Osmotic Properties of the Calcium-Regulated Actin Filament†

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ABSTRACT: The diameter of the actin filament decreases with an increase of the protein osmotic pressure. This phenomenon is accompanied by a decrease of the angle (α) formed between the long axis of the actin monomer and the pointed end of the filament axis. At 1.8×10^5 dyn/cm^2 (the protein osmotic pressure in frog muscle) the diameter is 8.34 nm and the angle (α) is 61.5°. The interfilament distance of tropomyosin-decorated actin filaments, at a set of different osmotic pressures, is larger than that of F-actin filaments. This suggests that the two tropomyosin helices project out of the contour of the actin filament. The tropomyosin-decorated actin filament is more rigid than F-actin. At 1.8×10^5 dyn/cm^2, the angle (α) is 76.4°, as compared to the value of 61.5° for F-actin. The interfilament distance of troponin−tropomyosin-decorated actin filaments is sensitive to Ca^{2+}: in the physiological range of protein osmotic pressure it decreases from 13.3 nm, in the presence of 2 mM EGTA, to 12.2 nm in the presence of 0.2 mM $CaCl_2$. Two alternative models are proposed to explain the decrease in interfilament distance. (a) Calcium shifts tropomyosin along the actin monomer, toward the filament axis (the classical model). (b) Calcium releases the rigidity of the tropomyosin-decorated filament and restores the original plasticity of F-actin. The consequent decrease of the angle (α) brings the tropomyosin helices nearer to the filament axis, without any real movement of tropomyosin along the actin monomer.

The characterization of the osmotic properties of charged, nonspherical, interacting proteins is difficult. Fortunately, actin and tropomyosin assemble spontaneously into helical structures, which, under proper conditions, undergo association into hexagonally packed bundles of filaments (De Rosier & Tilney, 1981). This behavior allows one to relate osmotic pressure, albeit indirectly, to the geometric parameters of the supramolecular structure. With increasing osmotic pressure, the removal of protein-bound water and a change in the volume of the system occur only if the latter change is accompanied by a suitable change of the geometric parameters of the supramolecular structure. The effects of osmotic pressure may thus provide information on the geometry of the supramolecular assembly, even though the geometry depends in a complex manner on the concentration and charge of the protein.

Exploiting this technique we have shown previously that osmotic pressure influences the diameter of the actin filament (Grazi et al., 1993) and have proposed an active role for the actin monomer in the generation of the power stroke in the binary actomyosin system (Grazi et al., 1994). We now extend these studies to the osmotic behavior of the calcium-regulated actin filament, with the aim of gaining further information on the mechanism of regulation of skeletal muscle contraction.

MATERIALS AND METHODS

G-actin (Spudich & Watt, 1971), tropomyosin (Smillie, 1982), troponin (Potter, 1982; Reisler et al., 1980), and myosin (Margossian & Lowey, 1982) were prepared from rabbit muscle. Molar concentrations were calculated on the basis of M_r values of 42 kDa for actin (Collins & Elzinga, 1975), of 65 kDa for tropomyosin (Weber & Osborn, 1969), and of 90 kDa for troponin (Sperling et al., 1979). The absorption coefficients used were the followings: actin, $A_{290}^{1\%} = 6.2$ (Gordon et al., 1976); tropomyosin, $A_{276}^{1\%} = 3.85$ (Wegner, 1979); troponin, $A_{280}^{1\%} = 4.5$ (Sperling et al., 1979); myosin, $A_{280}^{1\%} = 5.3$ (Margossian & Lowey, 1982).

The regulation by calcium of the ATPase activity of myosin was studied in the following system: myosin 0.3 mg/mL, actin 0.18 mg/mL, tropomyosin 0.14 mg/mL, troponin 0.017 to 0.05 mg/mL; 13 mM Tris-HCl buffer, pH 7.7, 5 mM $MgCl_2$, 12 mM KCl, 2.66 mM ATP, and either 0.2 mM $CaCl_2$ or 1.5 mM EGTA. Temperature was 25 °C. At the end of the incubation the reaction was stopped with 5% trichloroacetic acid. Inorganic phosphate was assayed according to Tashima and Yoshimura (1975). In the complete system, at the larger troponin concentration tested, myosin rate was 6.39 s^{-1} in the presence of calcium and 0.9 s^{-1} in the presence of EGTA.

