STIFFNESS OF SKINNED RABBIT PSOAS FIBERS IN MgATP AND $MgPP_i$ SOLUTION

B. BRENNER,* J. M. CHALOVICH,‡ L. E. GREENE,‡ E. EISENBERG,‡ AND M. SCHOENBERG*
Laboratory of Physical Biology, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, and ‡Laboratory of Cell Biology, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892

ABSTRACT The stiffness of single skinned rabbit psoas fibers was measured during rapid length changes applied to one end of the fibers. Apparent fiber stiffness was taken as the initial slope when force was plotted vs. change in sarcomere length. In the presence of MgATP, apparent fiber stiffness increased with increasing speed of stretch. With the fastest possible stretches, the stiffness of relaxed fibers at an ionic strength of 20 mM reached more than 50% of the stiffness measured in rigor. However, it was not clear whether apparent fiber stiffness had reached a maximum, speed independent value. The same behavior was seen at several ionic strengths, with increasing ionic strength leading to a decrease in the apparent fiber stiffness measured at any speed of stretch. A speed dependence of apparent fiber stiffness was demonstrated even more clearly when stiffness was measured in the presence of 4 mM $MgPP_i$. In this case, stiffness varied with speed of stretch over about four decades. This speed dependence of apparent fiber stiffness is likely due to cross-bridges detaching and reattaching during the stiffness measurement (Schoenberg, 1985. *Biophys. J.* 48:467). This means that obtaining an estimate of the maximum number of cross-bridges attached to actin in relaxed fibers at various ionic strengths is not straightforward. However, the data we have obtained are consistent with other estimates of cross-bridge affinity for actin in fibers (Brenner et al., 1986. *Biophys. J.* In press.) which suggest that ~60–90% of the cross-bridges attached in rigor are attached in relaxed fibers at an ionic strength of 20 mM and ~2–10% of this number of cross-bridges are attached in a relaxed fiber at an ionic strength of 170 mM.

INTRODUCTION

Solution studies have shown that in the presence of ATP, myosin subfragment-1 (S-1) can bind to the actin-tropon-in-tropomyosin complex even under relaxing conditions where the ATPase activity is very low (Chalovich et al., 1981; Chalovich and Eisenberg, 1982; Wagner and Ginig-er, 1981; Wagner, 1984). The chemical states which bind to actin under these conditions are M·ATP and M·ADP·P_i (Chalovich et al., 1981) and their binding is characterized by a rapid equilibrium between attachment and detachment (Stein et al., 1979). Similarly, it has also been shown that at low ionic strength and in the absence of Ca^{++}, conditions similar to those used in the solution studies, single skinned rabbit psoas fibers are completely relaxed; they do not develop active force, have no unusual resting tension, and do not shorten actively (Brenner et al., 1982; Schoenberg et al., 1984). Nevertheless, when stretched very rapidly, these fibers appear quite stiff. This apparent fiber stiffness is approximately proportional to filament overlap, suggesting that it is due to attached cross-bridges (Brenner et al., 1982). Since the stiffness is not detectable when slow stretches are used, it appears as if the bridges attach to and detach from actin very rapidly, just as S-1 does in solution (Stein et al., 1979).

The apparent stiffness of relaxed fibers is much smaller at an ionic strength (μ) of 170 mM than at μ = 20 mM, which suggests that fewer cross-bridges might be attached to actin at the higher ionic strength. This again, is similar to the behavior of S-1 in solution (Greene et al.,1983). However, an alternative explanation for the low apparent fiber stiffness observed at high ionic strength is that a substantial number of cross-bridges are still attached but the attachment/detachment rates are much higher than at low ionic strength. Such an increase in rates would allow more cross-bridges to detach and reattach during the stiffness measurement, thereby offering an alternative explanation for the decrease in apparent fiber stiffness.

Recently equatorial x-ray diffraction patterns were obtained from single skinned rabbit psoas fibers, and these studies also suggest that a significant number of cross-bridges might be attached to actin under relaxing conditions at μ = 20 mM (Brenner et al., 1984). The results are consistent with at least some detachment as ionic strength is increased from μ = 20 mM to μ = 100 mM. However, at still higher ionic strength, no conclusion could be drawn

Dr. Brenner's present address is Institute of Physiology II, University of Tübingen, Federal Republic of Germany.
Dr. Chalovich's present address is Department of Biochemistry, East Carolina University School of Medicine, Greenville, NC 27834.
Reprint requests should be addressed to Dr. Schoenberg.

because significant filament disorder obscured signs of further cross-bridge detachment.

