The Effect of Polyethylene Glycol on the Mechanics and ATPase Activity of Active Muscle Fibers

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ABSTRACT We have used polyethylene glycol (PEG) to perturb the actomyosin interaction in active skinned muscle fibers. PEG is known to potentiate protein-protein interactions, including the binding of myosin to actin. The addition of 5% w/v PEG (MW 300 or 4000) to active fibers increased fiber tension and decreased shortening velocity and ATPase activity, all by 25–40%. Variation in [ADP] or [ATP] showed that the addition of PEG had little effect on the dissociation of the cross-bridge at the end of the power stroke. Myosin complexed with ADP and the phosphate analog V_i or AlF_4 binds weakly to actin and is an analog of a pre-power-stroke state. PEG substantially enhances binding of these states both in active fibers and in solution. Titration of force with increasing $[P_i]$ showed that PEG increased the free energy available to drive the power stroke by about the same amount as it increased the free energy available from the formation of the actomyosin bond. Thus PEG potentiates the binding of myosin to actin in active fibers, and it provides a method for enhancing populations of some states for structural or mechanical studies, particularly those of the normally weakly bound transient states that precede the power stroke.

INTRODUCTION

Large polymers are excluded from a solvent region surrounding proteins and from crevices in proteins. The binding of one protein to another reduces the volume from which the polymers are excluded, providing an entropic effect that favors protein-protein association (Bhat and Timasheff, 1992; Timasheff, 1993; Parsegian et al., 1986). One such polymer, polyethylene glycol (PEG), has been used by numerous investigators to potentiate the interactions of macromolecules and in particular to favor the interactions that crystallize proteins. The mechanism by which PEG induces protein aggregation has been studied extensively, mainly for its use in inducing crystallization for structural studies. The PEG polymer adopts a random coil configuration in solution, acting as if it were a sphere with an effective radius. The effective radii of the two polymers used here, with molecular weights of 300 and 4000 (PEG 300 and PEG 4000), are 7 Å and 25 Å, respectively (Bhat and Timasheff, 1992). The center of mass of the PEG polymer cannot come closer to a protein than its effective radius. Thus the PEG is excluded from a volume around the protein defined by the surface area of the protein and the effective radius of the PEG. Removal of the protein surface area formed by protein-protein interactions effectively decreases the volume that is not available for solvating the PEG. This provides an entropic effect promoting protein-protein association. The greater the volume that becomes accessible for PEG solvation upon formation of the protein complex, the greater the effect of PEG. The volume change is related to the surface area involved in the complex, so the effect of PEG on a protein-protein interaction can provide information on the topology of their interface. In the same fashion, the presence of a polymer that is excluded from a cavity in a protein favors closing of the cavity. For example, PEG has been shown to promote the binding of glucose to hexokinase, because binding involves closure of a crevice, resulting in a decrease in the volume that is inaccessible to PEG (Rand et al., 1993).

Because PEG is excluded from a volume surrounding a protein, its presence can be thought of as exerting an osmotic pressure, which attempts to shrink the inaccessible volume. The change produced in the equilibrium between two states can be related to the change in the volume inaccessible to PEG, ΔV, and to the osmotic pressure exerted by the PEG, Π, by the following:

$$RT \ln (K_1/K_0) = -(\textstyle\prod_1 - \prod_0)\Delta V \qquad (1)$$

Here K_1 and K_0 refer to the equilibrium constants in the presence of osmotic pressures Π_1 and Π_0. The osmotic pressure exerted by PEG has been determined as a function of PEG molecular weight and concentration (Reid and Rand, 1997; Cohen and Highsmith, 1997). Thus the measurement of an equilibrium constant for a protein-protein association as a function of PEG concentration can be used to calculate ΔV and from this to estimate the size of the interface formed during the association.

Previous work has shown that the addition of PEG can potentiate the formation of the actomyosin complex in solution (Highsmith et al., 1996; White et al., 1995). During actin-activated ATP hydrolysis, addition of PEG decreased

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the value of the K_m for actin by a factor of 10–25. Addition of PEG had little effect on the ATPase activity of myosin by itself under conditions where the myosin is not precipitated by the PEG (Highsmith et al., 1998). Low-molecular-weight PEGs in the range of 300-4000 MW readily diffuse into the interfilament space and do not compress the fiber lattice. Thus they can be used to perturb protein-protein interactions inside the filament array of a muscle fiber. We have used PEG to perturb the actomyosin interaction in skinned muscle fibers, and we have measured its effect on the mechanics and ATPase activity of the fibers. PEG may affect several transitions in active fibers, but it will certainly produce a more favorable actomyosin bond. A number of experiments have defined the cyclic actin, myosin, and ATP interaction in solution reviewed by Cooke (1986, 1997), Goldman (1987), and Geeves (1991). A central goal in the field of muscle physiology is now to understand the kinetics and energetics of this interaction as it occurs in the filament array of the muscle fiber. The effect of PEG on fiber mechanics and energetics can provide insight into the role of the actomyosin bond. The binding of a myosin head to an actin polymer results in the formation of a large protein interface, on the order of 1000 Å^2 (Schroder et al., 1993; Rayment et al., 1993a). The free energy released by removing an interface of this size from solution is considerable (Horton and Lewis, 1992), and formation of the actomyosin interface is hypothesized to be a major source of free energy driving the power stroke (see Cooke, 1997, for a review). While it is clear that a large protein interface is formed at the end of the power stroke, the extent to which a protein interface is formed for other interactions is not known, particularly for those that occur in the weaker bonds formed at the beginning of the power stroke.

The use of polymers to potentiate protein-protein interactions has another goal, that of attempting to simulate more closely the conditions that occur in vivo. Studies of the actomyosin interaction have been carried out both in living fibers and in skinned fibers from which the cell membrane has been removed. The latter preparation has several advantages. It provides a relatively intact filament array and allows the investigator to modify the solutions that perfuse the filament array. However, a number of questions remain concerning the validity of the skinned fiber preparation and whether the skinning process has perturbed the interactions of the proteins within the filament array. One difference between the skinned fibers and living fibers is the loss of ~100 mg/ml of soluble components. A considerable amount of both experimental and theoretical work has suggested that protein interactions occurring in the crowded interior of a living cell are perturbed by the presence of other soluble components (Minton, 1981). The effective osmotic pressure exerted by soluble components in the interior of a bacterial cell has been estimated by measuring the size and concentration of the components. The intracellular pressure is considerable and could increase the affinity of two moderately sized proteins by an order of magnitude or more (Minton, 1981). Evidence supporting the presence of a large effect of the intracellular osmotic pressure on protein-protein interactions comes from the observation that protein interactions that do not occur at a given concentration in the test tube do occur at the same concentrations inside a living cell (Zimmerman, 1988a). The use of a small polymer that can diffuse into the interfilament region of the skinned fiber can simulate in part the presence of the soluble components that are lost in the skinning procedure, providing an in vitro system that is closer to that in vivo.

MATERIALS AND METHODS

Fiber mechanics

Rabbit psoas fibers were harvested and chemically skinned as described previously (Cooke et al., 1988). For mechanical experiments, single fibers were dissected from a bundle of fibers and mounted in a well between a solid-state force transducer and a rapid motor for changing fiber length. The apparatus has been described more fully by Cooke et al. (1988). Briefly, one end of the fiber was attached to a glass capillary connected to a silicon beam force transducer (resonant frequency of 2.5 kHz when immersed in solution; Aksjelskapet Microelectronic, Horten, Norway). The other end was attached to a metal arm connected to the armature of a motor (response to a step position command with a time constant of 0.4 ms; model CX-660; General Scanning, Watertown, MA). Data were sampled at 1000–5000 s^{-1} by a 486-based PC interfaced to the force transducer and photodiode by an analog/digital board (Analogic HSDAS-12; Analogic, Wakefield, MA). During tension clamps the input to the motor was computed in real time and was proportional to the difference between command tension and the most recent measurement. The computer program that acquired data and drove the motor was written in PC/Forth 3.2.

