globular protein of equal molecular weight (for example, the hydrated radius of 11.6-kDa parvalbumin is ~1.5 nm (Maughan and Godt, 1989) whereas that of 9.4-kDa dextran is ~2.9 nm (Arrio-Dupont et al., 1996)), we independently measured the relative permeability of the 3.5-kDa dialysis membrane for parvalbumin and another low-molecular-weight cytosolic protein, adenylate kinase, using small bags made from the dialysis tubing. Each bag was filled with 0.25 ml of relaxing solution containing 2 mg L^{-1} of each purified protein (Sigma): parvalbumin (from frog, approximately equal amounts of isoform IVa (11.7 kDa) and isoform IVb (11.6 kDa) (Ogawa and Tanokura, 1986); Sigma P6393 and adenylate kinase (21.6 kDa (Callaghan, 1957); Sigma M3003).

The bags were sealed and dialyzed overnight in relaxing solution (without ATP or creatine phosphate) at 4°C. Samples of dialysate were combined with equal amounts of SDS buffer solution and loaded on 10% polyacrylamide slab gels for electrophoretic separation of proteins (see Maughan and Godt, 1999, for details). Samples at various dilutions were run on adjacent lanes to assure an optimal load. Calibration curves were generated using samples of the original solution run at various dilutions. Protein bands were stained with Coomassie Blue, and the band densities were measured with a laser densitometer (Image 7000, Bio-Rad, Hercules, CA). Experimental data fell within the calibrated range, which was quite linear (r = 0.92 for parvalbumin and 0.96 for adenylate kinase). As we assumed, virtually all of the parvalbumin (average reflection coefficient 1.11 ± 0.15 SD; $n = 10$) and adenylate kinase (average reflection coefficient 1.04 ± 0.20; $n = 8$) was retained by the 3.5-kDa bags.

To obtain an estimate of the reflection coefficient of the 12–14-kDa membrane for parvalbumin under conditions approximating those of the skinned fiber experiments, we performed additional tests using 4% agarose cylinders pre-loaded with 0.96 mM (11.2 g L^{-1}) parvalbumin. This concentration of parvalbumin is close to the reported value in frog muscle fibers (0.9 ± 0.1 mM (Maughan and Godt, 1999)). Using the procedures described above, a pair-wise comparison of pressure differences showed $P_{3.5}$ (3.3 ± 1.2 mOsm; mean ± SD, throughout) was significantly higher than P_{13} (1.7 ± 1.4 mOsm; $p < 0.05$), with an average pressure difference of 1.5 ± 0.1 mOsm. Because the 3.5-kDa membrane is virtually impermeable to parvalbumin (reflection coefficient 1.0), we interpreted $P_{3.5}$ = 3.3 mOsm as representing the osmotic pressure of 0.96 mM parvalbumin in the 4% agarose cylinder ($P_{3.5}$ of a protein-free 4% agarose cylinder is, by our method, not significantly different from zero). The corresponding osmotic coefficient of parvalbumin in the 4% agarose cylinder is 3.4 ± 1.3 (= 3.3 ± 1.2 mOsm/0.96 mM). Assuming that the osmotic coefficient for parvalbumin is the same on either side of each membrane, the ratio of the osmotic pressure across each membrane equals the ratio of the reflection coefficients. $P_{13}/P_{3.5}$ is 0.49 ± 0.19 (Eq. 6.35 of Cochran, 1977, was used to calculate the standard deviation from the variance expression for ratios); therefore, under our experimental conditions, the reflection coefficient of the 12–14-kDa membrane to parvalbumin is ~0.49.