In the present study we investigated the apparent stiffness of single relaxed rabbit psoas fibers as a function of speed of stretch. It was hoped to extend the data to a region where one could measure a "true stiffness," i.e., a stiffness independent of the speed of stretch. This would make it possible to obtain a clear measure of the number of attached cross-bridges. Somewhat surprisingly, we found that when the apparent stiffness was plotted as a function of the speed of stretch, it was not clear whether the apparent stiffness had reached a speed-independent value even when the speed of stretch was close to 10^5 (nm/half-sarcomere)/s, the fastest stretch we could apply. We also saw a speed dependence of stiffness in the presence of 4 mM $MgPP_i$. In this case, apparent stiffness varied with speed of stretch over at least 4 decades of speed. If this wide speed dependence of the apparent fiber stiffness observed in the presence of $MgPP_i$ also occurs in the presence of MgATP under relaxing conditions, it means that under most conditions, measurements of muscle stiffness in relaxed fibers underestimate the number of attached cross-bridges. It is possible, however, to estimate the number of attached cross-bridges from the calculated cross-bridge affinity for actin in fibers (Brenner et al., 1986) and the stiffness measurements were compatible with these estimates.

METHODS

Fiber Preparation and Solutions

Segments of single skinned rabbit psoas fibers, ~ 2–8 mm long, were dissected and mounted between a force transducer and displacement generator using cyano-acrylate glue (see Brenner et al., 1982). To avoid complications due to prolonged storage of skinned fibers, all experiments were done with fibers less than 7 d old. Table I shows the composition of the solutions used. All experiments were performed at 5°C. The pH was 7.00 ±0.05. Ionic strength was adjusted by adding or removing KCl.

Force Transducer

Force was measured with a modified AKERS strain gauge (AE 801, Aksjeselskapet Mikroelektronikk, Horten, Norway). The 5-mm-long silicon beam was ground to ~1.5 mm of free beam length and then extended by means of a light-weight epoxy-carbon fiber, ~1.2 × 0.1 × 0.15 mm, epoxied to the silicon beam. The natural frequency was ~60 kHz in air and between 25 and 30 kHz with the fiber mounted and placed in solution. The sensitivity ranged between 0.90 and 0.95 mV/mg and the resolution was around 1 mg. In the experiments, generally nine force traces were averaged to improve the resolution by a factor of three.

Sarcomere Length Calculation

To measure sarcomere length, the position of the first order of the laser diffraction pattern was monitored using a Schottky barrier position sensitive detector (PIN SC/10D, United Detector Technology, Inc., Santa Monica, CA) in a system similar to that described previously (Rüdel and Zite-Ferenczy, 1979; Brenner et al., 1982; Schoenberg and Wells, 1984). The position of the first order was converted to sarcomere length by an analog circuit using the thin grating equation. The frequency cutoff (-3dB) for the resulting sarcomere length signal was 30 kHz. The sensitivity was 1.7 mV/(nm/half-sarcomere) and the resolution was 0.5 nm/half-sarcomere. Again, averaging the signals of nine measurements improved the resolution threefold.

Displacement Generator

The displacement generator was a modified moving coil galvanometer (Brenner, 1980). It could stretch muscle fibers 5 nm/half-sarcomere in 75 to 150 μs, depending on the overall fiber length.

Stiffness Measurements

Both force and sarcomere length during a stretch were recorded using a Nicolet digital processing oscilloscope (Nicolet 1090; Madison, WI), stored on magnetic tape, and analyzed on a PDP-10 digital computer. (Digital Equipment Corp., Maynard, MA). Apparent stiffness was taken as the initial slope of the plots of force vs. change in sarcomere length.

Since rabbit psoas muscle fibers, like frog muscle fibers (Rüdel and Zite-Ferenczy, 1979), are not completely homogeneous with regard to sarcomere length and tilt of striation, it was important to orient the fiber so that the intensity of the first-order diffraction line used to monitor sarcomere length had maximal or near-maximal intensity (Fig. 1, *a* and *b*). This was necessary in order to sample a large fraction of the myofibrillar population, thus giving a more representative average of the sarcomere length (Fig. 1 *c*). When this precaution was followed, sarcomere length changes could accurately be measured along the length of the fiber as shown by the close agreement between sarcomere length and overall length, when the applied length changes were slow (Fig. 2).