Buffer solutions for the osmotic stress experiments contained per 1000 g of water: KCl, either 0.108 mol (buffers A and B) or 0.1 mol (buffer C); triethanolamine, 0.01 mol; $MgCl_2$, NaN_3, 2-mercaptoethanol, 2 mmol each; ATP 0.2 mmol. Either $CaCl_2$, 0.2 mol (buffer B) or EGTA, 2 mmol (buffer C) were further added. pH was taken to 7.45 with 6 N HCl.

The following protein solutions were prepared: (a) F-actin (3 mg/mL) in buffer A; (b) F-actin (3 mg/mL) and tropomyosin (0.66 mg/mL); 7:1 molar ratio, in buffer A; (c) F-actin (3 mg/mL), tropomyosin (0.66 mg/mL) and troponin (0.92 mg/mL), 7:1:1 molar ratio, in buffer B; (d) F-actin (3 mg/mL), tropomyosin (0.66 mg/mL) and troponin (0.92 mg/mL) in buffer C. Protein solutions were then dialyzed for 17 h at 2 °C against the same buffer solutions.

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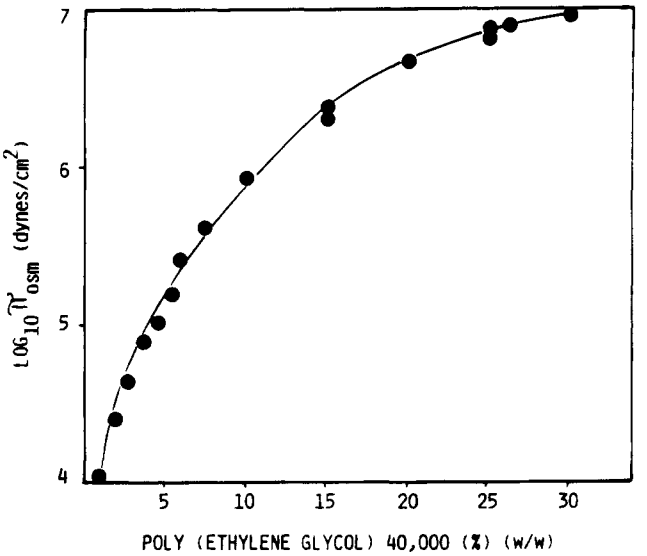

FIGURE 1: Plot of the osmotic pressure generated by poly(ethylene glycol) 40 000 (w/w) solutions. Osmotic pressure was measured as described in the methods section. Temperature was 22 °C.

The osmotic pressure of the protein systems was measured using a "secondary" osmometer: protein solutions (1 mL) were equilibrated by dialysis against buffer solutions supplemented with poly(ethylene glycol) 40 000 (100 mL). Cellulose dialysis tubing (M_r cutoff 6000) was purchased from Medicell International.

Osmotic pressure associated with poly(ethylene glycol) solutions (up to 5 g/100 g of water) was measured by means of osmometers equipped with UH 100/25 Schleicher and Schuell membranes, M_r cutoff 25 000 (Ito et al., 1987). At larger poly(ethylene glycol) concentrations measurements were made directly with a pressure gauge (Figure 1).

Equilibration of protein solutions was carried out for 48–96 h, at 22 °C, in stopped bottles, immersed in a shaker water-bath thermostatically controlled to within ±0.1 °C. At the end of the equilibration, a bag at that time was processed. It was withdrawn from the bottle, wiped off gently, and opened with a pair of scissors. The content was rapidly transferred, by means of a spatula, onto preweighed 2 × 2 cm cover glasses previously stored in a desiccator over P_2O_5.