Single fibers were dissected on a cold stage and mounted on the apparatus. The fiber ends overlapping the arms were fixed with 2.5% glutaraldehyde, following the methods of Chase and Kushmerick (1988). Excess glutaraldehyde was removed by vacuum. After fixation, the ends of the fiber were glued to the arms, using cellulose proprionate diluted with acetone. Excess glue was removed by vacuum. The fiber was then lowered into a relaxing solution, and fiber length and thickness were measured. The length of the unfixed portion of the fiber between the arms varied from 3 to 6 mm, the fiber diameter ranged from 50 to 80 μm, and the initial sarcomere length ranged from 2.2 to 2.4 μm.

A common protocol was to first immerse the fiber in a relaxing solution and allow complete perfusion of creatine kinase and nucleotides (~3 min). The apparatus had two wells, and fibers could be rapidly switched between solutions. The fiber was then switched to an activating solution in the other well, and mechanical measurements were made within 20–30 s. After measurements, the fiber was returned to the relaxing solution, and the solution in well 2 was replaced with the appropriate experimental buffer. Mechanical measurements were repeated, and the fiber was returned to the relaxing solution. The solutions in well 2 were changed again, and mechanical measurements were repeated under control conditions. The effect of PEG was then calculated as the percentage change in the presence of PEG relative to the average of the two control experiments. For half of the experiments, the order was reversed so that the mechanical measurements were made first and third in the presence of PEG and second under control conditions. Force-velocity relationships were fit to a linear form of the Hill equation (Cooke et al., 1988). Experiments on fibers were carried out at 10°C except where noted. The ATPase activities of small bundles of one to three fibers were measured by direct determination of phosphate, as described in more detail by Myburgh et al. (1997).

Sarcomere length

Initial sarcomere length was 2.2–2.4 um, determined by measuring the distance between the central and first maxima of a diffraction pattern, using a 20 mW He-Ne laser (0.632 μm wavelength; Uniphase Corp., Manteca, CA). Changes in sarcomere length were tracked by movement of the centroid of the first diffraction maximum across a 15-mm-wide position-sensing photodiode (model SL-15; UDT Sensors, Hawthorne, CA). Because the intensity of the diffraction pattern varied both from fiber to fiber and between trials, the change in photodiode output voltage corresponding to a specified change in position of the diffraction pattern was calibrated immediately before each measurement, as described in more detail by Burmeister-Getz et al. (1998). The position of the photodiode was moved by micrometer by 0.05 mm, and the voltage from the photodiode was sampled. The calibration varied from 5 to 10 analog-to-digital units per nanometer of sarcomere length. The sarcomere length calibration varied because of the difference in the ratio of the intensity of the diffracted line to the background. A factor of 2 represents the limits of the range of variation. Most calibrations were within ~25% of one another. Repeated measurements on rigor or relaxed fibers showed that the spatial resolution of the photodiode sarcomere length measurement was ~0.5–1.0 nm. The diffraction pattern remained stable for a longer period of time when the fiber was repeatedly slacked and restreched following the protocols of Brenner and co-workers (Brenner, 1983).

ActoS1 affinity

The affinity of S1 for actin in the absence of nucleotides was determined by a modification of the method of Kurzawa and Geeves (1996). The fluorescence of pyrene-iodoacetamide attached to Cys^{374} on actin has been shown to be quenched by ~65–70% by the binding of S1 (Kouyama and Hihashi, 1981). This quenching was used to measure the effect of PEG on the binding of S1 to actin in the absence of nucleotides. Actin was labeled following the methods of Kurzawa and Geeves (1996). Actin in rigor buffer (defined below) plus a 1.1 M excess of phalloidin was placed in a cuvette in a SPEX fluorimeter, and steady-state fluorescence was measured with excitation at 365 nm and emission at 405 nm. Phalloidin was included to ensure complete actin polymerization. Apyrase (0.1 mg/ml) was included to eliminate trace amounts of ADP that came in with the proteins. The change in fluorescence was monitored as increasing amounts of S1 were added to the solution, taking into account the dilution due to the addition. The bound S1 was calculated from the amount of quench observed relative to that observed at saturating S1. The free S1 was then calculated by subtracting the bound S1 from the total added S1.

The affinity of S1 for actin in the presence of nucleotides, P_i analogs, and varying concentrations of PEG was determined by sedimentation. The solution contained the basic rigor buffer described below without the KAc, plus 1 mM ATP and the appropriate phosphate analog, made up as described below for the fiber experiments. The proteins and nucleotides were mixed and allowed to come to equilibrium for 10 min at 0°C. The actin concentration was 50 μM, and S1 was 10 μM in all experiments. The actin, along with bound S1, was sedimented in an airfuge (Beckman) at 90,000 $\times$ *g* for 20 min at room temperature. The protein composition in the supernatant was then assayed by gel electrophoresis in the presence of sodium dodecyl sulfate, and protein concentration was determined by the Bradford assay. The amount of S1 remaining in the supernatant was then used to calculate the free and bound species. Virtually no actin was found in the supernatant for any of the conditions. Data were fit to Eq. 1, where the osmotic pressure, Π, exerted by PEG 4000 of concentration C is determined from the equations of Highsmith and Cohen (Cohen and Highsmith, 1997):

$$\prod = RT(2.5 \times 10^{-3} \times C + 5.4 \times 10^{-4} \times C^2 + 0.6 \times 10^{-5} \times C^3)\ \text{dyne/cm}^2. \quad (2)$$

Solutions

The basic rigor buffer contained 120 mM KAC, 5 mM $MgCl_2$, 1 mM EGTA, 3 mM P_i, and 50 mM 3-(*N*-morpholino)propanesulfonic acid (pH 7). A relaxing solution was achieved by the addition of 20 mM creatine phosphate, 1 mg/ml creatine kinase, and 4 mM ATP. Activating solution was obtained by further addition of 1.1 mM $CaCl_2$. Additional buffers resembling the above were made with the desired percentage of PEG (w/v). The phosphate analog AlF_4 was obtained by the addition of 10 mM KF and the desired concentration of the metal as $AlCl_3$. Solutions of vanadate were made up following the protocols of Goodno (1982). In some experiments where low phosphate concentrations were desired, a "phosphate mop" was included (Brune et al., 1994: Webb, 1992), consisting of 100–200 units/ml of nucleoside phosphorylase and 8 mM 7-methylguanosine, following the protocols of Pate et al. (1998). In some of these experiments, the fibers were split, producing diameters of ~25–30 μm. The split fibers have still lower internal concentrations of P_i, because of faster diffusion of P_i out of the fiber.

RESULTS

Addition of 1–5% PEG to the solution bathing a relaxed fiber produced no change in isometric tension or stiffness. Addition of PEG to an active fiber resulted in a reversible increase in isometric force (P_o), as shown in Fig. 1. The increase in force upon the addition of PEG occurred with a time to half-maximum of ~5–10 s. This is approximately the time required for complete perfusion of PEG through the fiber. The decrease in force occurring upon removal of PEG had a similar time course. Incubation of fibers in rigor buffer plus 5% PEG 4000 overnight at 0°C had no effect on