RESULTS

Validation of method using protein-loaded agarose cylinders

Experiments on protein-loaded 4% agar gels (surrogates for skinned muscle fibers) provided a positive control for the experimental approach. Fig. 3 shows a typical time course for the equilibration of G-200 Sephadex beads (one dry, the other prehydrated) on 3.5-kDa membranes placed on a 4%

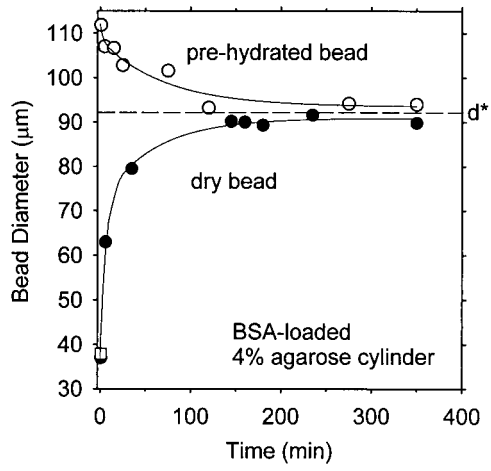

FIGURE 3 Typical time course for the swelling and shrinking of G-200 beads on a 3.5-kDa membrane on a 4% w/v agarose cylinder under oil, preloaded with 10% w/v bovine serum albumin. ●, data for dry beads; ○, data for hydrated beads (□, before hydration). Curves are least-squares fits of double-exponential functions to data points. The dashed line (d^*) is the average of the extrapolated values at $t = \infty$. Experiment 013087.

agarose cylinder containing, in this case, 10% w/v bovine serum albumin (see Materials and Methods), corresponding to 1.5 mM BSA. The final diameter of each bead was estimated by extrapolation of a least-squares curve fit. The two estimates were averaged, yielding d^* (dashed line). From calibration curves (Fig. 2), an interpolated concentration c^* corresponding to d^* was determined, from which an osmotic pressure P^* was calculated (using Eq. 1). The results are summarized in Table 1.

Our expectations were 1) the measured osmolarity should be the same using either membrane because the molecular mass of BSA (66.2 kDa (Brown, 1976)) is well above the cutoff of both filters (i.e., the reflection coefficients for BSA are ~1.0) and 2) given that the reflection coefficients of both membranes are ~1.0, the measured osmolarity should be in accord with previous values. The osmotic pressure of 10% BSA was measured in 10 agarose cylinders. As expected, the values obtained using the 3.5-kDa filters (3.8 ± 1.3 mOsm, mean ± SD) were not significantly different from those using the 13 kDa filter (3.7 ± 1.0 mOsm; $p > 0.05$). The osmotic pressure of 10% BSA in relaxing solution was therefore taken as the mean of both sets of measurements, rounded to the first decimal place, i.e., 3.8 ± 1.1 mOsm (Table 1). In similar fashion, the osmotic pressure of 20% BSA was measured in another five agarose cylinders. Again, as expected, the values obtained using the 3.5-kDa filters (9.1 ± 0.9 mOsm) were not significantly different from those using the 13-kDa filter (9.2 ± 0.6 mOsm; $p > 0.05$). The mean was 9.1 ± 0.8 mOsm (Table 1). These values, obtained in the 4% agarose cylinder using our hybrid method (dialysis membrane-Sephadex bead osmometry), are in reasonable accord with values obtained from conventional membrane osmometry of BSA in bulk water (10% BSA, 3.2 mOsm; 20% BSA, 9.2 mOsm (Scatchard et al., 1946)). (Scatchard et al. (1946) showed that the osmotic pressure (P, in mm Hg) of BSA is equal to $Aw\ (1 + Bw)$, where w is the concentration of BSA in grams per kilogram of water (their Eq. 5). For 0°C, 0.15 M NaCl, and pH 7.1 (where the valence of albumin is −17), $A = 0.268$ mm Hg kg water g^{-1}, and $B = 0.0104$ kg water g^{-1} (their Eq. 8). Assuming a density of 1 g ml^{-1} for relaxing solution and 0.0589 mOsm per mm Hg at 4°C, the osmotic pressure of 10% BSA (100 g kg^{-1}, or 1.5 mM) is 3.22 mOsm and that of 20% BSA (200 g kg^{-1}, or 3 mM) is 9.17 mOsm. The corresponding osmotic coefficients (in mOsm mM^{-1}) are 2.15 (3.22 mOsm/1.5 mM) and 3.06 (9.17 mOsm/3 mM).)