Determination of the Wet Weight of the Sample. From the weight of the sample, determined at time intervals, in a 15 min period, the weight at 3 h is substracted (at this time evaporation is essentially complete). The plot, against time of the logarithm of these differences is linear and is extrapolated to zero time (the time of opening of the bag) to yield the wet weight of the sample.

Determination of the Dry Weight of the Sample. The sample is stored for 17 h at 80 °C and weighed and the weight of the cover glass is substracted.

Determination of the Water Weight of the Sample. The dry weight is substracted from the wet weight of the sample.

Determination of the Protein Weight. (a) The weight of salt is substracted from the dry weight. The weight of salt is calculated from the salt content of the equilibrating solution (9.29 mg/g of water) and from the water weight. (b) The cover glass is immersed for 4 h in a 1 M NaOH solution (10 mL). The absorbance at 290 nm of the resulting protein solution is then determined against a blank of 1 M NaOH. The original protein solution is utilized as a standard.

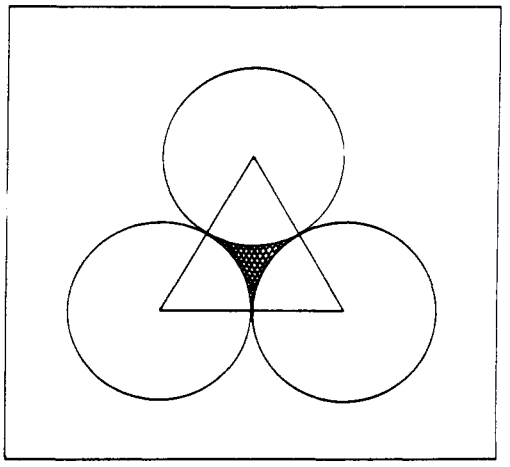

FIGURE 2: Cross section of hexagonally packed actin filaments: the elementary unit.

Measurements with the two methods were usually within 5% and were averaged.

Light scattering was determined at 500 nm with a 90° observation angle by using a Jasco FP spectrofluorimeter. In these experiments protein solutions and poly(ethylene glycol) 40 000 solutions were prepared as it was described for the osmotic stress experiments. The solutions were then mixed to give the desired protein and poly(ethylene glycol) concentrations.

THEORY

The Relationship between Actin Concentration and the Average Interfilament Distance of the Hydrated Actin Filaments. The progressive removal of the bulk water forces actin filaments to come in close contact, side by side and to form bundles (Suzuki et al., 1989; Grazi et al., 1990) of hexagonally packed (De Rosier & Tilney, 1981) actin filaments. Under these conditions the volume of the solutions is given by: volume of the hydrated filaments + volume of the interfilament spaces.

Inspection of Figure 2 shows that (a) the cross section of hexagonally packed actin filaments is composed by elementary units with the shape of an equilateral triangle of area $\sqrt{3}d^2/4$, where d is the interfilament distance; (b) the cross-sectional area of the gap between filaments is given by the area of the triangle minus the area of the semicircle $(\sqrt{3} - \pi/2)d^2/4$.

Inspection of Figure 2 reveals also that to each filament cross-sectional area is associated the cross-sectional area of two interfilament gaps. Total area (filament area + 2 × gap areas) being $2\sqrt{3}d^2/4$. The volume of the solution is thus equivalent to that of a solution of section $2\sqrt{3}d^2/4$ and of length, the total length l of the actin filaments in the solution. Since l is equal to

$$\text{moles of actin} \times N \times 2.73 \times 10^{-7}\ \text{cm}$$

where N is the number of Avogadro and 2.73×10^{-7} cm is the number of centimeters of filament per actin monomer (Hanson & Lowy, 1973), the volume of the solution is

$$V = \text{moles of actin} \times N \times 2.73 \times 10^{-7} \times 2\sqrt{3}d^2/4\ \text{cm}^2 \quad \text{(i)}$$