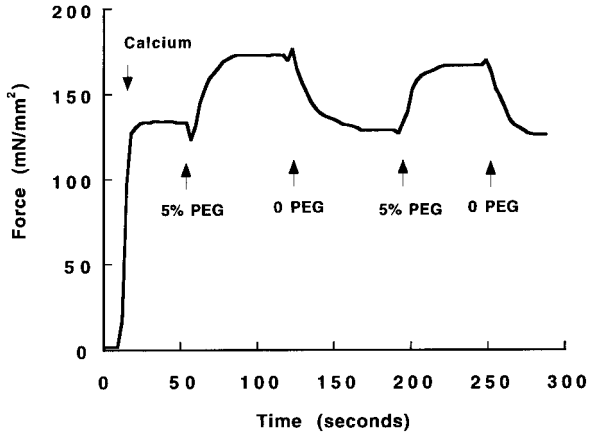


FIGURE 1 Fiber tension is shown as a function of time. The fiber was initially in a relaxing solution, and at 10 s calcium was added to activate the fiber. After mixing, 5% PEG4000 was added, generating an increase in isometric tension. The fiber is then switched to another bath that lacks PEG, resulting in a drop in tension. After tension equilibration in this bath, the fiber is switched back to the bath containing PEG. Tension is enhanced rapidly and reversibly by PEG, and the fiber tension remains relatively stable, with tension at 400 s (126 mN/mm^2) only 6.5% less than that at 65 s (135 mN/mm^2). In this fiber the addition of PEG increased tension by 31%, averaged over the four times that the solution was switched. The average change for a number of fibers was 35 ± 4%. Data were obtained from a single fiber with a diameter of 60 μm and a length of 4.5 mm.

either fiber force or velocity, measured in a PEG-free solution. Thus the effects of PEG appear to be rapidly reversible, and even long exposure to PEG causes no permanent alteration in protein function. The small polymers used here diffuse into the fiber interior and have an effect that is very different from that of the much larger polymers that are excluded from the fiber interior and have been used to shrink the fiber lattice (Godt and Maughn, 1981; Zhao and Kawai, 1994; Myburgh and Cooke, 1997). The polymers used here do not affect the fiber lattice. Fiber diameters observed by light microscopy under ×400 power did not change upon incubation in either PEG 4000 or PEG 300.

Activation of fibers in solutions that contain PEG results in a shift in the force velocity relation toward increased tensions and decreased velocities, as shown in Fig. 2. The effect produced by PEG on tension, velocity, or ATPase activity increased with increasing concentrations of PEG up to ~5% PEG, as shown for PEG 300 in Fig. 3. The isometric ATPase activity was inhibited by the presence of PEG, as also shown in Fig. 3. The decrease in ATPase activity approximately paralleled the decrease in the velocity of contraction. The effects produced by PEG 4000 were similar to those shown for PEG 300 in Fig. 3. Addition of 5% PEG 4000 increased isometric tension by 35% ± 4% (SEM, $n = 10$), decreased the maximum velocity of shortening by 37% ± 3% (SEM, $n = 10$), and decreased the ATPase activity by 24 ± 6% (SEM, $n = 12$). Two polymers with different molecular weights were used, because the comparison between them provides some additional information on the mechanism of PEG action. However, in a limited number of experiments measuring tension and velocity, the two polymers produced similar effects, and in most of the subsequent experiments PEG 4000 was used.

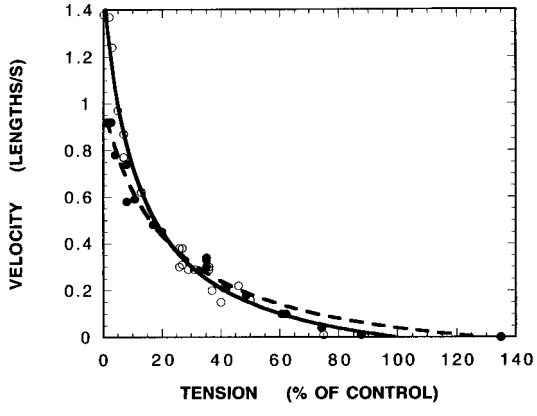


FIGURE 2 Fiber velocity is shown as a function of fiber tension in the absence (○) and presence (●) of 5% PEG 4000. Single fibers were mounted on the apparatus, incubated in relaxing solutions in either the presence or absence of PEG 4000. The fibers were then activated by the addition of calcium, and three to five velocities were obtained for each fiber, all within 20 s of activation. Fibers activated in the presence of PEG have increased isometric tension and decreased maximum velocity of shortening.

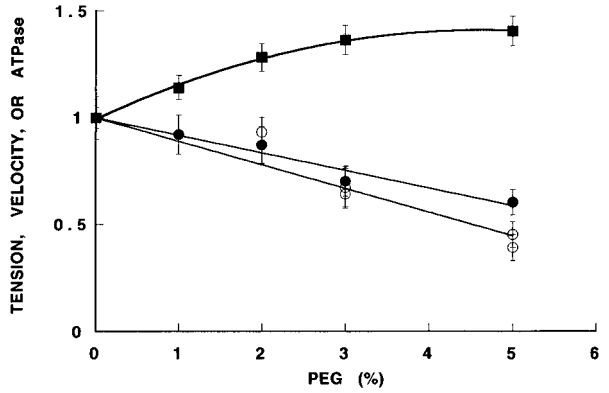


FIGURE 3 The isometric tension (■), ATPase activity (○), and maximum shortening velocity (●) are shown relative to their values in the absence of PEG as a function of the concentration of PEG 300. The tensions were measured as described in Fig. 1. The maximum shortening velocities were determined from extrapolation to zero force of force-velocity relations such as those shown in Fig. 2. The ATPase activities of isometric fibers were measured in bundles of one to three fibers. The fibers were mounted and incubated in a series of small aliquots of activating solution for 1–5 min, and the ATPase activity was measured by determination of the P_i released as a function of time. Different fibers were measured in different concentrations of PEG. Error bars represent the SEM of five to seven separate fiber bundles, with the ATPase activity determined from three to five different time points for each. In the absence of PEG the isometric tension was 120 mN/mm^2, the maximum velocity of contraction was 1.55 lengths/s, and the isometric ATPase activity was 1.3 s^{-1}.

The rate of actomyosin dissociation is governed by the release of ADP followed by the binding of ATP. To investigate whether either of these reactions is perturbed by PEG, we measured fiber tension and velocity as a function of ADP and ATP concentrations in the presence and absence of 5% PEG 4000. Shortening velocity is shown in Fig. 4 as

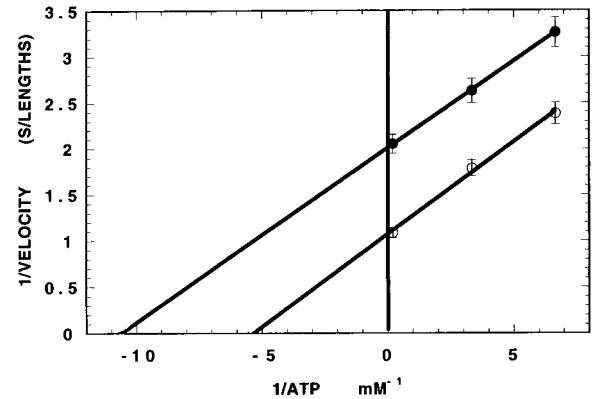


FIGURE 4 Fiber shortening velocities were measured at 10% of P_o as a function of the ATP concentration in the absence (○) and presence (●) of 5% PEG 4000. The reciprocal of the fiber velocity is shown as a function of $[ATP]^{-1}$. The data show that the velocity of shortening reached in saturating ATP and the value of K_m are both reduced by a factor of ~2 in the presence of PEG. Error bars represent SEM on four to six measurements.

a function of [ATP], in both the absence and presence of PEG. Both the velocity obtained from extrapolation to infinite [ATP], V_∞, and the value of K_m for ATP are decreased by a factor of ~2 in the presence of 5% PEG 4000. Model simulations show that the rate of ATP binding at the end of the power stoke can be estimated from the ratio of V_∞ and K_m (Pate et al., 1993a,b). This relationship is similar to the more familiar one used in enzyme kinetics, in which a second-order binding constant is estimated from k_{cat}/K_m (see Fersht, 1977, p. 105). This relationship has been experimentally verified for fibers, using a series of nucleotides (Pate et al., 1993a,b). The ratio V_∞/K_m is not changed by the addition of PEG, indicating that it does not influence the rate at which ATP binds to myosin heads at the end of the power stroke in isotonic contractions. When [ATP] is lowered from 4 mM to ~50 μM, the isomeric tension increases because of the reduced rate of cross-bridge dissociation by ATP, leading to an increased population of force-generating rigor bonds. However, the absolute change in tension produced by the addition of PEG was the same within experimental error at 4 mM ATP (42 ± 4 mN/mm^2) and at 50 μM ATP (38 ± 12 mN/mm^2). These data show that the rate of ATP binding to force-generating myosin heads in isometric or isotonic active fibers is changed very little by the presence of PEG.

