Ca^{2+}-SENSITIVE CROSS-BRIDGE DISSOCIATION IN THE PRESENCE OF MAGNESIUM PYROPHOSPHATE IN SKINNED RABBIT PSOAS FIBERS

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

ABSTRACT We find that at 6°C in the presence of 4 mM $MgPP_i$, at low or moderate ionic strength, skinned rabbit psoas fibers exhibit a stiffness and an equatorial x-ray diffraction pattern similar to that of rigor fibers. As the ionic strength is increased in the absence of Ca^{2+}, both the stiffness and the equatorial x-ray diffraction pattern approach those of the relaxed state. This suggests that, as in solution, increasing ionic strength weakens the affinity of myosin cross-bridges for actin, which results in a decrease in the number of cross-bridges attached. The effect is Ca^{2+}-sensitive. Assuming that stiffness is a measure of the number of cross-bridge heads attached, in the absence of Ca^{2+}, the fraction of attached cross-bridge heads varies from ~75% to ~25% over an ionic strength range where ionic strength in solution weakens the binding constant for myosin subfragment-1 binding to unregulated actin by less than a factor of 3. Therefore, this phenomenon appears similar to the cooperative Ca^{2+}-sensitive binding of S1 to regulated actin in solution (Greene, L. E., and E. Eisenberg, 1980, *Proc. Natl. Acad. Sci. USA,* 77:2616). By comparing the binding constants in solution and in the fiber under similar conditions, we find that the "effective actin concentration," that is, the concentration that gives the same fraction of S1 molecules bound to actin in solution as cross-bridge heads are bound to actin in a fiber, is in the millimolar range. An effective actin concentration in the millimolar range suggests that the strength of actin binding to cross-bridges in fibers may be several orders of magnitude weaker than the strength of ATP binding. Previously, it has been assumed that these two quantities were equal, as this gives the minimum energy loss when ATP dissociates the cross-bridge from actin (Morales, 1980, *J. Supramol. Struct.,* 3:105:1975; Eisenberg, E., Hill, T. L. and Y. Chen, 1980, *Biophys. J.,* 29:195).

INTRODUCTION

An important question in understanding the contractile mechanism is how tightly myosin cross-bridges bind to actin filaments under different conditions. Although much is known about the strength of binding of soluble myosin subfragment-1 (S1) and heavy meromyosin (HMM) to actin in solution, relatively little information is available concerning the strength of binding of myosin cross-bridges in fibers. One reason for this lack is that to obtain a binding constant one needs to find a condition where there is a known fraction of attached cross-bridges. Furthermore, because of the nonlinear relationship between the binding constant and the fraction bound, to insure any sort of accuracy this fraction must not be close to either 0% or 100%. Unfortunately, a large number of conditions are ruled out. For example, in rigor practically 100% of the cross-bridges are attached under a variety of conditions, making this not well suited for study. In an actively contracting muscle, cross-bridges cycle in a mixture of states that introduces too much complexity. In relaxed muscle, it is possible to vary the number of attached cross-bridges by varying the ionic strength (Brenner et al., 1984), but the absolute fraction of attached cross-bridges is not known (Brenner et al., 1986). Ideally, one would like to find a condition where one could vary the fraction of attached cross-bridges over the entire range of 0% to 100%, in which case one could accurately determine when half the cross-bridges were attached.

Although most studies report that in fibers nearly all the cross-bridges are attached in the presence of magnesium pyrophosphate ($MgPP_i$) or magnesium adenyl-5′-yl imidodiphosphate (MgAMP-PNP), in solution $MgPP_i$ and MgAMP-PNP have a considerable weakening effect on acto-S1 binding. In the presence of troponin-tropomyosin, this weakening effect is highly cooperative (Greene and Eisenberg, 1980*a*). If a similar cooperativity occurs in muscle in the presence of $MgPP_i$ or MgAMP-PNP, it might be possible to obtain complete detachment of cross-bridges simply by reducing the strength of binding slightly by, for example, raising the ionic strength.

Dr. Brenner's present address is the Institute of Physiology II, University of Tübingen, Federal Republic of Germany.
Address offprint requests to Dr. Schoenberg.

Along these lines, we examined the behavior of skinned rabbit psoas fibers in solutions containing 4 mM $MgPP_i$ at 6°C. This temperature was chosen to maximize the likelihood of finding cross-bridge dissociation, since solution studies suggest the strength of binding is less at 6°C than at room temperature (Greene and Eisenberg, 1980*b*). In agreement with previous studies (White, 1970; Schoenberg and Eisenberg, 1985), we found that at moderate ionic strength (80–120 mM), nearly all the cross-bridges are attached in 4 mM $MgPP_i$ solution. However, when cross-bridge binding is weakened somewhat by raising the ionic strength to 200–240 mM, nearly all the cross-bridges reversibly detach. This dissociation of cross-bridges in the presence of $MgPP_i$ is both Ca^{2+}-sensitive and seemingly cooperative, just like S1 binding to regulated actin in solution under similar conditions. By comparing the solution and fiber binding, we were able to express the strength of binding in the fibers in terms of the concentration of actin that is necessary in solution to mimic the binding in the fiber. This "effective actin concentration" was found to be in the millimolar range.