The volume of the same solution is also given by the water volume (1000 mL) plus the salt volume (in our conditions 5 mL) plus the protein volume. The volume of the salt is calculated by taking into account the partial molal volume of the salt. The protein volume is calculated from the mass and the partial specific volume. The partial specific volume of G-actin, at 20 °C, is 0.716 mL/g (Kay, 1960). The standard change of volume for the conversion of G-actin into F-actin, in 2 mM $MgCl_2$ and 0.1 M KCl, is +74 mL/mol (Garcia et al., 1992). The partial specific volume of F-actin is thus 0.718 mL/g, a value that agrees with the value of 0.72 mL/g, determined by Kobayashi et al. (1964). Thus, the volume, in mL, of a solution containing m moles of actin, 0.1 mol of KCl, 10 mmol of triethanolamine, 2 mmol of $MgCl_2$, and 1000 g of water is given by

$$V = 0.718 \times M_r \times m + 5 \text{ (volume of salt)} + 1000 \text{ cm}^3 \quad \text{(ii)}$$

By equating i and ii, the interfilament distance can be calculated:

$$d = \left\{ \frac{[(0.718 \times M_r \times \text{m}) + 1000 + 5] \times 4}{m \times N \times 2.73 \times 10^{-7} \times 2\sqrt{3}} \right\}^{1/2} \text{cm} \quad \text{(iii)}$$

where M_r is 42 000 for pure actin and the equivalent weight of (7 × 42 000 + 65 000)/7 = 51 285 for tropomyosin decorated F-actin or of (7 × 42 000 + 65 000 + 90,000)/7 = 64 142 for troponin−tropomyosin-decorated F-actin. Equation iii, of course, holds beyond the actin concentrations at which the hexagonal packing of the actin filaments occurs.

For pure actin, the interfilament distance equals the diameter of the filament; for troponin−tropomyosin-decorated F-actin the situation is, on the contrary, more complex. Troponin is located at regular intervals along the filament but is not helically arranged (Hanson et al., 1972). In the bundles, the calcium-regulated actin filaments are assembled in such a way that the troponin molecules of adjacent filaments are displaced axially relative to each other, both at low and at high calcium concentration (O'Brien et al., 1975). Thus, the interfilament distance equals the diameter ($2r$) of the actin filament plus the tickness (t) of that portion of troponin projecting out of the circumference of the actin filament: $d = 2r + t$.

It is clear that, by measuring only the change of protein concentration, it is not possible to decide whether the change of d is due to the change of r or to the change of t (troponin may penetrate more or less deeply in the actin filament "groove") or to both these reasons.

RESULTS

Osmotic Stress of F-Actin and of Tropomyosin-Decorated F-Actin Solutions. F-actin and tropomyosin-decorated F-actin solutions are equilibrated against poly(ethylene glycol) solutions of known osmotic pressure. As it is shown in Figure 3, the two mixtures reach approximately the same concentration (about 5 m*m*) at an osmotic pressure of 1.74 × 10^4 dyn/cm^2. At larger pressures, however, the behavior of the two solutions diverges: at the pressure of 5 × 10^5 dyn/cm^2 the concentration of F-actin is about 18 m*m* while the concentration of tropomyosin-decorated F-actin is only 8.5 m*m*. At larger osmotic pressures the trend becomes