However, when rapid length changes are applied to one end of a muscle fiber, especially when stiffness is low, the sarcomere length changes along the fiber no longer mirror the overall length changes because of propagation effects (Schoenberg et al., 1974; Sugi and Kobayashi, 1983). In order to minimize such effects, sarcomere length was recorded as near to the force transducer as practically possible, usually at a distance of ~0.5 to 1 mm.

Induction of Rigor

At 5°C, rigor was induced by quickly washing out the relaxing solution, replacing it with cold Quick Rinse solution (Table I). Since this solution was high in EDTA, rigor was induced quickly with little generation of tension. After several chamber volumes of Quick Rinse solution were

TABLE I
COMPOSITION OF SOLUTIONS*

	Imidazole	EGTA	EDTA	$MgCl_2$	ATP	PP_i	KCl	Ionic st.
MgATP solution	10	1	—	3	1	—	0–100	19–120
Quick rinse	20	5	5	—	—	—	80	120
Rigor solution	20	5	—	2	—	—	90	120
$MgPP_i$ solution	20	5	—	6	—	4	70	120

*All in mM, all solutions adjusted to pH 7.00 at 5°C. Ionic strength adjusted by adding appropriate amount of KCl.

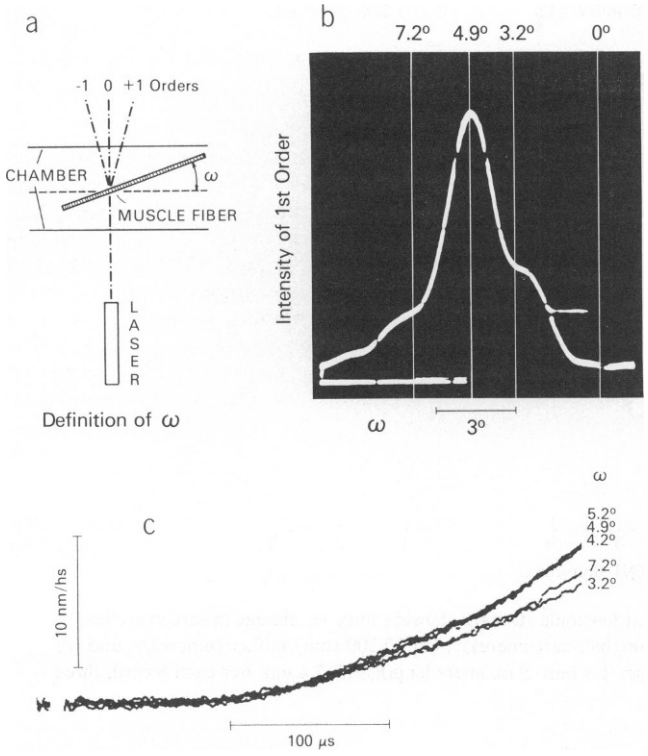

FIGURE 1 Performance of the laser diffractometer. (*a*) Definition of ω as the angle between incident laser beam and a normal to the fiber. (*b*) Effect of beam incidence on the intensity in the first order diffraction line. Abscissa, angle ω. Ordinate, light intensity in the first order of the laser diffraction pattern. (*c*) Output of the diffractometer during fast length changes. Abscissa, time in μs. Ordinate, displacement in nm/half-sarcomere. Calibration bars, 100 μs and 10 nm/half-sarcomere. Note that as long as the fiber orientation relative to the laser beam is within $\pm 1°$ of the position yielding maximum intensity in the first order, the variation in the output is within the noise of the signal. Care was taken to stay within this range.