Preliminary accounts of this work have been reported briefly (Brenner et al., 1983).

METHODS

Biochemical Studies in Solution

The binding of S-1 to regulated actin in the presence of $MgPP_i$ at 6°C was measured as described previously (Greene and Eisenberg, 1980*a*) in a solution containing 10 mM imidazole, 6 mM $MgCl_2$, 4 mM PP_i, 1 mM DTT, 0.2 mM Ap_5A, and either 0.5 mM CaEGTA or 1 mM EGTA. The total ionic strength was ~35 mM. The binding constant of S1 to the strong form (see Discussion) of troponin-tropomyosin-actin was obtained by fitting the data to the cooperative binding model of Hill et al. (1980). Fitting the data to the model of Hill et al. also permitted determination of the parameter, L', the equilibrium constant between the weak-binding and strong-binding forms of the troponin-tropomyosin-actin complex (see Discussion).

Stiffness and X-ray Diffraction Studies in Skinned Fibers

Single skinned rabbit psoas fibers were prepared and mounted as reported previously for both stiffness and equatorial x-ray diffraction measurements (Brenner et al., 1982; Brenner et al., 1984). The base solution contained 10 mM imidazole, 6 mM $MgCl_2$, 4 mM PP_i, 1 mM DTT, 0.2 mM Ap_5A, and either 1 mM EGTA or CaEGTA. Ionic strength was varied between 40–240 mM by the addition of KCl. The temperature was maintained at 6 ± 1°C, the pH at 7.00 ± 0.05. The fraction of cross-bridges attached in the various solutions was estimated by both stiffness and x-ray determination. Stiffness was taken as the initial slope of the instantaneous force–sarcomere length relationship during stretch of the fiber at a velocity of $\sim 5 \times 10^3$ nm/half-sarcomere/s. The ratio of x-ray intensities, I_{11}/I_{10}, was determined from the integrated intensities of the two innermost equatorial x-ray reflections, [1, 1] and [1, 0]. Previously it had been shown that an increase in this ratio indicates mass transfer from the thick filament region to the thin filament region, signifying cross-bridge attachment (Huxley, 1968; Yu et al., 1979; Brenner and Yu, 1985).

In $MgPP_i$ solution, when a region of the fiber was exposed to x-rays for >15 min, it was damaged by radiation in such a way that the patterns always looked like the rigor pattern regardless of ionic strength. To avoid this problem, we obtained each data point (sample time <10 min) from a new spot along the fiber. To be certain that this in no way comprised comparability between the x-ray and stiffness measurements, in four experiments we measured stiffness and x-ray diffraction patterns from the same fiber simultaneously. In this case, stiffness was measured based on overall length rather than sarcomere length.

In the x-ray studies, rigor, $MgPP_i$, and relaxed conditions were studied in random order (although the fiber was always put into $MgPP_i$ solution from rigor). In addition, at the beginning and end of the experiments, relaxed patterns at an ionic strength of 170 mM were recorded to further test reversibility.

RESULTS

S1 Binding to Regulated Actin in the Presence of 4 mM PP_i: Solution Studies

Fig. 1 shows the binding of S1 to regulated actin in 4 mM $MgPP_i$ solution at 6°C, as S1 concentration is varied in the presence and absence of Ca^{2+}. The ordinate gives the fraction of actin monomers having S1 bound and the abscissa is the product of the S1 binding constant to the strong form of troponin-tropomyosin-actin times the free S1 concentration. It is seen, as reported previously for experiments that took place at 25°C, that the binding of S1 to actin is Ca^{2+}-sensitive and cooperative, as evidenced by sigmoidicity in the binding isotherms. The cooperativity is not as pronounced as that observed at higher temperature. By fitting the data of Fig. 1 to the model of Hill et al. (1980), the apparent binding constant of S1 to the strong form of regulated actin in the presence of $MgPP_i$ under the above conditions was found to be 4.5×10^4 M^{-1}. As at 25°C, we found that the binding constant to the strong form was the same in the presence and absence of Ca^{2+} and about two- to threefold stronger than the binding constant of S1 to unregulated actin under the same conditions (data not shown).

The solution chosen for the above experiment was designed to match the conditions of the fiber experiments (below) as closely as possible, except that the ionic strength was significantly lower in the solution study since, at 6°C,

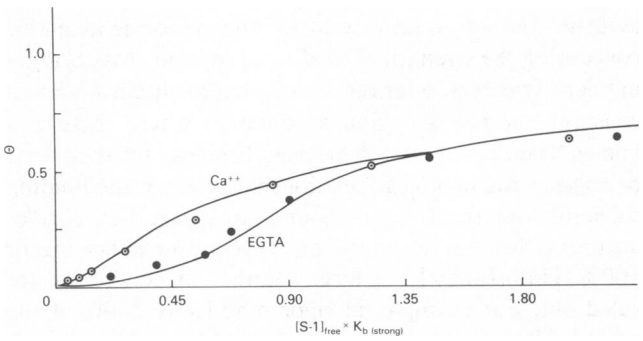

FIGURE 1 Binding of S1·PP_i to regulated actin at 6°C, μ = 35 mM, in the presence (○) and absence (●) of Ca^{2+}. ⊙. is moles of S1 bound per mole of F-actin monomer. Smooth curves are calculated from the model of Hill et al. (1980) assuming $K_{(strong)} = 4.5 \times 10^4$ M^{-1}, $K_{(weak)} = 5 \times 10^3$ M^{-1}, $Y = 5$, and $L' = 30$ for $+Ca^{2+}$ and 2 for $-Ca^{2+}$. Similar values were obtained from two other experiments.