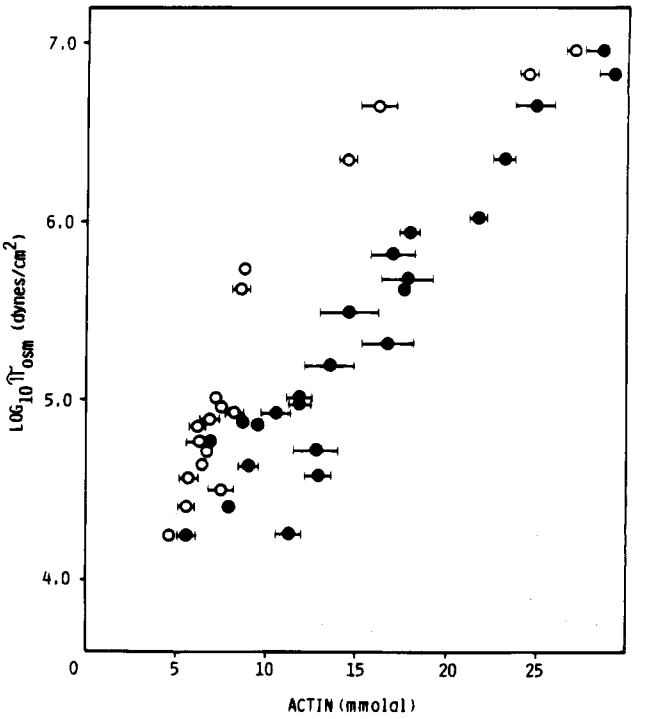

FIGURE 3: Osmotic pressure of F-actin (●) and of tropomyosin-decorated F-actin (○) solutions, as a function of actin concentration, measured by equilibration against poly(ethylene glycol) solutions Mean for two experiments. Measurements were performed at 22 °C as it was described under methods.

convergent and the two solutions reach approximately the same concentration 26−27 m*m* at 9.12 × 10^6 dyn/cm^2.

It is known that the increase of the concentration of F-actin solutions promotes the transition of actin filaments into bundles of filaments (Suzuki et al., 1989) and that tropomyosin counteracts this phenomenon (Grazi et al., 1990). At the concentration of $MgCl_2$ employed in these studies, however, the transition of filaments into bundles of filaments occurs also in the presence of tropomyosin (Grazi et al., 1992). This is confirmed by the experiments described in Figure 4, where the formation of actin bundles is monitored by the increase in light scattering of the solution. Light scattering begins to increase at 1.15% (w/w) poly(ethylene glycol) (i.e. at the osmotic pressure of 1.74 × 10^4 dyn/cm^2) and is essentially complete at 2.21% (w/w) poly(ethylene glycol) (i.e. at 2.63 × 10^4 dyn/cm^2). It is thus clear that below 1.74 × 10^4 dyn/cm^2 actin is present essentially in the filamentous form and above 2.63 × 10^4 dyn/cm^2 it is present essentially in the form of bundles of filaments. The increase of the osmotic pressure above 2.63 × 10^4 dyn/cm^2, by gradually overcoming the opposing elastic forces of the bundles, leads to the gradual conversion of the random stack of actin bundles into an ordered assembly of actin bundles lying side by side to each other. For reasons that will become clear in a later section, we think that the regular arrangement of the bundles is essentially complete at an osmotic pressure of about 10^5 dyn/cm^2.

Osmotic Stress of the Calcium-Regulated F-Actin Solutions. Troponin−tropomyosin-decorated F-actin solutions, either in the presence of 2 m*m* EGTA or in the presence of 0.2 m*m* $CaCl_2$, are equilibrated against poly(ethylene glycol) solutions of known osmotic pressure. As it is shown in Figure 5, the concentration of the two mixtures is approximately 3 m*m* at 1.74 × 10^4 dyn/cm^2. At pressures between 1.74 × 10^4 dyn/

