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Demembranated Muscle Fibers Catalyze a More Rapid Exchange between Phosphate and Adenosine Triphosphate than Actomyosin Subfragment 1†

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ABSTRACT: The rate of ATP $\rightleftharpoons$ P_i exchange, that is, the incorporation of medium P_i into ATP during the net hydrolysis of ATP, has been measured for rabbit psoas muscle fibers, myofibrils, and actomyosin subfragment 1 (acto-S1). The maximum exchange rate in fibers at saturating $[P_i]$ is 0.04 s^{-1} per myosin head at 8 °C, pH 7, and an ionic strength of 0.2 M. The dependence of the rate on P_i concentration can be approximated by a hyperbola with an apparent dissociation constant (K_m) of 3 mM. Myofibrils catalyze ATP $\rightleftharpoons$ P_i exchange with a similar K_m but at a slightly lower rate. In contrast, the soluble acto-S1 system, in which ATP hydrolysis is not coupled to tension generation, catalyzes exchange at a rate 500 times lower than that of fibers at low P_i concentration, and the K_m for P_i is greater than 50 mM. The difference between the ATP $\rightleftharpoons$ P_i exchange of fibers and of acto-S1 is discussed in terms of a model in which P_i binds to a force-generating state AM′·ADP and, due to mechanical constraint, the average free energy of this state is higher in the fiber than in acto-S1.

The kinetics of the hydrolysis of ATP by myosin subfragment 1 in the presence of actin are understood in moderate detail in solution. However, in intact muscle, movement of the myosin cross bridge while bound to actin results in work production, and the nature of the coupling between the chemical reaction and cross-bridge movement is less clear. Work on the thermodynamics of myosin and actomyosin (AM) ATPase (Goody et al., 1977; Cardon & Boyer, 1978; White & Taylor, 1976; White, 1977) led to the conclusion that the release of P_i from AM·ADP·P_i to give AM·ADP (the state formed by adding ADP to AM) has a dissociation constant, K_d, of about 300 M. The physiological concentration of P_i in unfatigued muscle is about 0.1 mM, and the free energy change associated with P_i release is about 35 kJ [RT ln

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(300/0.0001)]. The total free energy change of ATP hydrolysis under physiological conditions is about 60 kJ, and thus more than half the total free energy change of ATP hydrolysis is associated with P_i release. It has therefore been tempting to suppose that it is this part of the reaction sequence which leads to the generation of force and hence work. Conversely, if the dissociation of phosphate results in the force-generating state, an effect of phosphate on the mechanical properties of muscle would be expected. It has been known for some time that concentrations of P_i in the millimolar range have a significant effect on the mechanical properties of actively contracting insect fibers (White & Thorsen, 1972). More recently, Hibberd et al. (1985a) found that 10 mM P_i trebled the rate of tension loss of rigor rabbit psoas muscle fibers upon photolytic release of ATP from an inactive precursor. However, it is difficult to prove that these mechanical effects are due to phosphate binding at the active site, that is, the site from which phosphate, the product of ATP hydrolysis, is released and not due to binding at a secondary "control" site. Kawai (1986) has analyzed the effects of P_i on the mechanical behavior of rabbit psoas fibers and was unable to account for all the results in terms of a simple hydrolysis model. Phosphate binding at the active site during the hydrolysis of ATP has been demonstrated directly both by ATP ⇌ P_i exchange (Gillis & Marechal, 1974; Ulbrich & Ruegg, 1977) and by P_i ⇌ HOH oxygen exchange (Webb et al., 1986). In the latter study, it was found that the rate of P_i binding deduced from oxygen exchange was the same order of magnitude as the rate deduced from the effect of P_i on the rate of relaxation of rigor fibers in caged ATP experiments.