it was not technically feasible to measure the binding of S1·PP_i to regulated actin at ionic strengths of 80–240 mM; at these ionic strengths, at 6°C, the binding is too weak to measure directly. However, from the data in Fig. 1, we can readily estimate the binding constant at high ionic strength as follows. It has been observed that the ionic strength dependency of the binding of S1 to actin is relatively insensitive to nucleotide bound (Greene et al., 1983). For example, increasing the ionic strength from μ = 35 mM to μ = 170 mM weakens the binding of S1·AMP-PNP at 25°C by 80-fold (Greene et al., 1983). In a similar experiment done with ADP at 6°C (data not shown), we also obtained 80-fold weakening. Assuming that S1·PP_i behaves similarly, the binding constant in the presence of 4 mM $MgPP_i$ at μ = 170 mM, T = 6°C, is estimated from the data of Fig. 1 to be $4.5 \times 10^4/80$ or ~600 M^{-1}. Similarly, at μ = 240 mM the binding constant of S1 to actin in the presence of 4 mM $MgPP_i$ at 6°C is estimated as 300 M^{-1}. The significance of these numbers in relating the biochemical and physiological studies is discussed below.

Another parameter, useful in relating the biochemical and physiological results, is L', a parameter that is obtained by fitting the binding data to the model of Hill et al., 1980. L' is a measure of the equilibrium between the weak and strong binding forms of the troponin-tropomyosin-actin complex and was found to be 30 in the data of Fig. 1. Since we also obtained a value of L' = 30 for S1 binding in the presence of ADP at 6°C, μ = 170 mM (data not shown), we have assumed that the value of L' is not particularly sensitive to ionic strength (Greene, 1982), although, as discussed below, this is not a critical assumption.

Cross-bridge Binding to Actin in the Presence of 4 mM PP_i: Fiber Studies

Having shown that S1 binding to regulated actin in solution at 6°C is Ca^{2+}-sensitive and cooperative, we looked for similar behavior in fibers. Since S1 or, equivalently, cross-bridge concentration, is hard to vary in the fiber, we instead varied the strength of cross-bridge binding by varying ionic strength.

Fig. 2 shows a typical stiffness measurement trace (force vs. sarcomere displacement record) for a fiber bathed in 4 mM $MgPP_i$, with and without Ca^{2+}, both at μ = 80 and 220 mM. At the lower ionic strength, the fiber in $MgPP_i$ is nearly as stiff as in rigor regardless of the presence or absence of Ca^{2+} (Fig. 2 *A*). However, at the higher ionic strength there is a major difference in stiffness in the presence and absence of Ca^{2+}. In the presence of Ca^{2+}, the apparent fiber stiffness remains comparable to that of rigor, but in the absence of Ca^{2+} the apparent fiber stiffness is substantially decreased to <10% that of rigor (Fig. 2 *B*). These changes are reversible.

Fig. 3 shows a summary of four experiments in which stiffness was measured in the presence of $MgPP_i$ with and without Ca^{2+} as the ionic strength was varied from 40 to 240 mM. To be able to compare the different experiments, stiffnesses were expressed relative to the rigor stiffness at μ = 40 mM. The results clearly show that fiber stiffness decreases as a function of ionic strength and the decrease is strongly Ca^{2+}-sensitive. In the absence of Ca^{2+}, the relative stiffness decreases to ~ 0.5 at μ = 170 mM. In the presence of Ca^{2+}, the apparent stiffness also decreases as ionic strength increases, but the change at the highest ionic strength, μ = 240 mM, is only about one quarter as large as that found in the absence of Ca^{2+}. The simplest interpretation of these stiffness data is that increasing ionic strength decreases the number of attached cross-bridges in the

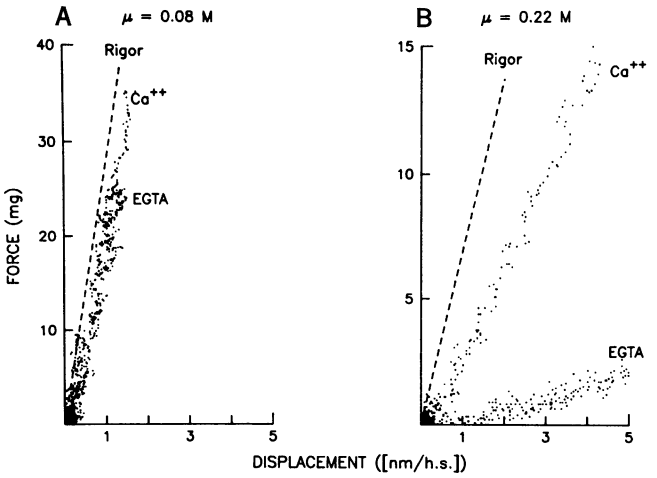

FIGURE 2 Original records showing force versus change in sarcomere length during stretches of a single skinned rabbit psoas fiber. The slope of the relationship gives the fiber stiffness. The speed of stretch was ~5 × 10^3 nm/half-sarcomere/s. The bathing solutions contained 4 mM $MgPP_i$, with or without Ca^{2+}; T = 6°C. Dashed line shows the rigor stiffness. In *A*, μ = 80 mM; in *B*, μ = 220 mM.