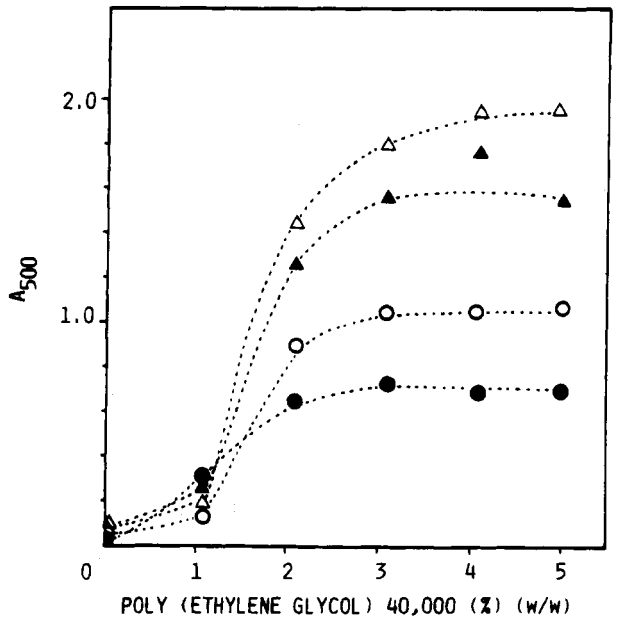

FIGURE 4: The increase in light scattering of F-actin solutions, as a function of poly(ethylene glycol) concentration. The incubation mixtures were prepared as described under methods. Temperature was 22 °C. F-actin, 10 μM as monomer (●); F-actin, 10 μM as monomer plus 1.43 μM tropomyosin (○); F-actin, 10 μM as monomer plus 1.43 μM tropomyosin plus 1.43 μM troponin, either in the presence of 0.2 mM $CaCl_2$ (△) or in the presence of 2 mM EGTA (▲). Poly(ethylene glycol) concentration was as indicated in the figure.

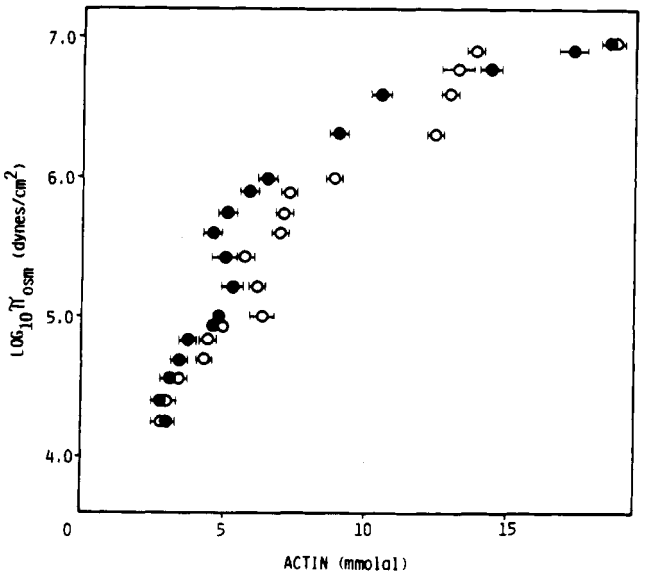

FIGURE 5: Osmotic pressure of troponin−tropomyosin-decorated F-actin either in the presence of 2 mM EGTA (●) or in the presence of 0.2 mM $CaCl_2$ (○), as a function of actin concentration, measured by equilibration against poly(ethylene glycol) solutions. Mean for two experiments. Measurements were performed at 22 °C, as it was described in the methods section.

cm^2 and 8 × 10^6 dyn/cm^2, the behavior of the two mixtures diverges, and concentration in the presence of calcium being always larger than the concentration in the presence of EGTA. Above the pressure of 8 × 10^6 dyn/cm^2 the concentration of the two mixtures tends to the same value.

DISCUSSION

Actin Concentration and the Average Radius of the Hydrated Actin Filament. With the progressive removal of the bulk water, actin filaments come in close contact, side by side, and form bundles of hexagonally packed (De Rosier & Tilney, 1981) actin filaments. Under these conditions, the interfilament distance equals the diameter of the filament, which, consequently, can be calculated according to eq iii. The method, however, is valid only at the osmotic pressure at which the filaments are hexagonally packed. To define the lower limit of this range we have first considered that pressure must be larger than 2.63 × 10^4 dyn/cm^2 (at this pressure filaments are completely assembled into bundles but are not regularly packed) and have then set the lower limit of validity of our method approximately at 7.94 × 10^4 dyn/cm^2. At this pressure, in fact, the radius of the hydrated actin filament, as calculated by our procedure (Figure 6), matches the value of 4.75 nm, determined by Holmes et al. (1990) from the X-ray fiber diagram from oriented gels of F-actin. We emphasize that the determination of Holmes et al. (1990) was performed at the approximate actin concentration of 1.84 m*m* (Popp et al., 1987), i.e. at an osmotic pressure lower than 10^4 dyn/cm^2. The coincidence of the value of the radius of the filament, determined at a pressure lower than 10^4 and at 7.94 × 10^4 dyn/cm^2, indicates that, in this range of pressures, the architecture of the water molecules in the core of the actin filament is not perturbed. When osmotic pressure is increased above 7.94 × 10^4 dyn/cm^2, the radius of the hydrated actin filament decreases progressively (3.4 nm at 9 × 10^6 dyn/cm^2, Figure 6). This indicates that water is progressively removed from the core of the filament.