In this paper, we report a detailed investigation of the ATP ⇌ P_i exchange in fibers, myofibrils, and actomyosin subfragment 1 (acto-S1). The greater sensitivity of ATP ⇌ P_i exchange measurements relative to oxygen exchange measurements has allowed the determination of the apparent P_i dissociation constant at the active site (K_m for P_i). In addition to the steps of P_i binding and reversal of the hydrolysis, ATP ⇌ P_i exchange also involves the release of ATP, and thus by combining the sets of data, an estimate of the rate of ATP release can be made.

Materials and Methods

Rabbit psoas fibers were skinned in a relaxing solution (Wood et al., 1975) and stored in a 50% glycerol relaxing solution for up to 3 weeks. Myofibrils were prepared as described by Knight and Trinick (1982) and cross-linked with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) to prevent contraction (Glyn & Sleep, 1985). Myosin subfragment 1 was prepared from rabbit back and leg muscle by the method of Weeds and Taylor (1975). Actin was prepared by the method of Spudich and Watt (1971). Subfragment 1 was cross-linked to actin with EDC using Rosenfeld and Taylor's (1984) modification of the method of Mornet et al. (1981).

Measurements of the rate of ATP ⇌ P_i exchange of fibers were normally done in 40–50-μL drops of solution which were placed on oval pieces (6 × 4 mm) of a no. 3 coverslip. Five such coverslips were glued via glass or aluminum pedestals (4 × 2 × 0.8 mm) to a large microscope slide below which cooling water circulated (see Figure 1). A sixth coverslip of somewhat larger size (9 × 7 mm) facilitated mounting of the fiber. The microscope slide could then be translated so as to bathe the fiber in the appropriate solution. This arrangement had a particular advantage for ATP ⇌ P_i exchange work because experiments could be done with samples as small as 25 μL, and this minimized the amount of labeled P_i needed to record adequate counts in the ATP. It had the secondary advantage

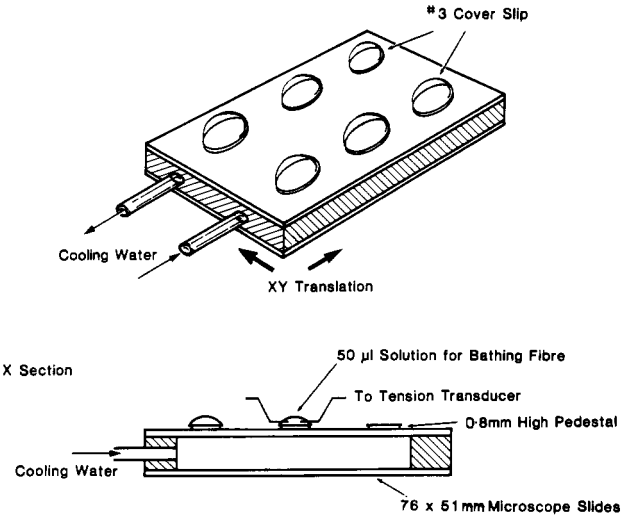

FIGURE 1: Arrangement used for changing solution bathing fiber.

that fibers entered and exited from drops along the fiber axis, and thus the stretching forces which occur on fiber transfer in a conventional multitrough system were much reduced.

The standard protocol was to dissect a bundle of two fibers in a 25% glycerol relaxing solution and to glue the fibers between two hooks using a solution of cellulose nitrate in acetone. The fibers were transferred to the standard relaxing solution [5 mM ATP, 7 mM magnesium chloride ($MgCl_2$), 100 mM *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES), 10 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 10 mM phosphocreatine, 90 mM potassium acetate (KOAc), and 1 mg/mL creatine kinase, pH 7, ionic strength 0.2 M, 8 °C]. The bundle was examined under the microscope (40× water immersion objective, 25× eyepiece) and the sarcomere length set to 2.5 μm. The widths of the two fibers and the length between gluing points were then measured. The fibers were immersed in the standard activating solution (15 mM ATP, 16 mM $MgCl_2$, 10 mM CaEGTA, 10 mM phosphocreatine, 100 mM TES, 40 mM KOAc, and 1 mg/mL creatine kinase, 0.2 M ionic strength, pH 7, 8 °C) to check that the tension per unit area was satisfactory and that the tension was stable. The fibers were then relaxed prior to transfer to a similar activating solution containing phosphate labeled with ^{32}P and a reduced [KOAc] to conserve ionic strength at 0.2 M. The drop in which the fiber was immersed was stirred by using a vibrating thermocouple probe. After a period of time, usually 5 min, the fibers were transferred to relaxing solution, and the drop of labeled activating solution was transferred to 2 mL of acid quench solution (0.5 M perchloric acid and 5 mM P_i). The coverslip was washed with a further 50 μL of quench solution.