The effect of PEG on the binding and release of ADP in active fibers was tested using high concentrations of ADP in the presence of moderate ATP. Fibers were activated in 2 mM ATP, and isometric tension and velocities at 10% and 5% of isometric tension were measured. ADP was then added to 4 mM, and the measurements were repeated. Tension rose by 15 ± 1.7%, in the absence of PEG (122–140 mN/mm^2) and by 15 ± 1.6% in the presence of PEG (135–155 mN/mm^2). Shortening velocity in the absence of PEG was 0.91 ± 0.02 lengths/s at 5% P_o. Addition of 4 mM ADP caused a decrease of 40% to 0.56 ± 0.03 lengths/s. In the presence of PEG, velocity at 5% P_o was 0.6 ± 0.02 lengths/s, and addition of ADP caused a decrease of 33% to 0.4 ± 0.03 lengths/s. A similar effect was seen for clamps to both 5% and 10% of P_o. We conclude that the presence of PEG did not produce a change in the effects of ADP on tension or on shortening velocity. Together, the results obtained upon variation of [ATP] and [ADP] showed that PEG had little effect on the release of ADP and subsequent binding of ATP, which together lead to dissociation of myosin from actin.

The addition of P_i to an active fiber inhibits tension by reversing the transition from the weakly binding, pre-power-stroke actin-myosin·ADP·P_i state to the force-producing actin-myosin·ADP state (Hibberd et al., 1985; Dantzig et al., 1992; Millar and Homsher, 1990; Pate and Cooke, 1989). The inhibition is approximately linear in log [P_i], with a shape that provides information on the energetics of the actomyosin interaction and, in particular, on the energetics of the force-producing actin-myosin·ADP state (Pate and Cooke, 1989). Increasing the concentration of P_i from 3 mM to 50 mM, and keeping the ionic strength constant, inhibited isometric tension in both the presence and absence of PEG (Fig. 5). Lower concentrations of P_i were obtained by using enzymatic removal of contaminating P_i and depletion of P_i generated within the fiber. Nucleoside phosphorylase catalyzes a reaction in which free phosphate and 7-methylguanosine are converted into 7-methylguanine and ribose-1-P_i (Brune et al., 1994; Webb, 1992). This system has been used previously to reduce the levels of P_i in muscle fibers (Pate et al., 1998). Reduction of [P_i] below ~100 μM produces a plateau in tension. Above this concentration, tension is inhibited approximately linearly with log [P_i]. The two curves describing tension as a function of log [P_i] obtained in the presence and absence of PEG have similar shapes, with the PEG curve elevated in tension by a constant amount, ~40 mN/mm^2. In the Discussion section we show that this relationship indicates that more free energy is available to drive tension generation in the presence of PEG.

The stiffness of active fibers was also increased by the addition of PEG, as shown in Figs. 6 and 7 and in Table 1. The changes in tension and sarcomere length after a step increase in fiber length of ~5 nm/half-sarcomere were monitored for a period of 40 ms. The increase in tension was greater in the presence of PEG than in its absence. Tension decayed following the step in length with a rate that was unchanged by the presence of PEG for control fibers or for fibers in the presence of phosphate analogs, although the

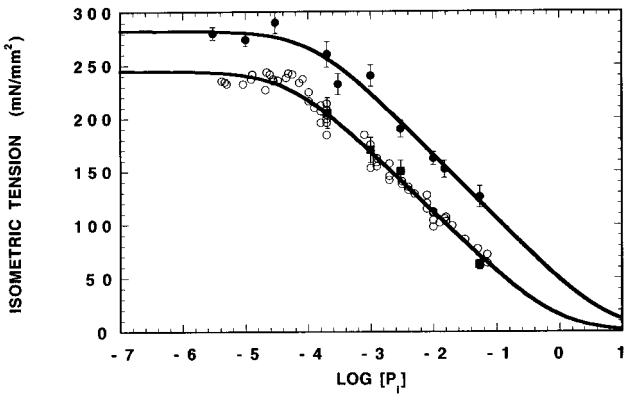


FIGURE 5 Isometric tension obtained in the presence (●) and absence (○) of 5% PEG 4000 is shown as a function of log [P_i]. Data in the absence of PEG are taken from Pate et al. (1998) (○) or from current measurements (■). The effect of PEG was to raise all tensions by the same amount, regardless of the concentration of phosphate. Data above 1 mM were obtained by addition of P_i, keeping the ionic strength constant. Lower concentrations of P_i were obtained by using nucleoside phosphorylase to scavenge contaminating P_i and P_i produced within the fiber. The internal level of P_i, which is determined by enzymatic reactions and by diffusion, was estimated by the method of Pate et al. (1998). The solid lines are fits to the data, using Eq. 8 of Pate and Cooke (1989), providing estimates of the free energy in the power stroke of 9.1 ± 0.2 *kT* in the absence of PEG and 10.7 ± 0.8 *kT* in the presence of PEG.

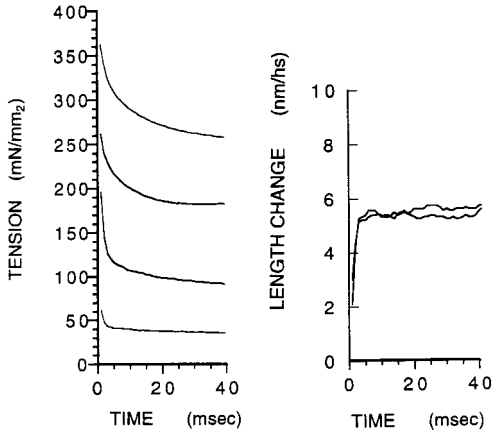


FIGURE 6 (*A*) Tensions are shown as a function of time after step changes in fiber length. The traces show, in descending order, fibers plus 5% PEG 4000; control fibers; fibers in 2 mM V_i pus 5% PEG 4000; fibers in the presence of 2 mM V_i. (*B*) The change in sarcomere length is shown as a function of time for control fibers and for fibers in the presence of V_i plus 5% PEG 4000, showing that sarcomere length control was the same in the two conditions. Sarcomere length was controlled as described in Materials and Methods.

rates for the former were slower than those for the later. Plots of peak tension as a function of the change in sarcomere length were linear, with a slope that defined the stiffness, as shown in Fig. 7. The increase in fiber stiffness (to 110% of control) was considerably greater than that of

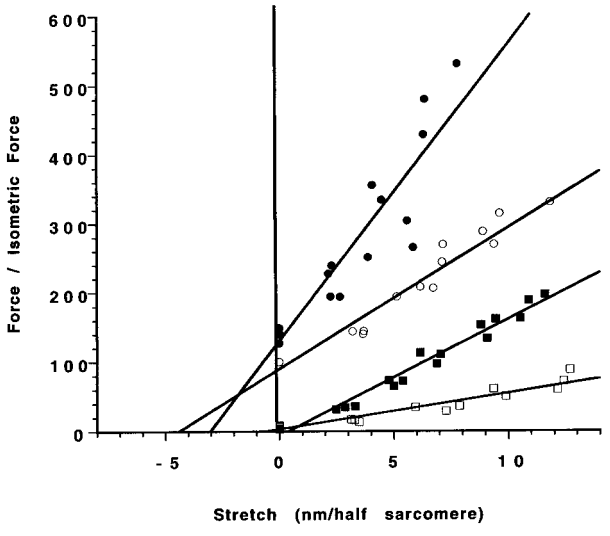


FIGURE 7 The peak tension attained after a length step is shown as a function of the size of the length step. The data were obtained as shown in Fig. 6 for control fibers (○), fibers in the presence of 5% PEG 4000 (●), fibers in the presence of 1 mM V_i (□), and fibers in the presence of 1 mM V_i plus 5% PEG 4000 (■). The phosphate analog inhibited both fiber tension (to 11 ± 3% of controls) and fiber stiffness (to 30 ± 12% of controls). In the presence of PEG there was a dramatic increase in the fiber stiffness (to 85 ± 10% of controls), with little change in isometric tension (to 10 ± 5% of controls).

the change in fiber tension (to 35% of control). We conclude that the relative change in stiffness is greater than the change in tension by a factor of almost 3. As discussed below, this proportionally greater increase in stiffness is probably due to an increased population of weakly bound cross-bridges.