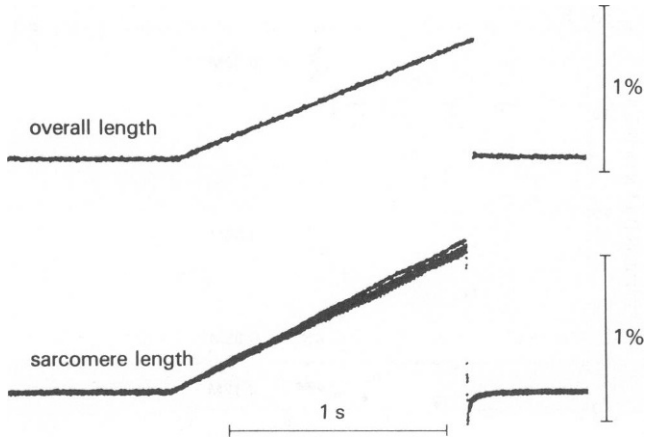

FIGURE 2 Superimposed sarcomere displacement from seven positions along a muscle fiber during slow stretch. Note that in this case the sarcomere length recordings at each location are quite similar and about the same magnitude as expected from the overall length change.

washed through, finally normal rigor solution was washed through. This produced fibers with more uniform striation spacing (Brenner et al., 1984) than when rigor was induced in the presence of Mg^{2+}. For the studies of fibers in $MgPP_i$, the normal rigor solution was further replaced by the $MgPP_i$ solution listed in Table I. Any small rigor tension existing prior to $MgPP_i$ addition then rapidly decayed close to zero.

The possibility of contaminant ATP influencing the rigor and $MgPP_i$ results was ruled out as in Schoenberg and Eisenberg, 1985. The contaminant ATP was found to be < 1 μM.

RESULTS

Apparent Fiber Stiffness in the Presence of ATP

When stretches are applied to one end of a fiber, plots of instantaneous force vs. change in sarcomere length show a characteristic shape, depending upon the speed of the applied stretch (Fig. 3). At slow speeds of stretch, the curves are concave downward. At higher speeds of stretch, the initial portion of the plot is more linear, and the slope of this initial portion increases. Apparent fiber stiffness was taken as the "initial slope." For fast stretches this was rather straightforward since the plots of instantaneous force vs. change in sarcomere length were essentially linear over the first 2–5 nm/half-sarcomere. For slower stretches the initial slope was estimated over the first 0.5 to 1 nm/half-sarcomere of displacement. This definition of apparent fiber stiffness is different from that defined in Schoenberg (1985), but this affects only the shape of the apparent stiffness-velocity of stretch curves and not the conclusions.

Fig. 4 shows plots of apparent fiber stiffness as a function of the speed of stretch for speeds up to 8×10^4 nm/(half-sarcomere/s) on both a linear and a log scale, for different ionic strength solutions. The plots of apparent stiffness vs. speed of stretch appear as if they are beginning to level off on the linear scale. However, with the same data plotted on a log scale, it is not at all clear that stiffness has reached a speed independent value at the highest velocities of stretch. What is clear, though, is that the apparent stiffness decreases greatly with increase in ionic strength.

Apparent Fiber Stiffness in the Presence of PP_i

We were able to obtain stiffness data in the presence of ATP over about 1.5 decades. To see whether it is reasonable to anticipate that apparent stiffness could continue to increase as the speed of stretch is varied more than 1.5 decades, we studied fiber stiffness in the presence of $MgPP_i$, where detachment rates are slower (Lymn and Taylor, 1971; Greene and Eisenberg, 1980; Marston, 1982) so that stiffness can be measured as speed of stretch is varied over more than 4 decades. We performed the experiments with 4 mM $MgPP_i$, $\mu = 120$ mM, a condition where the apparent binding constant of S-1 to regulated actin in solution in the presence of 4 mM $MgPP_i$ is about