The rate of ATP ⇌ P_i exchange catalyzed by each pair of fibers was measured over a range of P_i concentrations from 1 to 20 mM. Every third measurement was done at the standard [P_i] of 10 mM, and the results were used to normalize the intervening measurements. Each day several blank aliquots of activating solution were set up and not used in fiber experiments. These aliquots were analyzed in the usual manner, and any apparent ATP ⇌ P_i exchange was subtracted off the presented results to correct for imperfections of the P_i separation method and possible synthesis of labeled ATP due to contaminant enzymes in the creatine kinase. In general, the correction was less than 10%, in the worst case, it was 15%.

To measure ATP ⇌ P_i exchange, ATP must be separated from P_i with high efficiency, which was done by adsorption to a charcoal column and elution with EtOH–NH_3 followed

by a further step of purification on a Dowex-1 column (Sleep & Hutton, 1980).

The effect of temperature on the rate of ATP $\rightleftharpoons$ P_i exchange and tension was measured under similar conditions. In this case, alternate measurements were made at the standard temperature of 8 °C and used to normalize the intervening measurements at higher temperatures.

Control experiments were done with fibers stretched to a sarcomere length of >4.2 μm at which there should have been no overlap between myosin and actin filaments. In these experiments, fibers which had not undergone a contraction were slowly stretched to nonoverlap while in relaxing solution. Sarcomere length was monitored by using a 20× water immersion objective and observing the diffraction pattern with a phase telescope. The illuminating light was directed through a narrow band green interference filter. The fiber was scanned from end to end to ensure that there were no regions of the fiber at short sarcomere length. After a period of about 5 min, the transient increase of tension which occurs even on slow stretching had decreased to a constant value, and the fiber was transferred to the activating solution. In most cases, no increase in tension occurred. Although these fibers were able to shorten back to normal sarcomere length, they gave a negligible tension on activating at this length so that the same fiber could not then be used to measure ATP $\rightleftharpoons$ P_i exchange at full overlap. The loss of tension is presumably due to the failure of the filaments to interdigitate properly and may be due to degradation of connecting filaments. It is not observed with frog fibers, which are usually used without storing. It was no easier to do the experiment with and without overlap on the same fiber by inverting the procedure, for after sustaining tension for 5 min at full overlap most fibers had become sufficiently nonuniform in sarcomere length that some tension was generated after stretching the fiber to a length normally corresponding to no overlap.

Some experiments were done at 3 mM ATP, in which case the magnesium acetate concentration was 4 mM and the potassium acetate concentration changed to conserve the ionic strength at 0.2 M.

The directly measured parameter of the moles of [^{32}P]ATP produced per second was converted to moles of ATP per mole of S1 heads per second from the volume [π/4(width)^2] of the fiber, using a concentration of 154 μM for the S1 concentration within a fiber (Ferenczi et al., 1984; Yates & Greaser, 1983).

The effect of P_i on the steady-state ATPase activity of fibers was measured by using the apparatus and methods described by Glyn and Sleep (1985). The ATP $\rightleftharpoons$ P_i exchange catalyzed by acto-S1 and EDC-cross-linked acto-S1 was determined by using the methods of Sleep and Hutton (1980).