The effect of PEG on the formation of the weak actomyosin bond preceding the power stroke was investigated by incubating fibers in analogs of phosphate that are known to stabilize a state that resembles the myosin·ADP·P_i state, i.e., the state thought to occur at the beginning of the power stroke. We investigated two phosphate analogs, V_i and AlF_4 in active muscle fibers (Dantzig and Goldman, 1985; Chase et al., 1993, 1994; Phan et al., 1995, 1997). Both caused a dramatic inhibition of the isometric tension and stiffness to 10–15% of control isometric values. The further addition of 5% PEG 4000 induced small increases in tension, by ~5%; however, it induced dramatic increases in stiffness. As shown in Figs. 6 and 7 and in Table 1, stiffness returns to a value close (85–90%) to that of control fibers activated without the analogs. This shows that PEG has induced a dramatic increase in the number of myosin heads bound to actin in the presence of these phosphate analogs. In addition, tension decays more rapidly than for active fibers, showing that the states populated are transitory, as expected for a weakly bound state. The increase in stiffness produced by PEG was approximately the same for both of the phosphate analogs that we investigated (see Table 1). One possibility that could explain the increase in stiffness is that PEG is promoting the release of the phosphate analog, leading to a more rapid cycling of cross-bridges. To test this possibility, the return of tension after removal of the phosphate analog AlF_4 was monitored in the presence and absence of PEG. In the absence of PEG, force recovered with a half-time of ~10 ± 2 min, and the time course of tension recovery was not greatly altered by the presence of PEG. In addition, the isometric ATPase activity was inhibited completely in the presence of the phosphate analogs, and this inhibition was not relieved by the presence of 5% PEG 4000. Together these results show that PEG does not promote the release of the phosphate analog. There are two points in the cycle of the actomyosin interactions at which PEG is likely to exert a strong effect. One of these is the dissociation of myosin from actin at the end of the power stroke, which is considered above. The second of these is the association of myosin and actin at the beginning of the power stroke. The results discussed above show that the major effect of PEG is on the association steps, with little effect on dissociation.

The increase in fiber stiffness in the presence of PEG and phosphate analogs suggests that more cross-bridges are attached to actin, implying a tighter actomyosin bond under these conditions. A tighter bond between a myosin products complex and actin is also demonstrated by the decrease in the K_m for the actoS1 ATPase activity (Highsmith et al.,

TABLE 1 Tension and stiffness of active fibers with phosphate analogs

Condition	Control + PEG	AlF_4	V_i	AlF_4 + PEG	V_i + PEG
Tension	135 ± 4	9 ± 2	11 ± 3	10 ± 4	10 ± 5
Stiffness	210 ± 12	25 ± 7	30 ± 12	90 ± 15	85 ± 10

Values are given as a percentage of control values in the absence of either PEG or analogs. The isometric tension of control fibers was 122 ± 10 mN/mm^2. The stiffness of control fibers was 27 N/mm^2. Errors in tension represent the SEM for four to eight fibers for the experimental conditions listed in the last four columns, and for 22 measurements for control plus PEG. The errors in the stiffness represent the uncertainty in the fit to the data of Fig. 8, or of similar plots, each set of data representing two to six fibers. The concentration of PEG 4000 was 5% w/v, and the concentrations of AlF_4 and V_i were 1 mM and 2 mM, respectively.

1996; White et al., 1995). Alternatively, the cross-bridges could have become stiffer in the presence of PEG, but this would imply a stiffness exceeding that of rigor cross-bridges, which is unlikely. To further quantify the increased affinity of these states, the binding of S1 to actin was investigated in the presence of ATP and phosphate analogs and in the presence and absence of PEG. The unbound S1 was determined after sedimentation of actin along with bound S1 (see Fig. 8). In the absence of PEG a very small fraction of the S1 is sedimented along with the actin, indicating an apparent binding constant of 2×10^3 M^{-1}. With increasing levels of PEG a greater fraction of the S1 is sedimented, indicating tighter affinity for the actin. The precipitation of S1 by PEG is specific for actin. In the absence of actin a small fraction (10%) of the S1 is precipitated only at the highest levels of PEG (10–12%). S1 did not coprecipitate when an equal concentration of bovine serum albumin was added to the mixture instead of actin, showing that the precipitation reflects a specific bond with actin rather than a nonspecific association with another protein. The affinity constant of the S1 products complex for actin was calculated from the concentration of S1 remaining in the supernatant. The addition of 10% PEG 4000 increased the affinity constant from 2×10^3 to 5×10^4 M^{-1}. One possibility for explaining the precipitation of S1 with actin at the higher concentrations is that PEG has activated the ATPase activity by promoting release of the phosphate analogs. To assess this possibility we also measured the ATPase activity of actin, S1, and phosphate analogs in the presence and absence of PEG. In the absence of the phosphate analogs, the ATPase activity was 1.3 S^{-1}. In the presence of 2 mM AlF_4 or 2 mM V_i the ATPase activity was not significantly different from zero in either the presence or absence of PEG. This indicates that PEG does not activate the ATPase activity in the presence of phosphate analogs. Direct determination of the phosphate produced showed that ATP levels remained high throughout the assay. We conclude that increasing concentrations of PEG promote an increasing affinity of actin for S1 complexed with ADP and phosphate analogs.

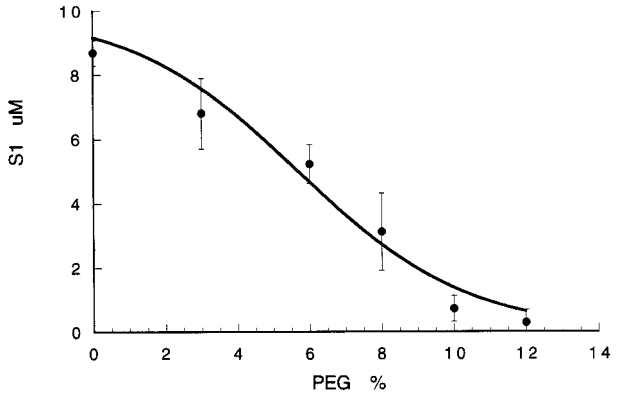


FIGURE 8 The effect of PEG on the affinity of actin for myosin in the presence of phosphate analogs. Actin (50 μM) was mixed with 10 μM S1 in rigor buffer (made without KAc) plus 1 mM ATP and either 2 mM AlF_4 or 2 μM V_i. The proteins were then sedimented and the S1 concentration remaining in the supernatant was assayed directly and by gel electrophoresis. The S1 concentration in the supernatant is shown as a function of the concentration of PEG 4000. The solid lines represent a fit to the data, assuming that the logarithm of the affinity constant varies linearly with the osmotic pressure exerted by the PEG, as described in the text.

The affinity of S1 for actin in the absence of nucleotides was also measured, using the fluorescence of pyrene attached to Cys^{374} of actin, following procedures similar to those of Kurzawa and Geeves, (1996). Addition of 5% PEG4000 increased the affinity by a factor of 4 ± 0.4 (see Fig. 9). This is a little smaller than the eightfold increase seen for the weakly bound states, described above; however, the difference may not be significant.