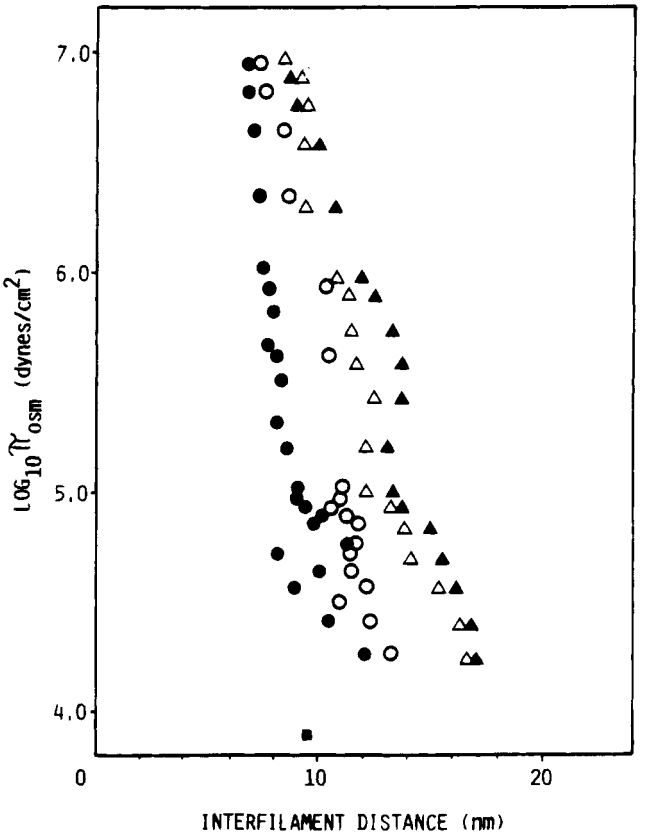

FIGURE 6: Interfilament distance as a function of protein osmotic pressure. Calculations were performed according to eq iii on the basis of the data of Figures 3 and 5. F-actin (●); tropomyosin-decorated F-actin (○); troponin−tropomyosin-decorated F-actin in the presence either of 2 m*m* EGTA (▲); or of 0.2 m*m* $CaCl_2$ (△). The diameter of the Heidelberg actin filament (Holmes et al., 1990) (■).

The Radius of the Hydrated Actin Filament and the Orientation of the Monomers in the Filament. The radius and, hence, the volume of the actin filament are a function of the protein osmotic pressure: both these parameters decrease with the increase of the pressure and vice versa. The volume decrease could be justified by a very large decrease of the partial specific volume of the protein. This explanation, however, is not at all reasonable, owing to the relatively small changes of pressure taking place in our experiments.

The change of the radius and, hence, of the volume could be, on the contrary, justified by a change of the orientation of the monomer in the filament, i.e. by the increase or the decrease of the angle (α) formed between the long axis of the monomer and the pointed end of the filament axis. In fact, the radius of the actin filament and the angle (α) are related by the formula:

$$\frac{\text{radius of the actin filament (nm)}}{4.75\ \text{(nm)}} = \frac{\sin(\alpha)}{\sin 90^\circ} \qquad \text{(iv)}$$

where 4.75 nm is the maximum radius of the actin filament (protein osmotic pressure lower than 10^4 dyn/cm^2) (Holmes et al., 1990) corresponding to the angle (α) = 90°.

At the protein osmotic pressure in frog muscle (1.8×10^5 dyn/cm^2, Maughan & Gorman, 1987) the hydrated filament radius becomes 4.17 nm (Figure 6) and, according to eq iv, the angle (α) becomes 61.5°. Simple trigonometric considerations indicate that, at the change of the angle (α) from 90° to 61.5°, the contact surfaces of the adjacent actin monomers slide past each other by about 13 Å and the interactions between the surfaces involve different amino acid residues in the two conditions. Hence, the amino acid interactions in the model of the actin filament at low protein osmotic pressure (below 10^4 dyn/cm^2) cannot be the same as those in the actin filament at the physiological protein osmotic pressure.