RESULTS

Time Course of Fiber ATP $\rightleftharpoons$ P_i Exchange. Fiber ATP $\rightleftharpoons$ P_i exchange was measured in 30–50-μL drops of activating solution containing about 2 μCi of [^{32}P]P_i. With this amount of labeled P_i, measurements could only be made at two time points. Experiments of this type showed that the rate of [^{32}P]ATP production was approximately linear over a 10-min time interval. To check the rate over a longer period, pairs of fibers were incubated in a series of activating troughs for 5 min each. For some fibers (about 30%), particularly at higher temperatures, there was initially a relatively rapid decline in tension and ATP $\rightleftharpoons$ P_i exchange rate. In some of these cases, the tension would decline by 30% in 5 min, and for all fibers, the rate of ATP $\rightleftharpoons$ P_i exchange showed a greater decline than tension. The majority of fibers maintained their tension and exchange rates well at 8 °C, and only these were included in Figure 2 when characterizing the P_i binding constant. On average, the counts exchanged into ATP with 10 mM P_i in the period 20–25 min were 80% of the counts exchanged in the period 0–5 min, and the tension at the later time was 95% of that initially.

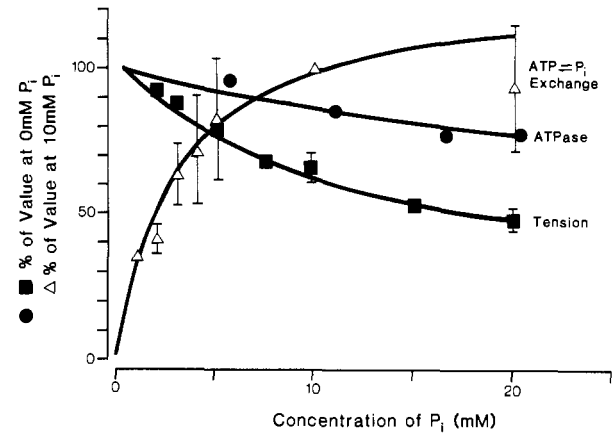

FIGURE 2: Dependence of fiber ATP $\rightleftharpoons$ P_i exchange, tension, and ATPase on [P_i]. Reaction conditions: 15 mM ATP, 16 mM $MgCl_2$, 100 mM TES, 10 mM CaEGTA, 10 mM phosphocreatine, the stated phosphate concentration, a potassium acetate concentration to give an ionic strength of 0.2 M, 1 mg/mL creatine kinase, pH 7, and 8 °C. Error bars represent ±1 SD. As described under Materials and Methods all ATP $\rightleftharpoons$ P_i exchange rates are relative to that at 10 mM, and thus there are no error bars on this point. 100% values are as follows: ATP $\rightleftharpoons$ P_i exchange = 0.034 s^{-1}; tension = 150 kN m^{-2}; ATPase = 1.2 s^{-1}. The tension losses at the end of the experiment for fibers contributing to this figure were less than 25%. (△) ATP $\rightleftharpoons$ P_i; (■) tension; (●) ATPase.

Dependence of ATP $\rightleftharpoons$ P_i Exchange, Tension, and ATPase of Fibers on P_i Concentration. The rate of ATP $\rightleftharpoons$ P_i exchange was measured as a function of [P_i] in the range 1–20 mM. Figure 2 shows the averaged results from five fibers, some of which were used more than once for any given P_i concentration so that the average number of measurements at each P_i concentration was typically eight. The best two of these fibers showed no significant decline in tension or ATP $\rightleftharpoons$ P_i exchange during the course of the experiment. The worst fiber showed a 25% loss of tension and a 30% loss of ATP $\rightleftharpoons$ P_i exchange. The effect of this deterioration was allowed for by having every third contraction in a standard 10 mM P_i solution. For fibers which held their tension satisfactorily, the sarcomere structure was quite well preserved and the sarcomere length unchanged at the end of the experiment. Fibers in which the tension loss was more marked had a less regular pattern of sarcomeres and significant differences in sarcomere length along the fiber. While the sequence of P_i concentrations used was not varied systematically, several different procedures used in the course of these experiments gave similar results. In order to characterize the dependence of exchange on [P_i], a hyperbola was fitted by using a least-squares routine, giving a K_m of 3.0 ± 0.8 mM (mean ± SD) and a V_m of 0.04 ± 0.02 s^{-1}. The slight decline in rate on going from 10 to 20 mM P_i was often observed although not statistically significant.