Osmotic pressure of muscle fiber proteins

The time course of volume changes of dry and hydrated beads on the dialysis membrane placed on skinned fibers (Fig. 4) resembled that obtained using agarose cylinders (Fig. 3). The skinned fibers remained relaxed during the entire time course (in some cases, minor contractions occurred at the fiber ends). For each membrane/fiber combination, extrapolated bead diameters (d_∞) were measured, and the average value was used to determine P^*, as above. Eleven skinned fibers were examined. The average osmotic pressure measured using the 3.5-kDa membrane/fiber system ($P_{3.5}$) was 9.7 ± 1.1 mOsm (Table 1). The average osmotic pressure measured using the 12–14-kDa membrane/fiber system (P_{13}) was 7.7 ± 1.4 mOsm. Pair-wise comparison of $P_{3.5}$ and P_{13} measured in the same fiber showed $P_{3.5}$ was significantly greater ($p < 0.05$) than P_{13}. The mean of the differences ($P_{3.5} - P_{13}$) was 2.1 ± 1.0 mOsm.

DISCUSSION

Highly structured water molecules occur in the monolayer adjacent to hydrophobic domains of intracellular solutes (Urry, 1995). However, the extent to which this monolayer and the several layers of (less) structured water extending beyond the first monolayer affect the osmotic properties of muscle cells remains an open question (Cameron et al., 1997). Resolution of this compelling problem is important because muscle water is commonly assumed to confer the same motional and colligative properties, including the same osmotic properties, to diffusible myoplasmic proteins

TABLE 1 Summary of results and values deduced from the results: osmotic pressures and osmotic coefficients

Molecule	System	Concentration (mM)	Osmotic pressure (mOsm)	Osmotic coefficient (mOsm mM^{-1})
BSA (10%, 100 g/L)	4% Agarose	1.51	3.8 ± 1.1	2.5 ± 0.8
BSA (20%, 200 g/L)	4% Agarose	3.02	9.1 ± 0.8	3.0 ± 0.3
Parvalbumin (11.2 g/L)	4% Agarose	0.96	3.3 ± 1.2	3.4 ± 1.3
Parvalbumin	Muscle fiber	0.9 ± 0.1*	3.4 ± 2.0	3.7 ± 1.8
All proteins† >3.5 kDa	Muscle fiber		9.7 ± 1.1	
Cytosolic proteins	Muscle fiber		8.2	
Cytomatrix proteins	Muscle fiber		1.5‡	

Results are expressed as means ± SD.
*Maughan and Godt, 1999.
†Both cytosolic and cytomatrix.
‡Matsubara et al., 1984.

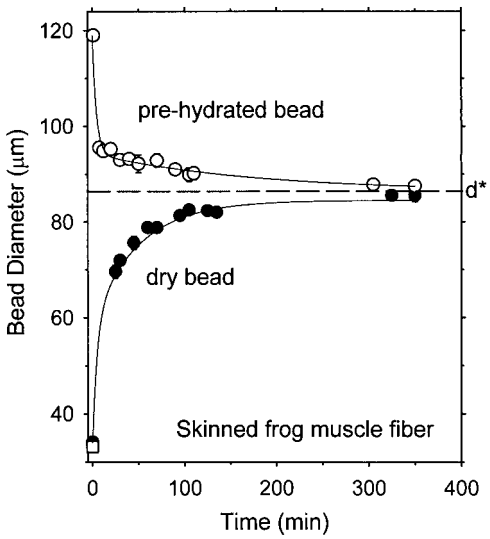

FIGURE 4 Typical time course for the swelling and shrinking of G-200 beads on a 3.5-kDa membrane on a skinned frog muscle fiber under oil. ●, data for dry beads; ○, data for hydrated beads (□, before hydration). Curves are least-squares fits of double-exponential functions to data points. Experiment 062987A.