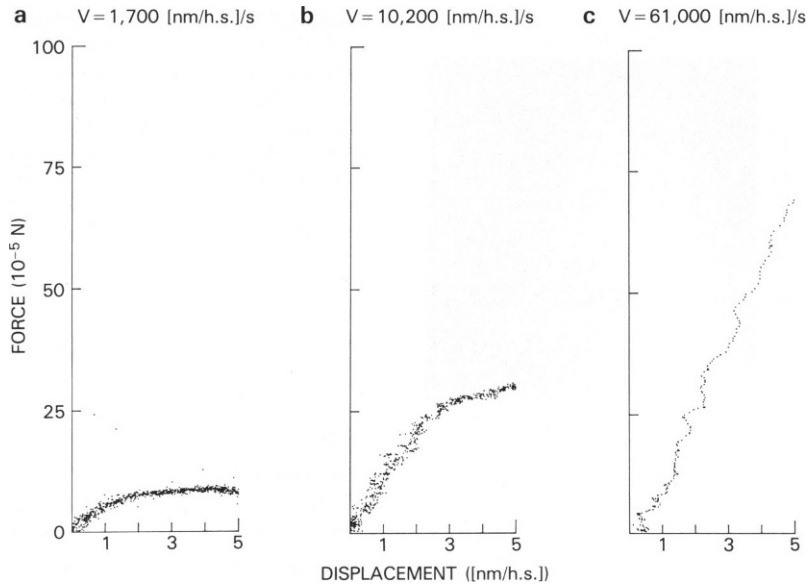

FIGURE 3 Original records from experiments in ATP relaxing solution at low ionic strength showing force vs. change in sarcomere length during stretches of different velocity (V). Speeds of stretch: (*a*) 1,700 (nm/half-sarcomere)/s, (*b*) 10,200 (nm/half-sarcomere)/s, and (*c*) 61,000 (nm/half-sarcomere)/s. Fiber diameter, 70 × 130 μm. Fiber length, 1.8 mm. Sarcomere length, 2.3–2.4 μm. For each record, three measurements were averaged to improve the signal to noise ratio.

the same as the binding constant of S-1 · ATP to regulated actin in solution at μ = 20 mM.

The first point of analogy we found between the fiber in $MgPP_i$ and in MgATP is that plots of instantaneous force vs. change in sarcomere length have a similar shape in the two cases (cf. Figs. 3 and 5). However, this similarity of shape as well as similarity of apparent fiber stiffness is observed only when the applied speed of displacement is about two to three orders of magnitude slower with $MgPP_i$ than with MgATP.

Also similar to the case with MgATP, when we plotted the apparent stiffness vs. speed of stretch in the presence of 4 mM $MgPP_i$, the apparent stiffness on a linear plot appeared to approach a speed-independent value when stiffness was measured over a range of speeds spanning 1.5 decades (Fig. 6 *a*). However, when the maximum speed of stretch was increased by another two decades, there was a further significant increase in apparent fiber stiffness (Fig. 6 *b*). This demonstrates that, when apparent stiffness is plotted vs. speed of stretch on a linear scale, the apparent approach to a speed independent stiffness value can be deceiving. In the presence of $MgPP_i$, apparent fiber stiffness continued to increase with speed of stretch over 4–5 decades (Fig. 7). If a similar effect is occurring in MgATP, then the apparent leveling off of apparent stiffness in the linear plot is artifactual and the true stiffness in MgATP

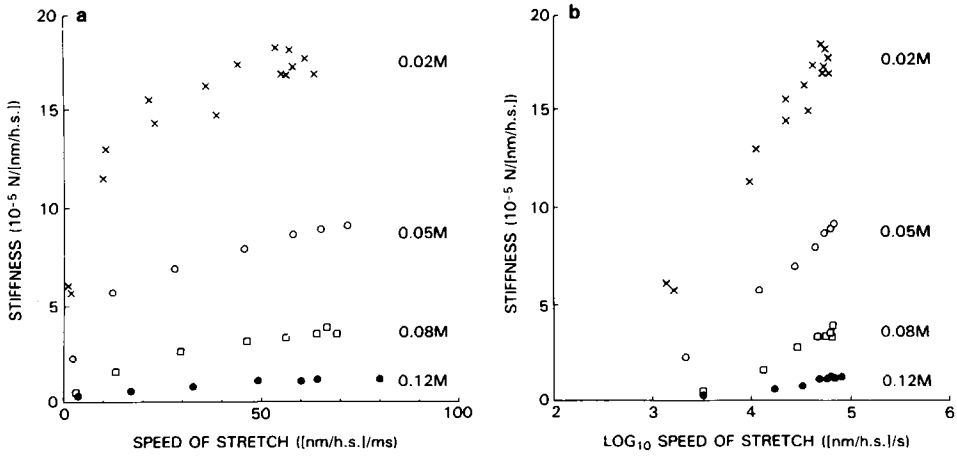

FIGURE 4 Effect of speed of stretch on apparent fiber stiffness in relaxed fibers at different ionic strengths. Apparent fiber stiffness taken as the initial slope of the force - sarcomere length relationship (Fig. 3). Abscissa, (*a*) speed of stretch in (nm/half-sarcomere)/ms, (*b*) $\log_{10}$ speed of stretch in (nm/half-sarcomere)/s. Ordinate, apparent fiber stiffness in N/(nm/half-sarcomere). Fiber diameter, 70 × 130 μm, Fiber length, 1.8 mm. Sarcomere length, 2.3–2.4 μm. (x) μ = 20 mM; (○) μ = 50 mM; (□) μ = 80 mM; (●) μ = 120 mM.