Plots of tension and ATPase against [P_i] are included in Figure 2. These results were from two different sets of experiments. Tension reached a steady value more rapidly than either the ATPase or the ATP $\rightleftharpoons$ P_i exchange rates could be measured, and thus any effect of fiber deterioration could be minimized by a separate experiment. However, the plots of tension versus [P_i] taken from the ATP $\rightleftharpoons$ P_i and ATPase experiments were very similar to that in Figure 2. At 20 mM P_i, the tension has declined to about 50% of the value in the absence of P_i and the ATPase to about 85%. For both ATPase

Table I: Control Experiments

experiment	conditions	tension[a]	ATP ⇌ P_i
no overlap	sarcomere length >4.2 μm, [P_i] = 10 mM	0	<5
+oligomycin	[P_i] = 2 mM	90 ± 5	79 ± 5
+oligomycin and quercetin[b]	[P_i] = 20 mM	92 ± 3	76 ± 2
relaxed fiber	pCa < 8	0	<1

[a] Percent of that obtained under standard conditions. Standard conditions are 10 mM P_i, 8 °C, pH 7, 15 mM MgATP, and an ionic strength of 0.2 M. [b] Oligomycin and quercetin were added from stock solutions in ethanol. The solution also contained 10 μM P^1,P^5-di-(adenosine-5′) pentaphosphate and 0.02% Triton, the latter to give quercetin access to both sides of the SR membrane (Shoshan & MacLennan, 1981). The concentration of ethanol in the final solution was 2%, and an equivalent amount was added to the solution in the control experiment in the absence of inhibitors.

and tension experiments, measurements were made alternately in a standard 10 mM P_i solution and the P_i concentration under test, and the results were normalized for any loss of tension or ATPase during the course of the experiment.

ATP ⇌ P_i Exchange in Fibers with No Filament Overlap. The most direct test of whether the observed ATP ⇌ P_i exchange is due to actomyosin is to carry out the experiment at a sarcomere length at which there is no overlap between myosin and actin filaments. Such experiments were done under the standard activating conditions at 10 mM P_i. The extent of exchange after 20 min was measured for three fibers at no overlap and after 10 min for three fibers at full overlap. After different fiber sizes were accounted for, the average rate at no overlap was less than 5% of that at full overlap, which indicates that ATPase other than actomyosin make a negligible contribution to the observed ATP ⇌ P_i exchange.

Effect of Inhibitors of Ion Pumps. The contribution of ion pumps to ATP ⇌ P_i exchange was also tested by using the sarcoplasmic reticulum Ca^{2+} pump inhibitor quercetin (100 μM; Shoshan & MacLennan, 1981) and the mitochondrial proton pump inhibitor oligomycin (1 μg/mL; Lardy et al., 1958). Tension and ATP ⇌ P_i exchange rates were first measured by using standard activating solutions and then in the presence of inhibitors. At both 2 and 20 mM P_i, the inhibitors caused a 10% loss of tension and a 25% loss of ATP ⇌ P_i exchange. These results are summarized in Table I. It seems likely that much of the apparent inhibition of ATP ⇌ P_i exchange is due to fiber deterioration because it was observed that in untreated fibers which did not maintain active tension well the exchange rate fell more rapidly than tension. Because of the deleterious effect on tension and exchange caused by quercetin and oligomycin, this line of investigation was not pursued, but the results obtained were consistent with actomyosin being responsible for most of the observed exchange.