as does bulk water. This assumption is made in virtually all studies of volume regulation, solute solubility, and other phenomena (Wiggins, 1990; Cameron et al., 1997, and references therein). Although no measurements of osmotic coefficients of proteins in a muscle cell have been made to date to corroborate this assumption, a variety of other motional and colligative properties of muscle solutes have been investigated. For example, in one early study Hill (1930) determined the weight of water in 1 g of muscle tissue that dissolved urea with a normal depression of vapor pressure (or freezing point). In frog muscle (resting or in rigor), this free, or bulk, water fraction (~0.77) turned out to constitute nearly all of the total water fraction (~0.80), suggesting that virtually all of the fluid myoplasm resembles bulk water in the sense that nearly all the fluid myoplasm dissolves conventional substances added to it with a normal depression of vapor pressure.

More recent frog muscle studies using quasi-elastic neutron scattering in whole muscle (Rorschach et al., 1987) and determinations of diffusion coefficients of ions (Kushmerick and Podolsky, 1969) and proteins (Maughan and Lord, 1988; Maughan and Godt, 1999) in isolated muscle fibers bolstered the conclusion that fluid myoplasm resembles bulk water. However, other studies using whole frog muscle (Ling and Negendank, 1970) and concentrated solutions of isolated proteins (Cameron et al., 1997) produced estimates of bulk water fractions in frog muscle that were considerably less than Hill's widely accepted value of ~0.77. This was taken to support the view that a large proportion of the solvent myoplasm interacts strongly with the myoplasmic proteins. In fact, measurements of water self-diffusion by quasi-elastic neutron scattering or nuclear magnetic resonance in a variety of cells (including frog skeletal muscle (Rorschach et al., 1987)) suggest that most, if not all, of the cellular water is slowed in its motion compared with bulk water (Cameron et al., 1997), suggesting strong interaction between protein and solvent water in the cell. According to this view, the osmotic coefficients of the proteins in the cell should be substantially greater than their values measured in bulk water.

The present study was designed to measure the osmotic coefficient of proteins in their native environment as an additional, as yet untested, approach to assess the colligative properties of proteins in the fluid cytoplasm. To measure the osmotic pressure of diffusible myoplasmic proteins in frog skeletal muscle fibers, we devised a hybrid method of single Sephadex bead osmometry and dialysis membrane filtration. To establish the validity of our method, we performed Sephadex bead osmometry on 4% agarose cylinders containing given concentrations of BSA, whose osmotic coefficients are known in bulk solution from conventional membrane osmometry. For conditions approximating those of the fibers, the osmotic pressures of 10% and 20% w/v (1.5 and 3.0 mM) BSA measured in cylinders was well within 1 SD of the expected values based on the data of Scatchard et al. (1946). Given this correspondence, Sephadex bead osmometry appears to be a valid method with which to estimate the osmotic pressures of diffusible proteins in the milliosmolar range.

Preliminary experiments using dialysis bags of the Spectra/Por membranes showed that the 3.5-kDa cutoff membrane was impermeable to parvalbumin and adenylate kinase (reflection coefficients ~1.0) and thus would be impermeable to the larger myoplasmic proteins as well. Preliminary experiments using parvalbumin-loaded 4% agarose cylinders produced a lower estimate for the reflection coefficient of the 12–14-kDa membrane for parvalbumin, namely, 0.49. Thus, for conditions that closely approximate those of the skinned fiber experiments, we assume that the reflection coefficient of the 12–14-kDa membrane is ~0.49 for parvalbumin and adenylate kinase.

In the skinned fiber experiments the average osmotic pressure measured using the 3.5-kDa membrane/fiber system ($P_{3.5}$) was 9.7 ± 1.1 mOsm. Assuming all myoplasmic proteins have a reflection coefficient of ~1 for the 3.5-kDa membrane, we conclude that 9.7 mOsm represents the osmotic pressure exerted by all myoplasmic proteins in the muscle fiber.