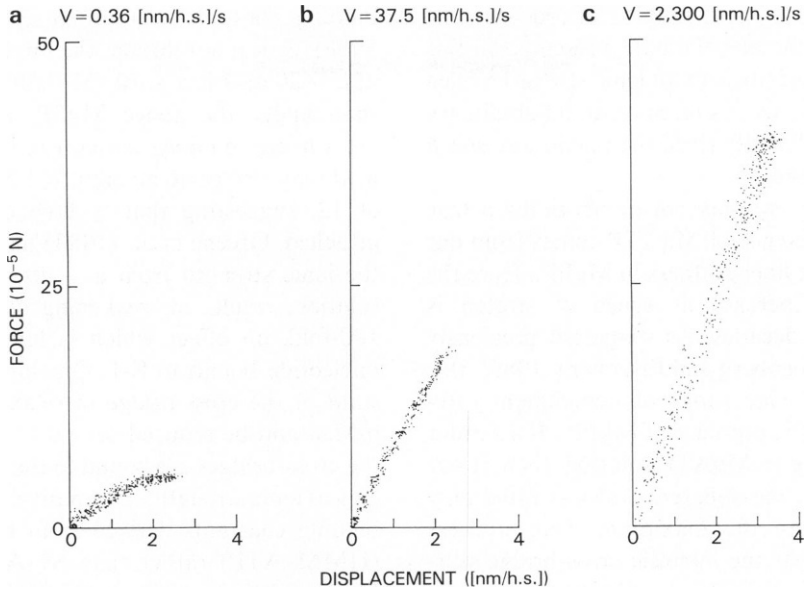

FIGURE 5 Original records showing force vs. change in sarcomere length during stretches of different velocity (V) in the presence of 4 mM $MgPP_i$. Ionic strength, 120 mM. Speeds of stretch: (*a*) 0.36 (nm/half-sarcomere)/s; (*b*) 37.5 (nm/half-sarcomere)/s; (*c*) 2,300 (nm/half-sarcomere)/s. Fiber diameter, 70 × 110 μm. Fiber length, 7.9 mm. Sarcomere length, 2.3–2.4 μm. No signal averaging.

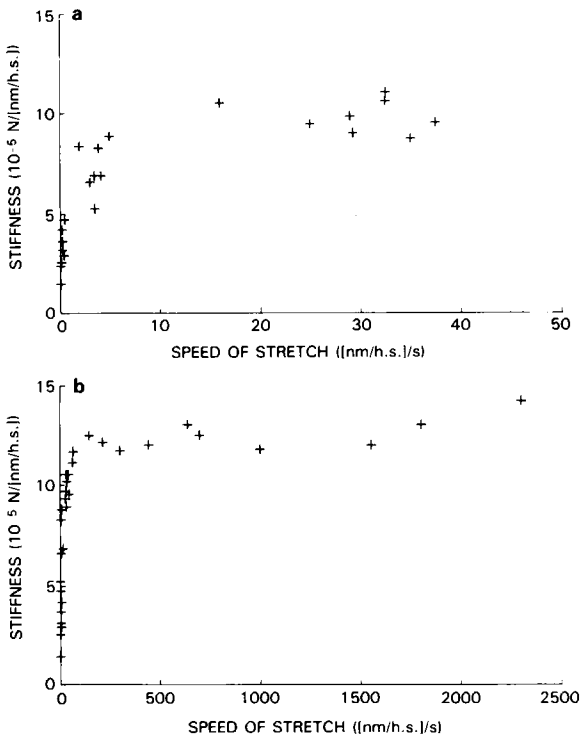

FIGURE 6 Effect of speed of stretch on apparent fiber stiffness in the presence of 4 mM $MgPP_i$. Ionic strength, 120 mM. (*a*) speeds of stretch up to 36 (nm/half-sarcomere)/s. (*b*) speeds of stretch up to 2,300 (nm/half-sarcomere)/s. Abscissa, speed of stretch in (nm/half-sarcomere)/s; ordinate, stiffness in N/(nm/half-sarcomere). Fiber diameter, 70 × 110 μm. Fiber length, 7.9 mm. Sarcomere length, 2.3–2.4 μm. Note, in *a*, that one appears to approach a speed-independent apparent fiber stiffness for speeds > 10 (nm/half-sarcomere)/s. In *b*, again it appears that a speed-independent stiffness has been obtained, but now the stiffness value is significantly higher.

solutions may be considerably higher than the highest measured apparent stiffness.