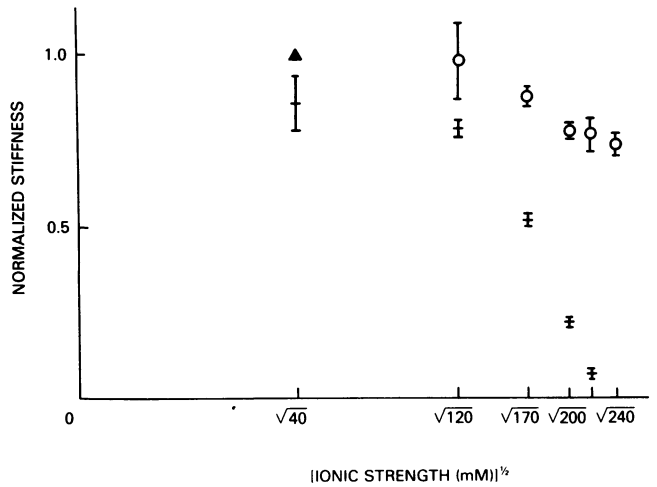

FIGURE 3 Summary of the ionic strength dependence of fiber stiffness in the presence of 4 mM $MgPP_i$ obtained from four fibers at 6°C. O, in the presence of PP_i and Ca^{2+}; +, in the presence of $MgPP_i$ but absence of Ca^{2+}. Data normalized to the rigor stiffness at μ = 40 mM (▲). Sarcomere length = 2.3 μm. Temperature = 6°C. Error bars show ±SEM.

presence of 4 mM $MgPP_i$, and that the decrease is Ca^{2+}-sensitive and possibly cooperative (see Discussion).

Since in certain cases changes in rate-constants, as opposed to changes in the number of cross-bridges attached, can produce an apparent stiffness change (Schoenberg, 1985; Brenner et al., 1986), we also examined the equatorial x-ray diffraction pattern of single, skinned psoas fibers under the same set of conditions as the stiffness measurements to be certain that the stiffness changes were, in fact, due to cross-bridge detachment. As Fig. 4 shows, at low ionic strength (*A* and *D*) all the patterns were rigor-like (*F*); in the presence of 4 mM $MgPP_i$ with or without Ca^{2+}, the intensity ratio, I_{11}/I_{10}, was only 20% lower than that of rigor. The rigor I_{11}/I_{10} ratio was insensitive to an increase in ionic strength from 80 to 240 mM. In $MgPP_i$ solution in the presence of Ca^{2+}, an increase in ionic strength to 240 mM also caused only a relatively small decrease in I_{11}/I_{10} (cf, Fig. 4, *D* and *E*). In contrast, increasing the ionic strength to 240 mM in $MgPP_i$ solution without Ca^{2+} (Fig. 4 *B*) caused the diffraction pattern to become very similar to that of a relaxed fiber at μ = 170 mM (C), not only in the intensity ratio, but also in the shape of the diffraction peaks. Like the stiffness measurements, these results suggest that as ionic strength is increased in the presence of 4 mM $MgPP_i$, cross-bridges detach from actin.

Fig. 5 shows a summary of five x-ray experiments in which the ionic strength was varied as in Fig. 3. It can be seen that the equatorial x-ray ratio, I_{11}/I_{10}, parallels the stiffness measurements. In the absence of Ca^{2+}, the midpoint of the intensity decrease occurs at ~180 mM and, in the presence of Ca^{2+}, at μ = 220 mM the total decrease is about one-third that observed in the absence of Ca^{2+}. Simultaneous stiffness measurements made during x-ray exposure using overall (rather than sarcomere) length control are shown in Fig. 5 *B* and provide further evidence that results from these two approaches correlate.

The effects of ionic strength are reversible. Diffraction patterns of relaxed and rigor fibers at μ = 170 mM at the end of experiments were indistinguishable from those obtained at the beginning of experiments. This was true despite the fact that a typical x-ray experiment generally lasted >5 h and involved 20 to 30 solution changes. We did not measure active isometric tension following these experiments, but we did find, in a separate series of experiments calling for somewhat briefer exposure to high ionic strength solution, that fibers were capable of exerting full isometric tension after exposure to μ = 240 mM solution.