The average osmotic pressure measured using the 12–14-kDa membrane/fiber system (P_{13}) was 7.7 ± 1.4 mOsm. A pair-wise comparison of values between the two sets of measurements yielded a significant difference of 2.1 ± 1.0 mOsm, a reduction of pressure we attribute to the fractions of parvalbumin and adenylate kinase molecules that penetrate the 12–14-kDa membrane.

Given estimates for the reflection coefficient γ of each myoplasmic protein for each membrane, we can estimate the contributions of parvalbumin and adenylate kinase to the total protein osmotic pressure using the following expressions:

$$P_{3.5} = \phi_{parv}\gamma_{parv,3.5}[\text{parvalbumin}] + \phi_{adk}\gamma_{adk,3.5}[\text{adenylate kinase}] + \Sigma\phi_{other}\gamma_{other,3.5}[\text{other proteins}] \text{ and}$$

$$P_{13} = \phi_{parv}\gamma_{parv,13}[\text{parvalbumin}] + \phi_{adk}\gamma_{adk,13}[\text{adenylate kinase}] + \Sigma\phi_{other}\gamma_{other,13}[\text{other proteins}],$$

where ϕ_{parv} and ϕ_{adk} are the osmotic coefficients of parvalbumin and adenylate kinase, respectively, and ϕ_{other} is the osmotic coefficient of other proteins. From the above discussion, we can let $\gamma_{parv,13} = \gamma_{adk,13} = 0.49$ and $\gamma_{parv,3.5} = \gamma_{adk,3.5} = \gamma_{other,3.5} = \gamma_{other,13} = 1.0$. Assuming that the concentration of adenylate kinase is at least an order of magnitude less than that of parvalbumin (Godt and Maughan, 1988), and further assuming that the osmotic coefficient of adenylate kinase is approximately equal to that of parvalbumin ($\phi_{adk} \approx \phi_{parv}$), then

$$P_{3.5} = \phi_{parv}\{1.0 + (1.0)(0.1)\}[\text{parvalbumin}] + \Sigma\phi_{other}(1.0)[\text{other proteins}] \text{ and}$$

$$P_{13} = \phi_{parv}\{0.49 + (0.49)(0.1)\}[\text{parvalbumin}] + \Sigma\phi_{other}(1.0)[\text{other proteins}].$$

Subtracting P_{13} from $P_{3.5}$, the pressure difference attributable to parvalbumin is

$$P_{3.5} - P_{13} = \phi_{parv}(1.1 - 0.54)[\text{parvalbumin}] = \phi_{parv}(0.56)[\text{parvalbumin}].$$

Given $P_{3.5} - P_{13} = 2.1 \pm 1.0$ mOsM and [parvalbumin] = 0.9 ± 0.1 mM, the deduced osmotic coefficient of parvalbumin (ϕ_{parv}) is 3.7 ± 1.8 mOsm mM^{-1}. The corresponding osmotic pressure of parvalbumin ($\pi_{parv} = \phi_{parv}$[parvalbumin]) is 3.4 ± 2.0 mOsm ($(3.7 \pm 1.8$ mOsm mM$^{-1}) \times (0.9 \pm 0.1$ mM)).

