*J. Biochem.* **140**, 39–42 (2006)
doi:10.1093/jb/mvj124

# Protein Osmotic Pressure and Cross-Bridge Attachment Determine the Stiffness of Thin Filaments in Muscle *Ex Vivo*

**Enrico Grazi* and Cristina Di Bona**
*Dipartimento di Biochimica e Biologia Molecolare, Univerità di Ferrara, Via Borsari 46, 44100 Ferrara, Italy*

Received April 19, 2006; accepted May 5, 2006

**The properties of some models of the actin filament are compared with those of the thin filament in muscle. The greater stiffness of thin filaments *ex vivo* with respect to F-actin *in vitro* is attributed to the effect of both protein osmotic pressure and the attached cross-bridges. By comparing the stiffness of thin filaments *in vitro* and in isometric and rigor muscles the stiffness of thin filaments in relaxed muscle is computed. The upper limit of thin filament stretching is deduced to approach ~10 nm μm$^{-1}$. It is also calculated that, on stretching by 2.02 nm of the fully non-overlapped thin filament or by 1.59 nm of the thin filament on isometric contraction, the energy released on the hydrolysis of one molecule of ATP is fully used up.**

**Key words: cross-bridge, *ex vivo*, protein osmotic pressure, stiffness, thin filament.**

Most of our knowledge on the mechanics of muscle was attained by measuring X-ray diffraction and force development in intact muscle fibres and by integrating the relative findings. The elastic properties of contractile components, thin filaments, thick filaments, cross-bridges were computed and their importance in muscle contraction was assessed. It happens however that not all the questions are answered with the above mentioned techniques and it turns out that, sometimes, even *in vitro* experiments may make important contributions. We provide here some examples of the usefulness of the integration of the *ex vivo* and *in vitro* approaches. Our present aim is to describe the effects of *ex vivo* conditions, particularly of the macromolecular osmotic pressure, on the stiffness of the actin filament. Thus to relate the actin chemical potential to the stiffness of the actin filament through the macromolecular osmotic pressure, we show here that the effect of macromolecular osmotic pressure and attached cross-bridges explains the greater stiffness of thin filaments *ex vivo* with respect to that of F-actin *in vitro*; we tentatively compute the stiffness of thin filaments in relaxed muscle and assess the upper limit of thin filament stretching *ex vivo*.

## PHYSICO-CHEMICAL PARAMETERS

*Macromolecular Osmotic Pressure*—Osmotic pressure ($\pi$) is the rate of change of energy with respect to the volume of all the exchangeable species. Thus changing the volume fraction or concentration of the macromolecular species by applying osmotic pressure is physical work done on that species. This work can be expressed as the chemical potential of the macromolecules subject to stress at fixed values of the intensive thermodynamic variables pertaining to the particular preparation (temperature $T$, hydrostatic pressure $P$, and moles $n_i$ of small molecules):

$$\Delta\mu(T,P,n_i) = -\pi\Delta V(\text{Joule}) \quad (1)$$

where $V$ is the total volume (essentially the water volume) that moves to or from the protein phase (1).

The solvent phase, s, is composed of water plus the small solutes. The solute phases are composed of a macromolecule [as an example poly(ethyleneglycol)], m, and F-actin, p. These three quantities are related by the Gibbs-Duhem equation for the solvent, the protein and poly(ethyleneglycol),

$$n_s d\mu_s = -n_m d\mu_m$$
$$n_s d\mu_s = -n_p d\mu_p$$

the chemical potential of each is related to the osmotic pressure of water in the protein and poly(ethyleneglycol) solutions. In the experiments of Tellam *et al.* (*2*) the two solute phases were contained in the same compartment. In the experiments of Grazi *et al.* (*3*) the two solute phases were physically separated by a dialysis membrane.

*Stiffness*—The stiffness of a system with respect to deformation (*e.g.*, the stiffness of a spring with respect to stretching) is the second derivative of the energy with respect to the corresponding displacement. The compliance is the reciprocal of the stiffness. In this paper stiffness (pN nm$^{-1}$) is defined as the force required to elongate by 1 nm a filament of 1 μm in length.

*Relationship between Protein Osmotic Pressure and the Stiffness of Thin Filaments*—Protein osmotic pressure is more simply related to the stiffness of thin filaments when the system is treated at the equilibrium, namely the polymerization starts from ADP-G-actin. In this case the monomer and polymers reach an equilibrium,

$$\text{ADP-G-actin} \leftrightarrow \text{F(ADP)actin}$$

At the equilibrium the standard free energy of the monomer association reaction, $\Delta G^{c_c}$, is related to the critical concentration, $c_c$, by,

$$RT\ln[c_c^{-1}] = RT\ln K_{ASS} = -\Delta G c^c \quad (2)$$

where $K_{ASS}$ is the G-actin–F-actin association constant.