It was also found that not only were the x-ray intensity changes seen in fibers bathed in $MgPP_i$ very sensitive to Ca^{2+} and ionic strength, they were also very sensitive to temperature. Lowering the temperature from 6°C to 2°C

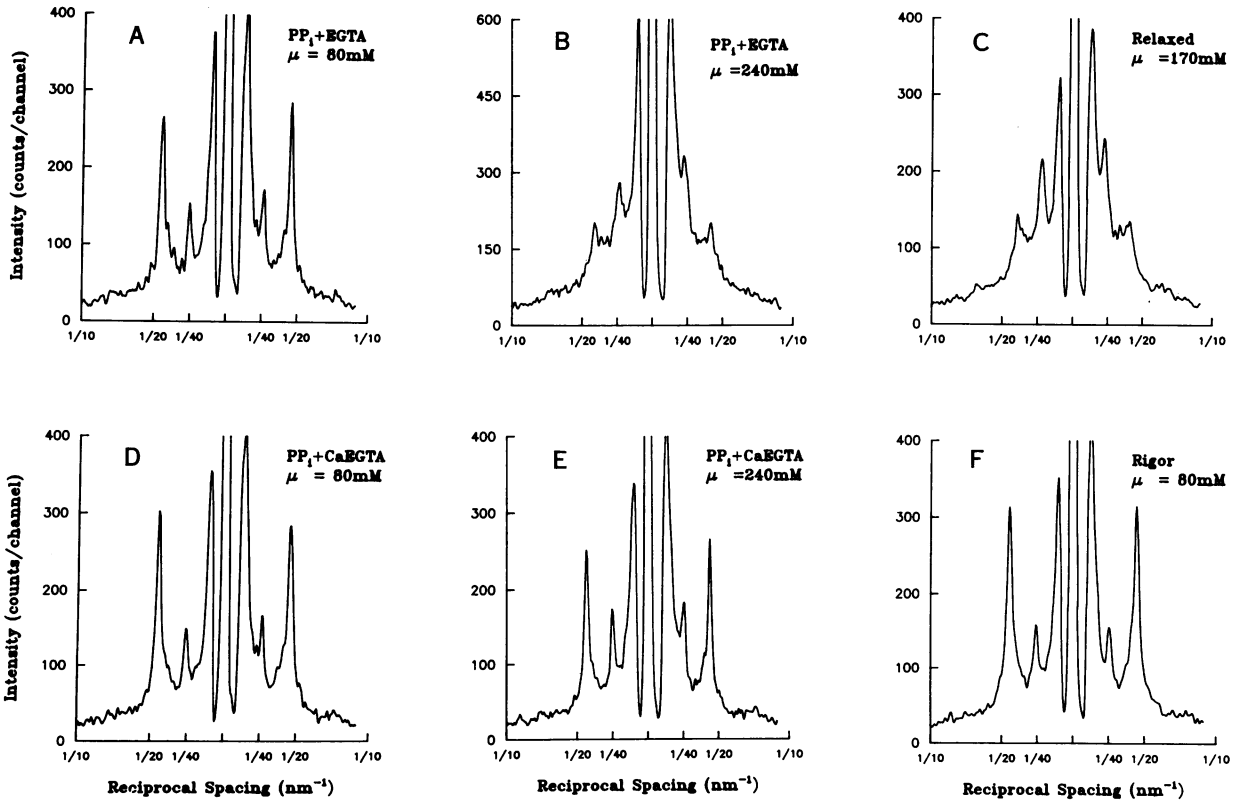

FIGURE 4 Equatorial x-ray diffraction patterns obtained from single skinned psoas fibers in the presence of 4 mM $MgPP_i$ at 6°C. Exposure time = 500 s. Sarcomere length = 2.3 μm. (*A*) $-Ca^{2+}$, μ = 80 mM; (*B*) $-Ca^{2+}$, μ = 240 mM; (*C*) relaxed control, μ = 170 mM; (*D*) $+Ca^{2+}$, μ = 80 mM; (*E*) $+Ca^{2+}$, μ = 240 mM; and (*F*) rigor control, μ = 80 mM.