Fiber ATP ⇌ P_i Exchange in the Absence of Ca^{2+}. The rate of ATP ⇌ P_i exchange of relaxed fibers at 10 mM P_i was less than 1% that of contracting fibers. This reduction in rate under relaxing conditions is even larger than that observed for the ATPase (Glyn & Sleep, 1985). The slow ATP ⇌ P_i exchange rate of relaxed fibers is relevant to the question of exchange from non-actomyosin sources but does not help to exclude the sarcoplasmic reticulum ATPase because of the Ca^{2+} dependence of the latter.

Table I summarizes the experiments on fibers stretched to nonoverlap, in the presence of ion pump inhibitors, and under relaxed conditions.

Loss of Exchanged ATP through Hydrolysis. There are two ways in which ATP that has become labeled through exchange can be hydrolyzed before the reaction is quenched: first, exchanged ATP which has diffused out of the fiber into the drop of activating solution could diffuse back in again and be hydrolyzed; second, exchanged ATP could be hydrolyzed before it gets outside the fiber. Only about 0.5% of the 15 mM ATP in the stirred 50-μL drop is hydrolyzed by the two fibers in 5 min, and thus the first source of loss is negligible. However, if ATP did not diffuse in from the exterior, a fiber initially containing 3 mM ATP would go into rigor after 30 s (assuming a turnover rate of 0.5 s^{-1}), and this loss of exchanged ATP, which will be proportional to 1/[ATP], is potentially more serious. The rate of ATP exchange can be expressed as $k_{exch} = k_{hydr}/[ATP] + k_{meas}$ where k_{exch} and k_{hydr} are constants and k_{meas} and [ATP] are variables. The measured rate, k_{meas}, was observed to be 30% more at 15 mM ATP than it was at 3 mM, and solving the equation gives an estimate of the rate of exchange as 1.07 times the measured rate at 15 mM. Part of the increased exchange at 15 mM is probably due to the better performance of fibers at the higher ATP concentration, and for this reason and the fairly small size of correction, it has not been applied to the results.

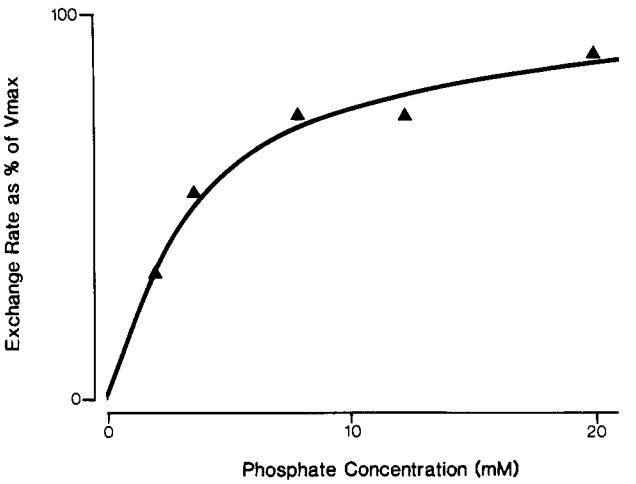

FIGURE 3: Dependence of EDC-cross-linked myofibrillar ATP ⇌ P_i exchange on P_i concentration. Conditions: 15 mM ATP, 16 mM $MgCl_2$, 50 mM imidazole, 10 mM phosphocreatine, 0.1 mM $CaCl_2$, stated P_i concentration, and a KCl concentration to give an ionic strength of 0.2 M, pH 7, 25 °C. The maximum rate corresponds to 0.03 s^{-1}.

Myofibrillar ATP ⇌ P_i Exchange Experiments. The dependence of the rate of ATP ⇌ P_i exchange on [P_i] for myofibrils cross-linked with EDC to prevent shortening was measured for three preparations, and a representative plot is shown in Figure 3. The dependence on P_i concentration is quite similar to that of fibers, the K_m being 3.5 mM. However, there is no sign of the apparent decrease in exchange rate between 10 and 20 mM P_i shown by many fibers. The rate at saturating [P_i] was 0.03 s^{-1} at 25 °C.