Given the uncertainties, the osmotic coefficient of $\phi_{parv} = 3.7 \pm 1.8$ for 0.9 mM parvalbumin is not significantly different from that measured in the 4% agarose cylinder ($\sim 3.4 \pm 1.3$; see Materials and Methods), suggesting that the bulk of the fluid interior of muscle is similar to that of a simple salt solution in a 4% agarose gel. Osmotic coefficients well above unity are expected of concentrated proteins in simple salt solutions, especially if the mobile protein is strongly charged and surrounded by numerous fixed charges. Such is the situation in muscle and 4% agarose. Skinned frog muscle fibers exhibit Donnan potentials of about -3.1 mV (Maughan et al., 1995), attributable to a net negative charge ($\sim$42 meq L^{-1} (Godt and Maughan, 1988)) on the cytomatrix proteins. Agarose gels of 4% w/v exhibit Donnan potentials of ~ -7.2 mV (Aldoroty and April, 1984), attributable to the negatively charged sulfated agaropectins comprising part of the gel. Parvalbumin IVa and IVb have isoelectric points of 4.75 and 4.97, respectively (Ogawa and Tanokura, 1986), reflecting their net negative charge. Thus, osmotic coefficients of 3.4 and 3.7 for parvalbumin in 4% agarose and muscle, respectively, probably reflect, in part, interactions with fixed charges (Scatchard et al., 1946, footnote 2 in Results). Steric and other short-range interactions may also contribute to elevating the osmotic coefficients (Neal et al., 1998).

A high osmotic coefficient could also result if a substantial portion of the myoplasmic fluid was a poor solvent for parvalbumin; that is, a condition in which parvalbumin is effectively excluded from much of the fluid volume. In muscle fibers, the osmotically inactive volume is most likely the water of hydration of the cytomatrix and cytosolic proteins (Troschin, 1966; Hinke, 1970; Clegg, 1984; Wiggins, 1990; Cameron et al., 1997). In 4% agarose, the osmotically inactive fluid volume is most likely the water of hydration of the neutral polysaccharides and charged agaropectins. According to this hypothesis, the ratio of the osmotic coefficient of BSA in bulk water to that observed in the 4% agarose cylinder yields an estimate of the osmotically active fluid volume fraction in the agarose cylinder. For both 1.5 and 3.0 mM BSA, the ratio is roughly 1 (i.e., 2.53/2.15 = 1.18 and 3.03/3.06 = 0.99). Thus, within the limitations of our measurements, virtually the entire fluid interior of a 4% agarose gel appears to be accessible to BSA. By the same token, the ratio of the osmotic coefficient of parvalbumin in the 4% agarose cylinder to that observed in the skinned muscle fiber yields an estimate of the osmotically active fluid volume fraction in the muscle fiber. For $\sim$1 mM parvalbumin, the ratio is also roughly 1 (3.4/3.7 = 0.92), again suggesting that most of the fluid interior of muscle (outside the cytomatrix and intracellular organelles) is accessible to parvalbumin.

The total osmolarity of the myoplasm in frog muscle is equal to the osmolarity of the solution in which it is bathed (in frog blood or standard frog Ringer's solution, $\sim$235 mOsm (Hill, 1965; Gordon and Godt, 1970)). We have shown here that parvalbumin contributes approximately 3.4 mOsm, or $\sim$1.4% of the total. The osmotic pressure of all intracellular proteins above 3.5 kDa is 9.7 mOsm, or $\sim$4.1% of the total. This 4.1% includes the sum of the osmotic pressures produced by all diffusible cytosolic proteins as well as the osmotic pressure produced by the cytomatrix.

To determine the contribution of only the diffusible proteins to the total osmotic pressure, one must subtract the pressure contributed by the cytomatrix, which is small but

not negligible. Upon removal of the sarcolemma in aqueous relaxing solutions and evacuation of all diffusible proteins from the skinned fiber, the cytomatrix pressure causes the myofilament lattice to swell (Maughan and Godt, 1980). This pressure is equal to the pressure required to compress the otherwise swollen myofilament lattice to its in vivo spacing. Using a long-chain neutral polymer that is excluded from the lattice, Matsubara et al. (1984) found that 1.5 mOsm colloidal pressure (~4% w/v Dextran T500) was sufficient to restore the lattice to its in vivo spacing in skinned skeletal muscle fibers of *R. temporaria*. Thus, the total osmotic pressure of the intracellular proteins, including parvalbumin but excluding cytomatrix proteins, is 8.2 mOsm (9.7 mOsm − 1.5 mOsm), or 3.5% of the total.