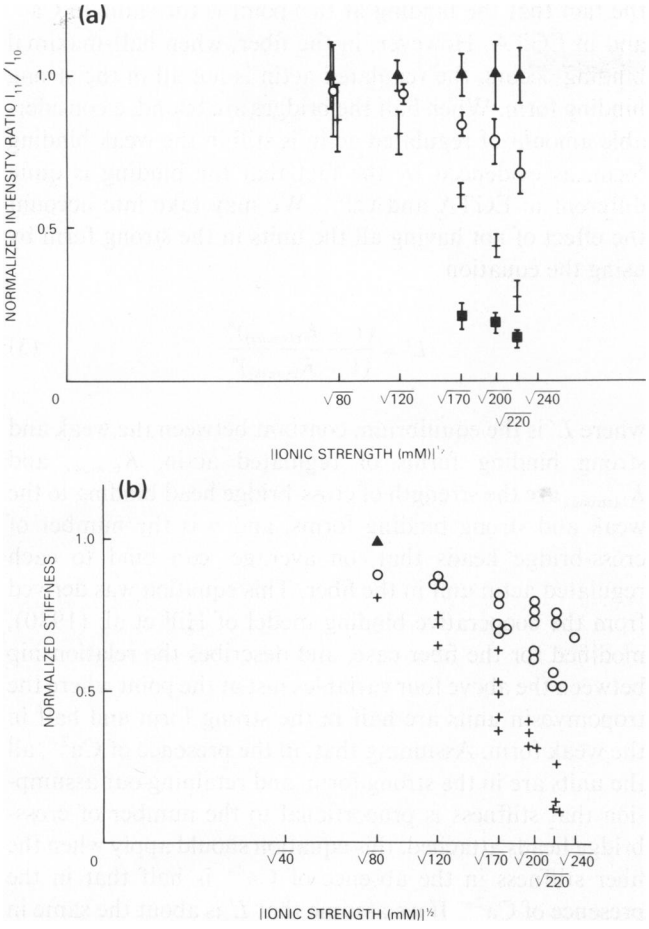

FIGURE 5 (*a*) Summary of the ionic strength dependence of the equatorial x-ray intensity ratio, I_{11}/I_{10}, obtained from five fibers in 4 mM $MgPP_i$ at 6°C. I_{11}/I_{10} is normalized with respect to the rigor value for each individual fiber. Error bars show ±SEM. (▲) Rigor. (○) 4 mM $MgPP_i$, $+Ca^{2+}$. (+) 4 mM $MgPP_i$, $-Ca^{2+}$. (■) Relaxed. (*b*) Apparent fiber stiffness measured simultaneously with x-ray exposure. The stiffness was calculated from the force change and the overall length change during stretch at a speed of ~10^3 nm/half-sarcomere/s. Normalization and symbols, as for *a*.

in Ca^{2+}-free $MgPP_i$ solution at μ = 200 mM resulted in the x-ray pattern becoming very much more relaxed. This change in the equatorial pattern with temperature also was reversible.

DISCUSSION

In 4 mM $MgPP_i$ solution, the binding of S1 to regulated actin (actin + troponin + tropomyosin) is Ca^{2+}-sensitive and cooperative. This behavior has been analyzed by Hill et al. (1980) in terms of a model in which each regulated actin unit, which consisted of seven actin monomers and one troponin-tropomyosin molecule, can exist in two forms, the weak-binding form and the strong-binding form. At low concentrations of S1, most of the regulated actin units are in the weak binding form so that S1 binds very weakly to actin. However, when enough S1 molecules bind, they shift the regulated actin units into the strong-binding form, the form where S1 binds strongly. It is this shift in the form of regulated actin with increasing S1 concentration that causes S1 to bind cooperatively. This model also suggests that Ca^{2+} affects the S1 binding by shifting the equilibrium between the two forms of regulated actin toward the strong binding form. This occurs without any change in the actual strength of binding of S1 to the strong form. Hence S1 binding in the presence of Ca^{2+} is much less cooperative than in the absence of Ca^{2+}, but the strength of binding at high S1 concentration is the same in the presence and absence of Ca^{2+}.

In many respects, cross-bridge binding in fibers in the presence of 4 mM $MgPP_i$ appears to be similar to S1 binding to regulated actin in solution, that is, high ionic strength in the fiber, where binding is weak, corresponding to low S1 concentrations in solution. In the fiber at low-to-moderate ionic strength, both in the presence and absence of Ca^{2+}, all or most of the cross-bridges are attached to actin, as evidenced by the high stiffness and high I_{11}/I_{10} ratio. Here presumably all of the regulated actin is in the strong form. However, as the ionic strength is increased from 150 to 220 mM in the absence of Ca^{2+}, fiber stiffness decreases >50%. Here presumably regulated actin units are shifting into the weak form, just as they do in solution in the absence of Ca^{2+} at low S1 concentration. Furthermore, just like in solution at low S1 concentration, in the fiber at high ionic strength the fraction of bridges bound is Ca^{2+}-sensitive.

The dissociation of cross-bridges in the absence of Ca^{2+} at high ionic strength appears to be cooperative. Over the range of ionic strengths where the stiffness and presumably the number of attached cross-bridges varies from ~75% to ~25% (150 to 220 mM, Fig. 3), ionic strength in solution weakens the binding constant of S1 to unregulated actin less than threefold. If a similar change in cross-bridge head binding constant occurred in the fiber between μ = 150 and μ = 220 mM, then this would imply that there must be some cooperativity in cross-bridge binding, since as much as a 10-fold change in binding constant would be required to produce that amount of detachment in a noncooperative system. One should be cautious about this conclusion, however, since if in the fiber one needs to dissociate both cross-bridge heads before getting a stiffness or x-ray change, then monitoring stiffness or x-ray will tend to make the system appear more cooperative than directly monitoring head detachment would. In this regard, it should be noted that in solution the cooperativity is less at 6°C than it is at 25°C.

Recently, Ishiwata et al. (1986) have reported that increasing the ionic strength of the solution bathing myofibrils in the presence of $MgPP_i$ also reversibly dissociates cross-bridge heads. Their findings are very similar to those reported here, and suggest that with regard to head dissociation in the presence of $MgPP_i$, myofibrils and fibers behave very similarly.