*To whom correspondence should be addressed. Phone: +39 0532 291421, Fax: +39 0532 202723, E-mail: gre@dns.unife.it

 

The problem is now to relate $c_c$ to water activity. This problem was solved by Tellam *et al.* (*2*) who showed that the critical concentration of actin is decreased by the addition of macromolecules to the solution, thus the increase in macromolecular osmotic pressure. In turn the decrease of critical concentration is related to the increase in the free energy intrinsic to the protein–protein interface and, consequently, to the stiffness of the filament.

## RESULTS

*Cross-Bridge Stiffness: The Effect of Protein Osmotic Pressure*—We showed previously that cross-bridge stiffness increases with both protein osmotic pressure (*3*) and the process of cross-bridge attachment (*4*). In their work Linari *et al.* (*5*) disregarded these effects: (a) they assumed the same cross-bridge stiffness in active and rigor muscle fibers; and (b) they compared intact (active muscle) and permeabilized (rigor muscle) fibers even though the protein osmotic pressure could be different in the two cases. However, even though Linari *et al.* (*5*) assumed the same stiffness for active and rigor cross-bridges, there is at least qualitative agreement on the fact that cross-bridge attachment, both *in vitro* and *ex vivo*, increases the stiffness of thin filaments. Concerning point (a), we observed that, *in vitro*, decoration with myosin subfragment-1 influences the stiffness of the actin filament (*6*). The increase is related to the saturation of the filament with myosin subfragment-1, and the stiffness is 0.5 pN $nm^{-1}$ $\mu m^{-1}$ at a myosin subfragment-1–F-actin of molar ratio 0.2:1 and 2.22 pN $nm^{-1}$ $\mu m^{-1}$ at a myosin subfragment-1–F-actin of molar ratio 1:1 (*7*). Concerning point (b), the consequence of the possible difference in protein osmotic pressure between permeabilized and intact fibers in the experiments of Linari *et al.* (*5*) cannot be discussed owing to the lack of quantitative data.

*Comparison of the Stiffness of Thin Filaments* Ex Vivo *and* In Vitro—From the overall compliance of the frog muscle half sarcomere, 1.05 μm in length, Linari *et al.* (*5*) computed the compliance of the remaining free part of the thin filament. At 4°C, the estimated thin filament stiffness was 58.46 pN $nm^{-1}$ $\mu m^{-1}$ in isometric contraction and 87.69 pN $nm^{-1}$ $\mu m^{-1}$ in rigor. These values were obtained from an isometric tension of 226 $kN \cdot m^{-2}$ in the intact fiber and on the assumption that there are $10^{15}$ thin filaments$\cdot m^{-2}$.

It is illuminating to compare these values with the stiffness of two models of the actin filament: tropomyosin-decorated tetramethyl-rhodamine-phalloidin F-actin, stiffness 65.3 ± 6.3 pN $nm^{-1}$ $\mu m^{-1}$ Kojima *et al.* (*8*) and F-actin decorated with alexa-fluor tropomyosin, stiffness 11.26 ± 2.4 pN $nm^{-1}$ $\mu m^{-1}$ Adami *et al.* (*9*). The stiffness of the model of Kojima *et al.* (*8*) approaches that of thin filaments in muscle while that of the model of Adami et al. (*9*) is 5–7.8 times lower even though both the species are composed of actin decorated with tropomyosin.

*Possible Reasons for the Different Behavior of the Two Models*—We have repeatedly criticized the use of phalloidin F-actin as a model for thin filaments (*7*, *10–12*). In this particular case it is sufficient to observe that phalloidin F-actin exhibits an extremely low critical concentration as compared to F-actin (*13*, *14*) and that the critical concentration is inversely related to the stiffness of the actin filament (*15*). It is therefore not a surprise that the model of Kojima *et al.* (*8*) exhibits a stiffness as great as 65.3 ± 6.3 pN $nm^{-1}$ $\mu m^{-1}$.