Other cytosolic proteins, in addition to parvalbumin and adenylate kinase, include creatine kinase (~82 kDa) and a number of glycolytic enzymes (~36.5 to 372.8 kDa). The total concentration of creatine kinase and the glycolytic enzymes is roughly 2 mM (Godt and Maughan, 1988). Together these proteins exert an osmotic pressure of ~4.5 mOsm; i.e., 8.2 mOsm (total proteins) − 3.4 mOsm (parvalbumin) − 0.3 mOsm (adenylate kinase, assuming 1/10 that of parvalbumin). Thus the composite osmotic coefficient of creatine kinase and the glycolytic enzymes is also roughly 2.3, close to the osmotic coefficient of 2.15 for 1.5 mM BSA in simple salt solution. However, creatine kinase and many (if not all) of the glycolytic enzymes are probably complexed to one another or to cytomatrix proteins in vivo, as numerous studies suggest (Clarke and Masters, 1975; Minton, 1981; Fulton, 1982; Maughan and Wegner, 1989; Wiggins, 1990). If so, the concentration of the complex, and therefore the total concentration of the uncomplexed enzymes, is probably much less than 2 mM. Given this assumption, the osmotic coefficients of the uncomplexed diffusible proteins would have to be substantially greater than 2 to account for a composite osmotic coefficient close to 2. In any event, the present results indicate that the contribution of the muscle proteins to the intracellular osmotic pressure is small compared with the contributions of inorganic and small organic molecules (Maughan and Godt, 1989, and references therein). Freely diffusible parvalbumin contributes a tiny but measurable fraction of the total osmotic activity of the cell. Virtually all of the fluid myoplasm resembles bulk water in the sense that the distribution of parvalbumin in the fluid phase resembles that of parvalbumin in a simple salt solution in a 4% agarose gel.

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REFERENCES

Aldoroty, R. A., and E. W. April. 1984. Donnan potentials from striated muscle liquid crystals: A-band and I-band measurements. *Biophys. J.* 46:769–779.

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.

Brown, J. R. 1976. Structural origins of mammalian albumin. *Fed. Proc.* 35:2141–2144.

Callaghan, O. H. 1957. The purification and properties of rabbit-muscle myokinase. *Biochem. J.* 67:651–657.

Cameron, I. L., K. M. Kanal, C. R. Keener, and G. D. Fullerton. 1997. A mechanistic view of the non-ideal osmotic and motional behavior of intracellular water. *Cell Biol. Int.* 21:99–113.

Clarke, F. M., and C. J. Masters. 1975. On the association of glycolytic enzymes with structural proteins of skeletal muscles. *Biochem. Biophys. Acta* 381:37–46.

Clegg, J. S. 1984. Intracellular water and the cytomatrix: some methods of study and current views. *J. Cell Biol.* 99:167s–171s.

Cochran, W. G. 1977. Sampling Techniques. John Wiley and Sons, New York.

Edmond, E., S. Farquhar, J. R. Dunstone, and A. G. Ogston. 1968. The osmotic behaviour of Sephadex and its effect on chromatography. *Biochem. J.* 108:755–763.

Fulton, A. B. 1982. How crowded is the cytoplasm? *Cell.* 30:345–347.

Godt, R. E., and D. W. Maughan. 1988. On the composition of the cytosol of relaxed skeletal muscle of the frog. *Am. J. Physiol. (Cell Physiol.).* 254:C591–C604.

Gordon, A. M., and R. E. Godt. 1970. Some effects of hypertonic solutions on contraction and excitation-contraction coupling in frog skeletal muscles. *J. Gen. Physiol.* 55:254–275.

Hill, A. V. 1930. The state of water in muscle and blood and the osmotic behaviour of muscle. *Proc. R. Soc. Lond. B.* 106:477–505.

Hill, A. V. 1965. Trails and Trials in Physiology. Edward Arnold, London.