In an earlier paper, Ishiwata et al. (1985) reported that increasing the total ionic strength to values higher than

those used in the present study results in irreversible dissociation and decrease of length of the thick filaments. We, too, see irreversible filament dissociation at very high ionic strength (B. Brenner and M. Schoenberg, unpublished observations), but this does not seem to be a factor in the experiments reported here, as evidenced by the reversibility of these effects.

Estimating the Strength of Cross-bridge Binding in Fibers

The strength of cross-bridge binding in fibers can be expressed in terms of the "effective actin concentration," the actin concentration that is necessary in solution to produce the same fraction of S1 molecules bound as cross-bridges are bound in the fiber. The effective actin concentration will be large if cross-bridges tend to bind strongly in fibers and small if they tend to bind weakly.

In solution, the binding constant of S1 to actin is defined as

$$K_{\text{solution}} = \frac{[\text{fraction of attached S1}]}{[A][\text{fraction of unattached S1}]}, \quad (1)$$

where $[A]$ is the free actin concentration. In muscle, since each cross-bridge sees only one, or at most, a few actin monomers, the reaction is first order, and the binding constant is defined as

$$K_b = \frac{[\text{fraction of attached cross-bridges}]}{[\text{fraction of unattached cross-bridges}]}.$$

With K_b and K_{solution} measured under identical conditions, the effective actin concentration is simply K_b/K_{solution}.

If we, for the moment, ignore the influence of tropomyosin on cross-bridge binding, and we assume that fiber stiffness is a measure of the number of cross-bridge heads attached, then calculating an effective actin concentration in the presence of 4 mM $MgPP_i$ is straightforward. If we assume that at moderate ionic strength (e.g., 80 mM) nearly all of the cross-bridges are attached (White, 1970; Schoenberg and Eisenberg, 1985), then when the stiffness is reduced by 50%, 50% of the cross-bridges are attached, implying $K_b = 1$. Fig. 3 shows that this occurs in the absence of Ca^{2+} at an ionic strength of ~170 mM. Since K_{solution} at μ = 170 mM is estimated to be ~600 M^{-1} (see Results), the effective actin concentration in the presence of 4 mM $MgPP_i$ and the absence of Ca^{2+} is calculated to be ~1.5 mM.

In determining the effective actin concentration in the simple calculation used above, it was implicitly assumed that the troponin-tropomyosin complexes were all in the strong binding form at the point where half-maximal binding of the cross-bridges to actin occurs. In our solution studies, the troponin-tropomyosin-actin complex is indeed completely shifted over to the strong binding form at the point where half the SI heads are bound, as illustrated by the fact that the binding at this point is the same in Ca^{2+} and in EGTA. However, in the fiber, when half-maximal binding occurs, the regulated actin is not all in the strong binding form. When half the bridges are bound, a considerable amount of regulated actin is still in the weak binding form, as evidenced by the fact that the binding is quite different in EGTA and Ca^{2+}. We may take into account the effect of not having all the units in the strong form by using the equation

$$L' = \frac{(1 + K_{b(\text{strong})})^n}{(1 + K_{b(\text{weak})})^n} \quad (3)$$

where L' is the equilibrium constant between the weak and strong binding forms of regulated actin, $K_{b(\text{weak})}$ and $K_{b(\text{strong})}$ are the strength of cross-bridge head binding to the weak and strong binding forms, and n is the number of cross-bridge heads that, on average, can bind to each regulated actin unit in the fiber. This equation was derived from the cooperative binding model of Hill et al. (1980), modified for the fiber case, and describes the relationship between the above four variables just at the point where the tropomyosin units are half in the strong form and half in the weak form. Assuming that, in the presence of Ca^{2+}, all the units are in the strong form, and retaining our assumption that stiffness is proportional to the number of cross-bridge heads attached, this equation should apply when the fiber stiffness in the absence of Ca^{2+} is half that in the presence of Ca^{2+}. If we assume that L' is about the same in the fiber and in solution, and that very few bridges bind to actin in the weak binding form, then, taking $L' = 30$ from Fig. 1, $K_{b(\text{weak})} \ll 1$, and $n = 4$ (since in the fiber there are about four myosin heads per tropomyosin unit), we then calculate an effective actin concentration of ~2 mM. The reason this value is only slightly different from that we obtained without correction for the state of the troponin-tropomyosin complex is because the value of L' is relatively small, implying that relatively little binding energy is expended in pushing the troponin-tropomyosin units into the strong form. It should be mentioned that although there is, in fact, little hard evidence concerning the value of L' in fibers, L' would have to be >10,000 before our estimate of the effective actin concentration would be in error by more than a factor of ten.

A third estimate of the effective actin concentration can be calculated from the data obtained in the presence of Ca^{2+}, assuming, as we did above, that in the presence of Ca^{2+}, all of the tropomyosin units are in the strong binding form. At μ = 240 mM, approximately ¾ of the cross-bridges are attached, which means that K_b is about 3. Under this condition, the binding constant in solution is estimated to be ~300 M^{-1} (see Results), resulting in an estimate of the effective actin concentration of ~10 mM.