The Tropomyosin-Decorated Actin Filament. The relationship between the interfilament distance of tropomyosin-decorated actin and osmotic pressure is shown in Figure 6. At 7.94×10^4 dyn/cm^2 this distance has the value of 10.8 nm, which compares with the value of 9.5 nm, in the absence of tropomyosin. The difference of 1.3 nm, between the two values, is probably due to the projecting of the two tropomyosin helices out of the contour of the actin filament. Between 7.94×10^4 and 4.17×10^5 dyn/cm^2, the interfilament distance decreases only from 10.8 to 10.5 nm, as compared to the decrease from 9.5 to 8 nm in the actin filament. If we assume that the sites of interaction between tropomyosin and actin do not change with osmotic pressure, at 4.17×10^5 dyn/cm^2 the value of the angle (α) of the tropomyosin-decorated actin filament becomes 76.4° (as calculated from eq iv, for an angle (α) of 90° at the interfilament distance of 10.8 nm). At the same pressure the value of the angle (α) is 57.3° for the actin filament (as calculated for an angle (α) of 90° at the interfilament distance of 9.5 nm). If we assume, on the contrary, that, with the increase of the osmotic pressure, the sites of interaction of tropomyosin with actin are displaced toward the filament axis, the decrease of the interfilament distance from 10.8 to 10.5 nm is compatible with a value of the angle (α) even larger than 76.4°.

Thus, in both cases, tropomyosin confers rigidity to the actin filament and counteracts the tendency of the adjacent monomers contact surfaces to slide past each other with the increase of the protein osmotic pressure.

The Calcium-Regulated Actin Filament. In the presence of 2 mM EGTA and at osmotic pressures between 10^5 and 5.37×10^5 dyn/cm^2, the interfilament distance of troponin–tropomyosin-decorated F-actin is practically constant, the average value being 13.3 nm. The interfilament distance then decreases up to 8.4 nm at a protein osmotic pressure of 9×10^6 dyn/cm^2 (Figure 6).

The interfilament distance is significantly reduced in the presence of 0.2 mM $CaCl_2$, being 12 nm at 10^5 dyn/cm^2 and 11.4 nm at 5.37×10^5 dyn/cm^2. The values of the interfilament distance converge to those found in the presence of EGTA, at pressures larger than 8×10^6 dyn/cm^2 (Figure 6).

At 1.8×10^5 dyn/cm^2, the interfilament distance of the troponin–tropomyosin-decorated filament (13.3 nm) is significantly larger than that of the tropomyosin-decorated filament (10.8 nm). The difference between the two values (3.6 nm) is likely to represent the thickness of the projection of troponin, out of the tropomyosin-decorated actin filament contour. This conclusion is supported by the observation that the interfilament distance of the troponin–tropomyosin-decorated filament is essentially invariant in that range of protein osmotic pressure. The addition of calcium to the system decreases the interfilament distance of the troponin–tropomyosin-decorated filament from 13.3 nm, in the presence of EGTA, to 12.2 nm, in the presence of calcium. This data is in keeping with the classical view that, in the presence of calcium, tropomyosin moves along the actin monomer, toward the center of the filament, by about 1.5 nm (Haselgrove, 1972; Kress et al., 1986) and that, as a consequence, troponin is pulled more deeply in the filament "groove", thus decreasing the thickness of its projection out of the contour of the filament. In this picture the angle (α) remains unchanged.

An alternative picture, however, must also be considered: that calcium releases the typical rigidity of the tropomyosin-decorated actin filament and restores the original plasticity of F-actin. This decreases the value of the angle (α) (i.e. the interfilament distance) and brings the tropomyosin helices nearer to the filament axis, without any real movement of tropomyosin along the monomer. Unfortunately, at present, we are unable to make a choice between these two models.

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