Hinke, J. A. M. 1970. Solvent water for electrolytes in the muscle fiber of the giant barnacle. *J. Gen. Physiol.* 56:521–541.

Kushmerick, M. J., and R. J. Podolsky. 1969. Ionic mobility in muscle cells. *Science* 166:1297–1298.

Ling, G. N. 1984. In Search of the Physical Basis of Life. Plenum Press, New York.

Ling, G. N., and W. Negendank. 1970. The physical state of water in frog muscle. *Physiol. Chem. Phys.* 2:15–23.

Matsubara, I., Y. E. Goldman, and R. M. Simmons. 1984. Changes in the lateral filament spacing of skinned muscle fibres when cross-bridges attach. *J. Mol. Biol.* 173:15–33.

Maughan, D. W., and R. E. Godt. 1980. A quantitative analysis of elastic, entropic, electrostatic, and osmotic forces within relaxed skinned muscle fibers. *Biophys. Struct. Mech.* 7:17–40.

Maughan, D. W., and R. E. Godt. 1989. Equilibrium distribution of ions in a muscle fiber. *Biophys. J.* 56:717–722.

Maughan, D. W., and R. E. Godt. 1999. Parvalbumin concentration and diffusion coefficient in frog myoplasm. *J Muscle Res. Cell Motil.* 20: 199–209.

Maughan, D., and C. Lord. 1988. Protein diffusivities in skinned frog skeletal muscle fibers. *In* Molecular Mechanism of Muscle Contraction. H. Sugi and G. H. Pollack, editors. Plenum, New York. 75–84.

Maughan, D. W., J. E. Molloy, M. A. P. Brotto, and R. E. Godt. 1995. Approximating the isometric force-calcium relation of intact frog muscle using skinned fibers. *Biophys. J.* 69:1484–1490.

Maughan, D., and C. Recchia. 1985. Diffusible sodium, potassium, magnesium, calcium and phosphorus in frog skeletal muscle. *J. Physiol.* 368:545–563.

Maughan, D., and E. Wegner. 1989. On the organization and diffusion of glycolytic enzymes in skeletal muscle. *In* Muscle Energetics. Alan R. Liss, New York. 137–147.

Minton, A. P. 1981. Excluded volume as a determinant of macromolecular structure and reactivity. *Biopolymers.* 20:2093–2120.

Neal, B. L., D. Asthagiri, and A. M. Lenhoff. 1998. Molecular origins of osmotic second virial coefficients of proteins. *Biophys. J.* 75: 2469–2477.

Ogawa, Y., and M. Tanokura. 1986. Steady-state properties of calcium binding of parvalbumins from bullfrog skeletal muscle: effects of Mg^{2+}, pH, ionic strength, and temperature. *J. Biochem.* 99:73–80.

Ogston, A. G., and J. D. Wells. 1970. Osmometry with single Sephadex beads. *Biochem. J.* 119:67–73.

Rorschach, H. E., D. W. Bearden, C. F. Hazlelwood, D. B. Heidorn, and R. M. Nicklow. 1987. Quasi-elastic scattering studies of water diffusion. *Scan. Microsc.* 1:2043–2049.

Scatchard, G., A. C. Batchelder, and A. Brown. 1946. Preparation and properties of serum and plasma proteins. VI. Osmotic equilibria in solutions of serum albumin and sodium chloride. *J. Am. Chem. Soc.* 68:2320–2329.

Tanford, C. 1961. Physical Chemistry of Macromolecules. John Wiley and Sons, New York.

Troschin, A. S. 1966. Problems of Cell Permeability. Pergamon Press, Oxford.

Urry, D. W. 1995. Elastic bimolecular machines. *Sci. Am.* 272:64–69.

Vink, H. 1971. Precision measurements of osmotic pressure in concentrated polymer solution. *Eur. Polymer J.* 7:1411–1419.

Wiggins, P. M. 1990. Role of water in some biological processes. *Microbiol. Rev.* 54:432–449.