DISCUSSION

We previously showed that at low ionic strength a significant number of myosin cross-bridges are bound to actin in relaxed fibers (Brenner et al., 1982; Schoenberg et al., 1984). If the myosin cross-bridges exhibit rapid equilibrium between attachment and detachment, as suggested by solution studies (Stein et al., 1979), one would expect that with speeds of stretch comparable to the rates of attachment and detachment, the apparent fiber stiffness would increase with increasing speed of stretch. However, when the speed of stretch becomes fast compared to the attachment and detachment rate-constants, the apparent fiber stiffness should reach a maximum, speed independent value since no cross-bridges will attach or detach during the stretch. Such a maximum, "true" stiffness value could

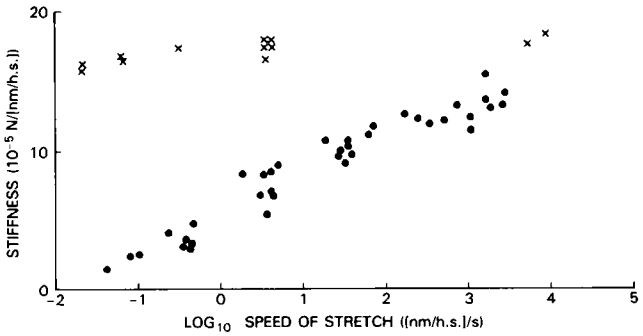

FIGURE 7 Apparent fiber stiffness at different speeds of stretch in rigor (x), and in the presence of 4 mM $MgPP_i$ (●). Ionic strength, 120 mM. Same fiber as in Fig. 6.

be used to get an estimate of the number of cross-bridges bound to actin. Unfortunately, the data presented in this paper show that in relaxed fibers at all ionic strengths, even at the fastest possible speeds of stretch we could obtain, we could not be certain we had obtained the maximum, speed independent stiffness value.

Further evidence that we may not be obtaining a true stiffness value in the presence of MgATP comes from our measurements of muscle fiber stiffness in $MgPP_i$. Here the stiffness continues to increase as speed of stretch is increased over about 4 decades. As suggested previously (Schoenberg, 1985; Schoenberg and Eisenberg, 1985), this result implies a rather wide range of detachment rate-constants in the fiber in the presence of $MgPP_i$. If a similar phenomenon is occurring in MgATP solution, then at our fastest speeds of stretch, the apparent stiffness value may be significantly less than the maximum, true stiffness value. If one assumes that the intrinsic cross-bridge stiffness is the same for rigor and relaxed fibers, then the number of attached cross-bridges in relaxed fibers at μ = 20 mM is estimated to be greater than 50% and perhaps even close to 100% of the number of cross-bridges attached in rigor fibers.

Comparison of Figs. 3 and 5 shows that the velocity of stretch necessary to attain a comparable apparent fiber stiffness is about three orders of magnitude slower in $MgPP_i$ solution than in MgATP solution. Even though a portion of this effect could be due to incomplete binding of $MgPP_i$ to the cross-bridges (Schoenberg and Eisenberg, 1985; Biosca et al., 1986), still, it appears that the attachment/detachment rates in MgATP solution are several orders of magnitude faster than in $MgPP_i$ solution, even under conditions where the solution binding constant of S-1 to actin is the same in the two solutions. These findings agree with the biochemical finding that, in solution, the rate-constant for dissociation of S-1·ATP (or S-1·ADP·P_i) from actin (Lymn and Taylor, 1971) is considerably faster than the rate-constant for dissociation of S-1·AMP-PNP and presumably S-1·PP_i (Marston, 1982). The data are also consistent with detachment rate constants of $> 1{,}000\ s^{-1}$ as found in MgATP solution (Lymn and Taylor, 1971).