Considering the uncertainty in each of the calculations, the three calculated values for the effective actin concentration are all about equal. In theory, the measurement in

the presence of Ca^{2+} should be the most reliable. However, to insure reversibility, we were hesitant to raise the ionic strength >240 mM. At this ionic strength, in the presence of Ca^{2+}, the stiffness decrease was only ~25%, making this estimate not so reliable as it might otherwise be.

Several assumptions are implicit in the above calculations. One is that the amount of $MgPP_i$ binding is the same in solution and in fibers. It is important to note that our calculation of the effective actin concentration does not depend on the assumption that acto-S1 is saturated with $MgPP_i$, it only requires that the level of saturation be the same in solution and in fibers. There has been considerable disagreement about the strength of binding of the ATP analogues, $MgPP_i$ and MgAMP-PNP, both in solution and in fibers. Early reports generally suggested tight binding of $MgPP_i$ and MgAMP-PNP in both solution and fibers (Hofmann and Goody, 1978; Marston et al., 1976). However, more recent reports suggest that much weaker binding occurs in both cases. In fibers, the studies of Pate and Cooke (1985) and the studies of Schoenberg and Eisenberg (1985), both suggest that MgAMP-PNP and $MgPP_i$ bind weakly to cross-bridges complexed with actin. In solution, recent biochemical studies with acto-S1 (Biosca et al., 1986*a*), with crosslinked acto-S1 (Sleep, 1986; Biosca et al., 1986*a*), and with myofibrils (Sleep, 1986; Johnson, 1986; Biosca et al., 1986*b*) all suggest that MgAMP-PNP and $MgPP_i$ bind weakly to actomyosin. If these more recent studies are valid, there is not a large difference in the affinity of $MgPP_i$ to acto-S1 in solution and to cross-bridges in fibers and the level of $MgPP_i$ saturation should be the same in both cases.

A second assumption in our calculation of the effective actin concentration is that the extent of analogue saturation is independent of ionic strength. This is true for MgAMP-PNP in solution (Greene, 1981; Konrad and Goody, 1982), and the similarity of the dissociation constants in fibers and in solution under many conditions (Biosca et al., 1986*a*; Schoenberg and Eisenberg, 1986) suggests that the same should be true for $MgPP_i$ in fibers.

A third assumption is that stiffness and equatorial x-ray ratio are quantitative measures of cross-bridge head attachment. Although it has been shown that muscle stiffness under many different conditions scales with filament overlap (Ford et al., 1981; Tawada and Kimura, 1984; Brenner et al., 1982), it has not been demonstrated clearly how, for example, dissociation of one of the two cross-bridge heads affects cross-bridge stiffness or equatorial x-ray ratio. We have made the simplest assumption that the stiffness and equatorial ratio are proportional to head number. If this assumption is invalid, clearly our estimates of effective actin concentration could be very much in error.

Nevertheless, if one merely assumes that very few cross-bridges are attached when the stiffness or equatorial x-ray ratio is similar to that of a relaxed fiber at normal ionic strength, then it seems likely that for most of the cross-bridge heads the effective actin concentration in the presence of $MgPP_i$ is in the millimolar rather than the molar range. If the effective actin concentration is also the same in the absence of analogue—that is, if removal of analogue has the same effect on the free energy of the cross-bridge in the fiber as on S1 in solution—then, an effective actin concentration in the mM range implies that, in fibers, actin binds about 3 to 4 orders of magnitude weaker to the cross-bridge than does ATP. This follows from the fact that at an ionic strength of 0.14 M, T = 22°C, pH = 7.0, S1 binds to actin in solution without nucleotide with a binding constant of $\sim 2 \times 10^7$ M^{-1} (Greene and Eisenberg, 1978). Multiplying this by an effective actin concentration of 3 mM yields a binding affinity in the fiber of 6×10^4. The binding constant of ATP to S1 in solution under similar conditions is $\sim 10^{10} - 10^{11}$ M^{-1} (Wolcott and Boyer, 1975; Goody et al., 1977). Assuming that the binding constant of ATP to a cross-bridge is similar to that to S1 (Biosca et al., 1986*a*; Schoenberg and Eisenberg, 1986), the binding affinity of ATP to a cross-bridge in the fiber at an ATP concentration of 6 mM would be from 6×10^7 to 6×10^8, or about 10^3 or 10^4 times stronger. Previously it had been assumed that these quantities were equal since equality would minimize the free energy loss when ATP dissociates the cross-bridge from actin (Morales, 1975; Eisenberg et al., 1980).

In summary, in fibers cross-bridges in the presence of 5 mM $MgPP_i$ and absence of Ca^{2+} are nearly all bound at an ionic strength of 100 mM and nearly all dissociated at an ionic strength of 250 mM. The binding is Ca^{2+}-sensitive, and quite possibly cooperative. Comparison of the fiber and solution binding suggests that the effective actin concentration for cross-bridge binding is in the millimolar rather than molar range. If the effective actin concentration is independent of nucleotide or analogue at the active site, this number should be useful in estimating the strength of cross-bridge binding under conditions other than those studied here, as for example, during relaxation (Brenner et al., 1986) or activation.

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