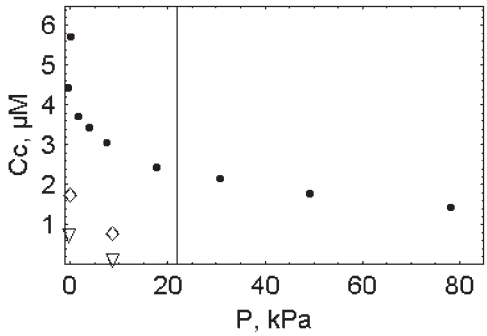


Fig. 1. **Effect of macromolecular osmotic pressure on the critical concentration of actin.** Total G-actin, 6.4 μM; temperature, 25°C; 0.4 mM $MgCl_2$ (filled circle); 1.11 mM $MgCl_2$ (open diamond); 1.68 mM $MgCl_2$ (open triangle). The vertical line indicates the physiological macromolecular osmotic pressure, 22.6 kPa. Data are taken from Figs. 2 and 3 of Tellam *et al.* (*2*).

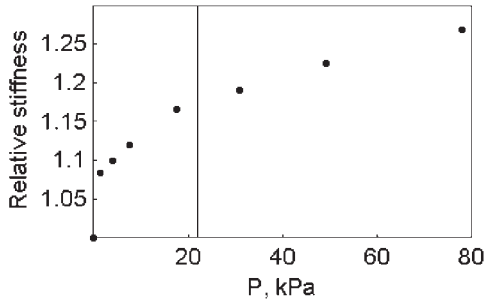


Fig. 2. **Relative stiffness of the actin filament as a function of macromolecular osmotic pressure.** In the calculation stiffness is assumed to parallel $-\Delta G c_c$ (Eq. 2). The critical concentration is expressed as mol $m^{-3}$. Relative stiffness is obtained by normalization as to the calculated value of the stiffness at ~0 osmotic pressure. The vertical line indicates the physiological macromolecular osmotic pressure, 22.6 kPa.

The model of Adami *et al.* (*9*), on the contrary, exhibits a lower stiffness than that of thin filaments. We propose that this is due to the effect of protein osmotic pressure. The protein osmotic pressure in muscle is ~22.6 kPa (*16*) while in a system composed of F-actin decorated with alexa-fluor tropomyosin (20 nM F-actin as the monomer) the protein osmotic pressure is much, much lower. The effect of protein osmotic pressure on the critical concentration of actin is shown in Fig. 1, which was obtained from Figs. 2 and 3 of Tellam *et al.* (*2*) by converting the concentration of poly(ethyleneglycol) 6000 into the corresponding macromolecular osmotic pressure. This was done by means of the equation (*17*),

$$\pi = -2.7 \times 10^{-4} c + 1.5 \times 10^{-5} c_2 (\mathrm{MPa}) \qquad (5)$$

As is clear from Fig. 1 there is an inverse relation between the actin critical concentration and macromolecular osmotic pressure, so that the greater protein osmotic pressure in muscle generates the greater stiffness of the thin filament. In the presence of 0.4 mM $MgCl_2$ as the

polymerizing agent the critical concentration is 5.7 μM in the absence of poly(ethylene glycol) and ~2.4 μM at 22.6 kPa, the physiological protein osmotic pressure of frog skeletal muscle (*16*). The effect of the macromolecule is even greater at higher $Mg^{2+}$ concentrations. At 1.6 mM $MgCl_2$ the critical concentration decreases from 0.738 μM in the absence of poly(ethyleneglycol) to 0.113 μM at 8.92 kPa.

Our explanation is apparently contradicted by the observation that an increase in protein osmotic pressure does not significantly increase the stiffness of the sarcomere in an intact fibre. In fact the stiffness of active intact fibres seems to be independent of the tonicity of the bathing solution in the range of 0.8–1.44 times that of a normal Ringer's solution (*18*, *19*), which alters the filament lattice spacing by 16% (*6*). However, the gap in protein osmotic pressure between that generated by 20 nM actin filaments *in vitro* and by protein in muscle is exceedingly greater than that caused by a 16% alteration of the filament lattice spacing. In fact, shown in Fig. 1, the decrease in critical concentration, and thus the increase in the stiffness of the actin filament, is larger at low values and levels off at larger values of the protein osmotic pressure. So the influence on the stiffness is expected to be minor in the physiologic range of protein osmotic pressure. This is so even though stiffness is exponentially related to $c_c^{-1}$. This is shown in Fig. 2 where the change in the relative stiffness in the range of physiological macromolecular osmotic pressure is estimated.

*Putative Stiffness for the Fully Non-Overlapped Thin Filament*—Linari *et al.* (*5*) determined the actin filament compliance from the slope of the relation between total half sarcomere compliance and sarcomere length between 2.00 and 2.15 μm. This is the length for which the number of myosin cross-bridges in the region of overlapping between the myosin filament and the actin filament remains constant and only the length of the non-overlapped region of the actin filament changes with sarcomere length. Under these conditions the stiffness assigned to the non-overlapped part of the thin filament was 58.46 pN $nm^{-1}$ $\mu m^{-1}$ in isometric contraction and 87.69 pN $nm^{-1}$ $\mu m^{-1}$ in rigor.