As pointed out, the apparent leveling off of apparent stiffness seen when stiffness is plotted versus velocity of stretch on a linear scale may be an artifact if the fiber in MgATP solution follows a similar logarithmic relationship between stiffness and velocity of stretch as it does in $MgPP_i$ solution. Unfortunately, this makes it difficult to determine with certainty, the number of attached cross-bridges in relaxed fibers as a function of ionic strength. However, it is of interest to determine if our current data is consistent with other estimates of cross-bridge binding. Recently we have been able to show that under conditions where, in solution, the apparent binding constant of S-1·PP_i to actin is estimated to be $6 \times 10^2\ M^{-1}$ (T = 6 °C, ionic strength 0.17 M, 4 mM $MgPP_i$), the cross-bridge binding constant is approximately 1.5 (Brenner et al., 1986). Since, in solution, the binding of S-1·ATP to actin at μ = 20 mM is $5 \times 10^3\ M^{-1}$, or about eightfold stronger than under the above $MgPP_i$ reference condition, the cross-bridge binding constant in relaxed muscle at μ = 20 mM may also be about eightfold stronger, i.e., have a value of 12, suggesting that > 90% of the cross-bridges are attached. Greene et al. (1983) have found that increasing the ionic strength from μ = 20 mM to μ = 170 mM in solution, results in weakening of acto-S1 binding about 100-fold, an effect which is largely independent of the nucleotide bound to S-1. On this basis, the binding constant of the cross-bridge in relaxing solution at μ = 170 mM would be reduced to ~ 0.12, implying that ~10% of the cross-bridges are bound to the actin filament at physiological ionic strength. Alternatively, one might use solution binding constants derived from heavy meromyosin·ATP (HMM·ATP) rather than S1·ATP. Since Wagner and Giniger (1981) and Chalovich and Eisenberg (1986) found that intact HMM·ATP binds about fivefold weaker than S1·ATP in the absence of Ca^{++}, the above estimates would be ~65% at low ionic strength and ~2% at μ = 170 mM. Our stiffness measurements are compatible with this range of estimates.

The above gives an estimate of the number of weakly bound cross-bridges attached in a relaxed fiber; how many weakly bound cross-bridges would there be expected to be in an active fiber? Biochemical studies (Wagner and Giniger, 1981; Wagner, 1984; Chalovich and Eisenberg, 1986) suggest that activation of the muscle fiber by Ca^{2+} should have only a small effect on the binding to actin of cross-bridges having bound ATP or ADP·Pi. The predicted effect is approximately fivefold. On this basis, the combined biochemical and physiological data suggest that ~10–20% of the cross-bridges should be attached in the weakly-binding ATP states in an activated psoas fiber. Huxley and Kress (1985) point out that the lack of a large change in the equatorial I_{11}/I_{10} x-ray ratio with either quick release or high velocity shortening (Podolsky et al., 1976; Huxley, 1979; Huxley et al., 1983) implies that, in activated intact frog muscle, there may be many more weakly bound cross-bridges. It is not clear whether this is in conflict with the above calculation or whether it represents a species difference. It is possible that activated skinned rabbit psoas fibers may have fewer weakly bound cross-bridges than frog. This comes from the observation (Brenner, 1983) that the equatorial x-ray ratio, I_{11}/I_{10}, appears to decay considerably more in the rabbit at high velocities of shortening than it does in the frog. Therefore, at present, the biochemical and physiological data with rabbit seem to be consistent, with increase of ionic strength greatly weakening the binding constant of cross-bridges in the weakly binding states and increase of $[Ca^{2+}]$ having only a smaller effect. This may not be true for the frog, but, at present, little biochemical data is available for comparison.

In summary, then, the finding that, in the fiber, the binding constant of the cross-bridges to actin in 4 mM $MgPP_i$ at $\mu = 0.17$ M is ~1.5 (Brenner et al., 1986) leads to the estimation that 65 to 95% of the cross-bridges are attached to actin in low ionic strength relaxing solution and 2–10% are attached in $\mu = 170$ mM relaxing solution. The stiffness data here, as well as recent x-ray data (Brenner et al., 1984), seem compatible with these estimates.

Received for publication 11 December 1984 and in final form 26 March 1986.

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