As discussed earlier, the formation of a protein-protein interface decreases the volume of solution from which PEG is excluded, leading to a higher affinity for the proteins in the presence of PEG. With Eqs. 1 and 2, the dependence of the affinity constant on the concentration of PEG defines the change in the size of the PEG-inaccessible volume, ΔV, which occurs upon formation of the protein complex. The magnitude of ΔV provides some information on the size of the interface that is formed. The value of ΔV can be obtained from the slope of the logarithm of the affinity constant as a function of the osmotic pressure exerted by the PEG. The osmotic pressure exerted as a function of the molecular weight of the PEG and its concentration have been determined experimentally, and algorithms have been fit to these data, allowing one to calculate osmotic pressures for any given concentration (Cohen and Highsmith, 1997; Reid and Rand, 1997; and also see the web site maintained

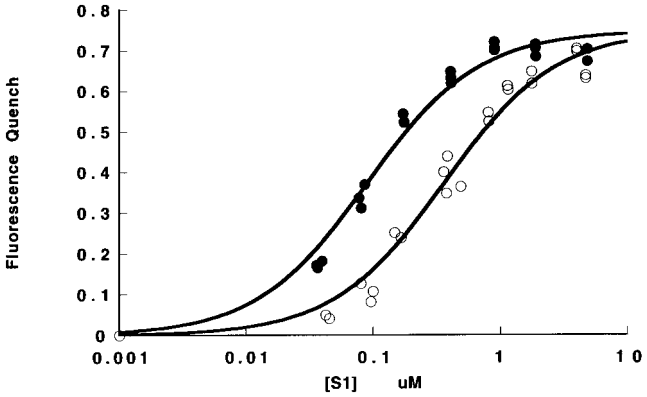


FIGURE 9 The effect of PEG on the strong binding of S1 to actin. The binding of S1 was measured by the quenching of the fluorescence of pyrene attached to Cys^{374} on actin in rigor buffer at 10°C, as described in Materials and Methods. The figure shows the amount of quenching observed as a function of the concentration of free S1 in the solution in the presence (●) and the absence (○) of 5% PEG 4000. The concentration of actin varied between 0.25 and 1 μM. The solid lines show fits to the data using a simple binding equation. The fits defined a K_d of 0.093 ± 0.005 μM and 0.37 ± 0.02 μM in the presence and absence of PEG, respectively.

by Dr. P. Rand at www.mgsl.dcrt.nih.gov/docs/Osmotic-Stress.html. The affinity of S1 for actin in the presence of phosphate analogs can be determined directly from the data shown in Fig. 8 and are plotted in Fig. 10 as a function of the osmotic pressure. The data shown in Fig. 10 show an approximately linear dependence of the logarithm of the affinity constant as a function of the osmotic pressure. This dependence is similar to that obtained previously for the apparent affinity of S1 for actin in the presence of ATP, determined by the value of the K_m for the acto-S1 ATPase activity (Highsmith et al., 1996). Determining an affinity constant from the stiffness of muscle fibers requires some assumptions. We first assume that fiber stiffness in the presence of the analogs is proportional to the fraction of attached myosin heads. We make the assumption that the stiffness of the weakly bound myosin heads is the same as that of the strongly bound heads. We measure stiffness by step changes, which would correspond to the stiffness measured in fast ramp stretches, where the stiffness of weakly bound cross-bridges is similar to that of active cross-bridges (Brenner et al., 1982). To convert these data to the actual number of attached heads also requires some estimation of the proportionality constant linking stiffness to population attached. One can assume that the highest stiffness reached corresponds to some fraction, X, of attached heads. If X is now allowed to vary from 25% to 75%, and the fractions of attached heads are converted to affinity constants, the change in affinity constant with PEG is found to be remarkably independent of X. This independence arises because a change in X shifts all of the calculated affinities by approximately similar amounts, resulting in similar slopes when plotted as in Fig. 10. The further assumption that one-half of the stiffness of active fibers is in the thin filament (for a review see Goldman and Huxley, 1994) also does not change the slope of the line appreciably, as it again tends to shift all of the states in the same direction. Making the assumption that $X = 50\%$, we plot the apparent affinity of myosin heads for actin in the presence of phosphate analogs and PEG inside the muscle fiber. These data are also shown in Fig. 10.

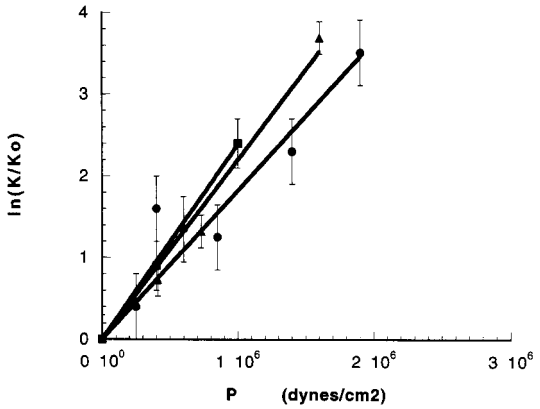


FIGURE 10 The presence of PEG 4000 increases the apparent affinity of actin for myosin in solution and in fibers. The logarithm of the apparent affinity of the actoS1 complex relative to the affinity in the absence of PEG is shown as a function of the osmotic pressure exerted by the PEG. The K_m for actoS1 ATPase activity is shown by the solid circles, taken from Highsmith et al. The apparent affinity of a myosin head for actin in fibers activated in the presence of V_i and in the presence of various concentrations of PEG was estimated from measurements of fiber stiffness as described in the text (■). The affinity of S1 for actin in the presence of AlF_4 and in the presence of various concentrations of PEG was determined by sedimentation, as shown in Fig. 8 (▲). All of the data were fit by straight lines constrained to pass through the origin, and the slopes of the lines are proportional to the change in the PEG-inaccessible volume that occurs upon formation of the complex. The slopes are similar, showing that the inaccessible volumes are similar.

The slopes obtained for all of the conditions investigated were similar: 1.8 ± 0.2 cm^2/dyne for K_m, 2.1 ± 0.1 cm^2/dyne for the affinity of S1 for actin in the presence of AlF_4, 2.0 ± 0.2 cm^2/dyne for the affinity of S1 for actin in the presence of V_i, and 2.2 ± 0.1 cm^2/dyne for the increase in the stiffness of fibers activated in either AlF_4 or V_i. The small differences in slopes between that for K_m of the ATPase activity and the other data may reflect a real difference, but better data would be required to confirm this. Essentially the slopes obtained for all sets of data shown in Fig. 10 are the same within experimental error, suggesting that a similar protein interface is formed in all three, as far as the volume inaccessible to PEG is concerned. These data suggest that PEG potentiates the binding of a myosin products complex to actin, and this accounts for the increase in stiffness in fibers incubated with the phosphate analogs as well as the K_m for the actoS1 ATPase activity.

DISCUSSION

Mechanism of PEG action

PEG will influence the kinetics and equilibrium of any transition in which the volume available for solvating the PEG either increases or decreases (Timasheff, 1993; Bhat and Timasheff, 1992; Parsegian et al., 1986). PEG alters protein-protein interactions through an entropic effect. The presence of PEG simply makes a particular transition more likely because of the presence of an external agent, not by a significant modification of the proteins themselves. Large changes in the PEG-inaccessible solvent volume will occur upon formation or dissociation of the actomyosin complex. Thus it provides a method of influencing the strength of the actomyosin interaction without perturbing the nature of the protein interfaces. However, PEG could also have an effect on other transitions. Myosin contains a large crevice that could open or close (Rayment et al., 1993b), or actomyosin could assume different conformations during the power stroke, altering the PEG-inaccessible volume.