Regarding intact muscle fibre X-ray diffraction, the periodicity increase in M6 myosin reflection provides evidence that the structure of the entire myosin filament (shaft included) changes on activation (*20*). It is thus likely that the thin filament behaves similarly and when the stiffness changes it changes concordantly for both the overlapped and non-overlapped portions. The problem is now what is the stiffness of the fully non-overlapped thin filament (or the stiffness of the filament in the case where the cross-bridges are all detached)? If we assume that stiffness increases linearly with the fraction of attached cross-bridges and that cross-bridge stiffness is equal in both rigor and the isometric condition, the stiffness, $x$, for the fully non-overlapped thin filament is obtained by solving the equation,

$$(58.46 - x)/0.43 = (87.69 - x)/1.00$$

The solution is, $x$ = 36.39 pN $nm^{-1}$ $\mu m^{-1}$.

However what happens in the more likely case that cross-bridge stiffness is greater in rigor than in the isometric condition? To answer this question a more general expression must be utilized,

$$(58.46 - x)/(0.43/n) = (87.69 - x)/1.00$$

where $n$ = (stiffness of the isometric cross-bridge/stiffness of the rigor cross-bridge). Analysis of this expression predicts that for $n$ = 0.7, the stiffness of the relaxed thin filament *in vivo*, $x$ = 11.91 pN $nm^{-1}$ $\mu m^{-1}$, approaches that of the model of Adami *et al.* (*9*), *i.e.* of alexa fluor tropomyosin F-actin *in vitro* (11.26 ± 2.4 pN $nm^{-1}$ $\mu m^{-1}$). This prediction contradicts the experimental evidence that shows that protein osmotic pressure increases the stiffness of the actin filament. We conclude, therefore, that *ex vivo* the ratio (stiffness of the isometric cross-bridge/stiffness of the rigor cross-bridge) must be lower than 1 and larger than 0.7.

*The Upper Limit of Thin Filament Stretching*—The models of thin filament so far described exhibit very different stiffness [0.38 pN $nm^{-1}$ $\mu m^{-1}$ (*15*) to 65 pN $nm^{-1}$ $\mu m^{-1}$ (*8*)] but their specific elongation at the yield point is very similar (7.1–9.7 nm $\mu m^{-1}$) and is not at all related to the stiffness (*8–10*, *15*, *21*, *22*). It is thus likely that also *ex vivo* the thin filament cannot afford stretching by greater than 10 nm $\mu m^{-1}$. Since the compliance of thin filaments in active muscle represents about 29% of the half sarcomere compliance (*5*) it seems safe to assume that stretching greater than 24–29 nm per half sarcomere is accompanied either by cross-bridge detachment or thin filament breaking.

*The Energy Required to Stretch the Thin Filament*—The elongation of the filament of Adami *et al.*, alexa-fluor-tropomyosin F-actin, is proportional to the force applied (*9*), thus the work performed on the filament while stretching is given by the expression,

$$W = \text{stiffness} \cdot \Delta l^2/2.$$

We thus calculate that stretching by 10 nm of a 1 μm filament of either alexa-fluor-tropomyosin F-actin, stiffness 11.26 pN $nm^{-1}$ (*9*), or a thin filament in isometric contraction, stiffness 58.46 pN $nm^{-1}$ (*5*), requires 563 and 2923 pN nm, respectively. Incidentally, the energy released on the hydrolysis of one ATP molecule under muscle conditions, 74.4 pN nm (*23*), is only sufficient to stretch by 1.59 nm the thin filament in isometric contraction and by 2.02 nm the fully non-overlapped thin filament (stiffness, 36.39 pN $nm^{-1}$ $\mu m^{-1}$).

## DISCUSSION

Muscle physiologists are doing an excellent work as to the elucidation of the mechanics of muscle fiber contraction and the determination of the behaviour of the different component of the contractile apparatus. The field, however, is more complex than it is usually believed. Water and the contractile apparatus undergo strong interactions that mutually influence the chemical potentials. As a result, in muscle, any single protein interaction, by modifying the water chemical potential, indirectly influences the state of all the other components of the system. This must be taken in to account when analyzing the mechanism of muscle contraction even though the introduction of a new variable makes it harder to come to *bona fide* conclusions.

This work was supported by grants from the Università di Ferrara and the Fondazione della Cassa di Risparmio di Ferrara.

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