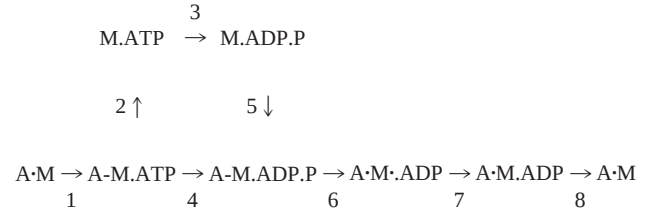
Does the action of PEG result from steric exclusion, as discussed above, or through some other property? The effect of PEG on protein structures has been examined in studies of protein function in solution and through comparison of protein structures obtained in PEG with those obtained via other precipitants. The general conclusion that has been reached is that PEG does not bind strongly to proteins and that it is relatively inert. For example, a number of structures obtained for trypsin in the presence or absence of PEG are similar, suggesting that PEG does not alter protein structure. The enzymatic properties of myosin by itself are not changed by the addition of PEG, unless the PEG precipitates the protein (Highsmith et al., 1998; Highsmith et al., 1996). The crystal structures of myosin also suggest that PEG does not greatly alter the structure of the catalytic domain. These structures fall into two categories. In one category, the structures of the catalytic domain of *Dictyostilium discoidum* myosin complexed with PP_i or BeF_3 and crystallized from 8.5% PEG 8000 are nearly identical to the catalytic domain of chicken S1 crystallized from ammonium sulfate (Rayment et al., 1993b; Smith and Rayment, 1995). It thus appears likely that PEG produces its effect in fibers through steric exclusion. The effect of PEG is very different from the effect of hydrostatic pressure, which also has been used to perturb fiber mechanics, but which affects protein-protein interactions due to the difference in the volumes occupied by bulk water and by water bound to proteins (Fortune et al., 1994). However, regardless of the mechanism, PEG provides a useful means of producing a significant perturbation of the actomyosin cycle, which can be applied both in solution and in the filament array of an active fiber.

The viscosity of the solution is also increased by PEG (Bhat and Timasheff, 1992), and it is possible that this property could explain some of its effects, especially those on fiber shortening velocity. Recent work with mono- and disaccharides has suggested that increased viscosity in these solutions inhibits both the tension and velocity of shortening (Chase et al., 1998). The viscosity of 5% PEG 4000 is almost twice that of water, and it is similar to that of a 20% w/v solution of glucose, which also inhibits the velocity by 40%. However, several results suggest that PEG is not acting through a change in viscosity. PEG potentiates tension instead of inhibiting it, as is seen with the small disaccharides (Chase et al., 1998). The inhibition of velocity by 5% PEG 300 is about the same as that by 5% PEG 4000, yet the viscosity of the former is only 15% greater than that of water, suggesting that viscosity is not the main determinant of PEG effects in our experiments. The concentrations (in mg/ml) employed here are about fourfold less than those employed by Chase et al. (1998). Although increased viscosity may play some role in perturbing the actomyosin interaction, and in particular in the inhibition of velocity, it is probably not the most dominant mechanism for PEG.

Effect of PEG on fiber mechanics

Addition of PEG enhances fiber tension and stiffness while inhibiting the rate of ATP hydrolysis and fiber velocity. The addition of 5% PEG 4000 or PEG 300 potentiates tension and stiffness and inhibits shortening velocity and ATPase activity, all by about the same amount, 30–40%. Thus in the broadest terms, addition of PEG leads to stronger, slower fibers. As argued below, many but not all of these effects could potentially be due to an increased affinity of myosin for actin. Below we attempt to explain these effects in more detail in terms of the known kinetic scheme of the cycle of force-producing interactions.

A considerable body of data obtained from purified proteins in solution and from the mechanics of active fibers has suggested that the actomyosin interaction can be described by the following kinetic scheme (reviewed in Geeves (1991), Cooke (1997), Goldman (1987), and Goldman and Brenner (1987)):



where A represents actin and M represents myosin. A-M represents a weak bond between actin and myosin, A·M represents a stronger bond, and A·M* represents a force-producing complex that has a conformation different from that of A·M·ADP or A·M.

Potentially PEG could affect any of the steps in the scheme given above; however, there is evidence that some transitions are not affected. The rate of ATP hydrolysis of

S1 by itself, 0.04 s^{-1}, is not significantly changed by the addition of PEG, nor is the phosphate burst seen after mixing ATP with S1 (White et al., 1995; Highsmith et al., 1996). These results show that the hydrolysis step of S1 itself, transition 3 above, is not altered by PEG. To investigate the possibility that PEG influences rate 1, we measured shortening velocity and isometric tension as a function of the ATP concentration. The binding of ATP dissociates actin from myosin rapidly at the end of the power stroke during high-velocity shortening at a rate that can be estimated from the dependence of velocity on [ATP]. The addition of PEG decreased the maximum shortening velocity by a factor of 2 at high ATP, and it decreased the K_m for ATP by a factor of 2, leading to the conclusion that rate 1 is not altered by PEG in shortening fibers. The dissociation of myosin heads in the middle of the power stroke by ATP in isometric fibers was probed by measurements of isometric tension as a function of [ATP]. The increase in tension as [ATP] is lowered is also not changed by PEG. Together the data suggest that the rate of ATP binding is not altered by PEG either at the end of the power stroke in shortening fibers or in the middle of the power stroke in isometric fibers. Similar experiments involving changes in [ADP] lead to the conclusion that PEG has not affected the binding or release of ADP from actomyosin (step 8). Thus we can conclude that the rates of transitions 1, 3, and 8 are not significantly perturbed by PEG.

Numerous studies of the interaction of PEG with proteins in other systems suggest that PEG is most likely to affect those steps that involve the formation or dissociation of protein-protein interfaces (Timasheff, 1993; Highsmith et al., 1996; Parsegian et al., 1986; Bhat and Timasheff, 1992). Such steps occur at the initial binding of myosin to actin step 5 or at the end of the power stroke, where ATP dissociates myosin from actin (step 2). Several observations in fibers and in solution support the conclusion that PEG does potentiate the binding of myosin to actin in transitions 2 and 5. The affinity of actin and myosin in these transitions is weak because of the bound nucleotides, and in an active fiber or during steady-state hydrolysis in solution, these states are transitory (Brenner et al., 1982; Chalovich et al., 1991a). It is important to understand the properties of these states, as their structure is different from that of the more strongly bound states, and they are a necessary step toward the force-producing states (Chalovich et al., 1991b). Thus the effect of PEG on these transitions was investigated using phosphate analogs, which increase the population of these weakly binding, putative, pre-power-stroke states. Addition of V_i or AlF_4 has been shown to produce a tightly bound myosin ADP complex that mimics the properties of the myosin ADP phosphate complex or of a transition state leading to this complex (Chase et al., 1993, 1994; Dantzig and Goldman, 1985). Addition of these phosphate analogs to active fibers decreased fiber tension to ~10% of control values and decreased muscle stiffness to ~20% of control values, as observed previously. Further addition of PEG produced very little increase in fiber tension, while producing a dramatic increase in fiber stiffness, returning stiffness to ~80% of that observed in the fully active fiber in the absence of the analogs (see Fig. 7 and Table 1). More directly, the presence of PEG potentiated the binding of S1-ADP-analog to actin (Fig. 8). These results suggest that formation of these complexes, in which myosin is weakly bound to actin, is accompanied by the formation of a large actomyosin interface. In active fibers in the absence of the phosphate analogs, the addition of PEG potentiated stiffness more than tension, by almost a factor of 3. As we discuss below, the tension exerted per force-generating cross-bridge is not lower in the presence of PEG. Therefore, the greater increase in stiffness shows that PEG has also potentiated the population of the weakly bound states in active fibers.

The size of the actomyosin interface formed in transitions 2 and 5 can be estimated from the dependence of the association constant on the osmotic pressure exerted by the PEG. This dependence, shown in Fig. 10, is similar for the binding of S1 to actin, for the K_m of the actoS1 ATPase activity, and for fiber stiffness. The K_m for the actoS1 ATPase activity is determined mainly by the binding of S1·ADP·P_i to actin (for a review see Taylor, 1979). Thus the changes in PEG-inaccessible volume are similar for all three analogs studied and for the S1·ADP·P_i complex formed during steady-state hydrolysis. The change in the PEG-inaccessible volume is a function of the shapes of the proteins, the shape of their complex, and the radius of the PEG and was calculated to be ~75,000 $Å^3$ for the effect of PEG 4000 on the K_m of the actoS1 ATPase activity (Highsmith et al., 1996). The slightly steeper slopes found for the binding of M·ADP·AlF_4 to actin or for the stiffness of fibers activated in the presence of AlF_4 would imply a slightly greater volume change, but the difference is not very significant. The exact size of the interface is difficult to determine from the volume change, because the contours of the protein surfaces are irregular and the radius of the PEG is large. However, a simple calculation by Highsmith et al. suggested it was of the magnitude expected (Highsmith et al., 1996). Although an exact determination of the area is difficult, it is clear that the volume change is large, implying that the interface is extensive. In addition, small changes in ΔV can be accurately determined, and these show that the volume change is similar for the three cases. In fact, the effect of PEG on the formation of the rigor actoS1 complex, a fourfold increase in affinity at 5% PEG 4000, is even a little less than that observed for the weakly bound state (see Fig. 9). Thus the changes in the PEG-inaccessible volume are large, suggesting that the interface formed for the weakly bound states is extensive and is probably as large as for the strongly bound states. This conclusion would exclude models where some type of tether between the proteins forms the weakly bound states, e.g., via some loops in the protein structures.

Potentiation of the weakly bound actomyosin complexes in transitions 2 and 5 can explain at least a part of the inhibition of ATPase activity by PEG. The isometric cycling rate is determined to a great extent by the rate of ATP hydrolysis. Dissociation is not an obligatory step for hydrolysis, and the pathway can proceed through either step 3 or step 4 (Stein et al., 1979). The rate of hydrolysis in the ternary complex with actin, step 4, is a factor of 10 slower than that of the free myosin, step 3 (White et al., 1993, 1997). Thus the tighter binding of M-ATP to actin in the presence of PEG will result in a decrease in the net rate of hydrolysis, leading to a decrease in the rate of cycling. An increased fraction of weakly bound heads could also account for some of the inhibition of shortening velocity. Inhibition of steps 1 and 8 would be likely candidates to explain the reduction in fiber velocity; however, these rates do not appear to be affected, as discussed above. Thus the reduced velocity must be due to other transitions. The inhibition of the ATPase activity by the binding of myosin·ATP to actin would result in the formation of A-M·ATP. This state is relatively stiff and could inhibit velocity. Inhibition of velocity by weakly bound myosin heads has been observed directly in in vitro motility assays (Cuda et al., 1997; Warshaw et al., 1990) and has been implicated in skeletal muscle fibers (Seow and Ford, 1993).

Information on the energetics of the power stroke can be obtained from the titration of tension by P_i. Below a certain concentration of P_i, tension reaches a plateau. As $[P_i]$ increases above this concentration, tension decreases linearly with log $[P_i]$. This inhibition of tension has been analyzed in terms of a model of cross-bridge energetics (Pate and Cooke, 1989; Pate et al., 1998). An increase in P_i decreases the free energy of the pre-power-stroke states that precede P_i release. When the free energy of the pre-power-stroke states becomes lower than that of the highest strongly bound state, transitions from the strongly bound state to the pre-power-stroke state will occur. At this point these transitions will begin to cause an inhibition in tension. As the phosphate concentration is increased beyond this point, tension will decrease approximately linearly with log $[P_i]$ and will extrapolate to zero when the free energy of M·ADP·P_i reaches the lowest energy available in the strongly bound states. This relationship was analyzed more quantitatively by Pate et al. (1998), and the data of Fig. 5 are fit to their Eq. 8. This equation describes the shape of the force-log $[P_i]$ curve in terms of two parameters, defining the highest and lowest free energies of the strongly bound states that are populated in active fibers. These fits define a free energy drop in the strongly bound states of 9.1 ± 0.2 *kT* in the absence of PEG and 10.7 ± 0.8 *kT* in its presence (Pate and Cooke, 1989; Pate et al., 1998). If the increase in force were due to the attachment of more myosin heads, each producing equal force on average, the titration of tension by $[P_i]$ would have been different, with a greater slope and reaching zero force at the same concentration of P_i in the presence or absence of PEG. In contrast, the absolute change in force induced by PEG is independent of the concentration of P_i. In the model presented by Pate and Cooke this result suggests that the increased tension cannot be explained by an increase in the fraction of force-generating heads. Thus the titration of force with $[P_i]$ suggests that the greater affinity of actin for myosin leads to a greater force per force-generating myosin head. The increase in free energy determined from the titration, 1.6 ± 0.9 *kT*, is close to the increase in the affinity of the actomyosin bond. It is close to the increase in the free energy of the strong actoS1 bond, 1.4 ± 0.1 *kT* at 5% PEG 4000, i.e., at an osmotic pressure of 1×10^6 dynes/cm^2. It is a little smaller than the free energy change seen in the weakly bound states at this osmotic pressure, 1.8–2.2 *kT*, but the difference is not outside experimental error. It appears from these data that the apparent free energy available to generate force in isometric fibers depends directly on the free energy of the actomyosin bond; however, data collected over a wider range of conditions would be required to verify this hypothesis.

As discussed above, the fraction of force-generating myosin heads does not appear to be greatly altered by PEG. This result is not easy to explain, because the cycling rate has changed. The inhibition of the ATPase activity must be due in part to the inhibition of the net rate of hydrolysis, as hydrolysis is one of the rate-limiting steps in the cycle. If the inhibition of the cycling rate by PEG involves only the inhibition of transitions that occur before the power stroke, the fraction of heads in the power stroke would decrease. An approximately constant fraction of force-generating heads could be maintained if PEG also potentiated the rate of some reaction that preceded the power stroke. In fact this does occur, as PEG potentiates the binding of the pre-power-stroke states. Thus PEG has shifted the weakly binding states in two ways: it has increased the fraction of the bound forms, as shown by the increasing stiffness in active fibers, and it has shifted states from the A-M·ADP·P and M·ADP·P form to the A-M·ATP and M·ATP form. Thus the population of the A-M·ADP·P state will remain approximately constant. The rate of the transition into the power stroke states would also be relatively constant if PEG does not affect this transition, which is an isomerization between two bound states and is unlikely to involve changes in PEG-inaccessible volume. In addition, the rate out of the power stroke states, governed by the rate of release of ADP and subsequent binding of ATP, is also not changed greatly by PEG, as discussed above. Although this kinetic argument rationalizes how the population could remain the same, the explanation may lie elsewhere. For instance, steric constraints could govern the fraction of myosin heads generating force, i.e., only those heads with actin in the right position would generate force, and all of those do so. We conclude that the fraction of attached force-generating myosin heads has not changed greatly, and the increased force is due to a greater force per attached head. Why would force

correlate with the strength of the actomyosin bond? A natural correlation is expected in models, such as that proposed by Huxley, in which the binding of actin to myosin stabilizes the stretching of a spring (Huxley, 1957). In this model, the stronger the actomyosin interaction, the further the spring can be stretched, and the greater the resulting isometric force.

In addition to producing some useful perturbations of the actomyosin cycle, PEG may also approximate more closely the crowded conditions that exist in vivo. The approximate osmotic pressure due to the presence of the soluble components in a living muscle cell can be produced by ~5% PEG 4000. Do weakly binding cross-bridges bind more tightly to actin in living muscle than in skinned fibers? In a direct comparison, it was found that the stiffness of stretched relaxed fibers did decrease upon skinning; however, the stiffness was not attributed to weakly bound cross-bridges (Bagni et al., 1995). More direct comparisons need to be made to answer this question.

SUMMARY

PEG potentiates the binding of actin to myosin complexed with ADP and P_i analogs both in solution and in fibers. It affects several transitions in active fibers, producing slower velocities and ATPase activities, and stronger forces per attached myosin head. Addition of PEG can produce a large population of actin-bound myosin heads complexed with ADP and phosphate analogs, providing a preparation that should facilitate the study of these putative pre-power-stroke states. Thus it perturbs the actomyosin interaction, in several useful ways, producing fiber preparations with enhanced populations of weakly binding states for mechanical and structural studies.

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