Dependence of the ATP ⇌ P_i Exchange of Acto-S1 on [P_i]. At the ionic strength (0.2 M) of the fiber experiments, the ATPase of S1 shows only a slight actin activation at experimentally accessible actin concentrations, and thus EDC-cross-linked acto-S1, which shows full activation (Brenner & Eisenberg, 1986), was used. The amount of [^{32}P]ATP increased essentially linearly with time during hydrolysis of the first 30–40% of ATP. The resulting plot of rate versus [P_i] is shown in Figure 4. There is little sign of deviation from a linear dependence upon P_i concentration. Two other experiments gave similar linear plots. It can be concluded that the apparent dissociation constant (K_m) of P_i is greater than 50 mM.

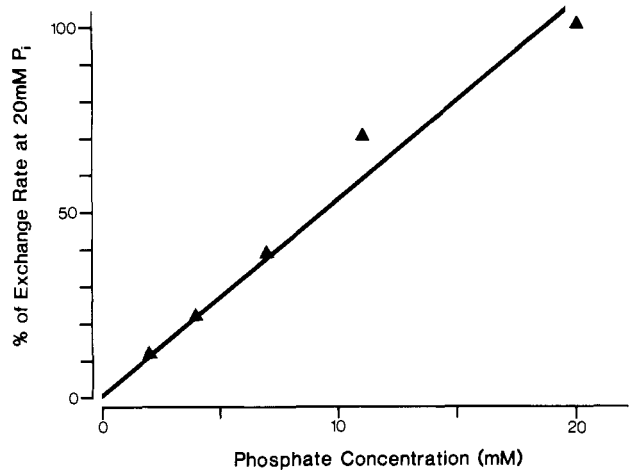

FIGURE 4: Rate of ATP $\rightleftharpoons$ P_i exchange catalyzed by EDC-cross-linked acto-S1 as a function of [P_i]. In a parallel experiment, the ATPase rate measured by using an NADH-linked assay system was found to be independent of [P_i].

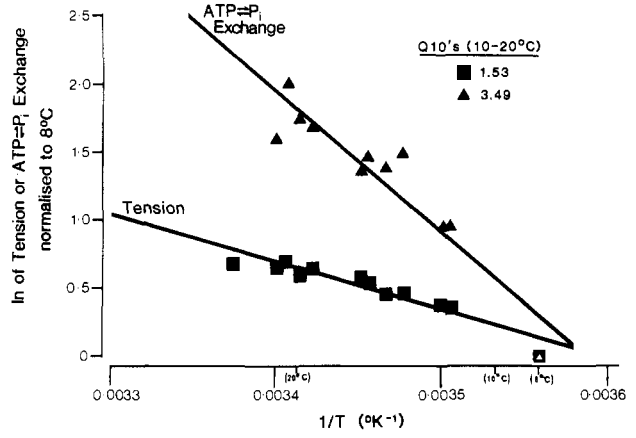

FIGURE 5: Arrhenius plot of ATP $\rightleftharpoons$ P_i exchange and tension. Conditions were as for Figure 2, and the [P_i] was 10 mM. (▲) ATP $\rightleftharpoons$ P_i; (■) tension.

Temperature Dependence of Fiber and Acto-S1 ATP $\rightleftharpoons$ P_i Exchange. The fiber ATP $\rightleftharpoons$ P_i exchange experiments were done at 8 °C because their success depended critically on getting several 5-min contractions without serious loss of tension from each pair of fibers. However, some measurements were made at higher temperature in order to compare the rates of ATP $\rightleftharpoons$ P_i exchange with the rates of P_i $\rightleftharpoons$ HOH exchange during ATP hydrolysis (Webb et al., 1986) which were measured at room temperature. Figure 5 is a plot of the log of the rate of ATP $\rightleftharpoons$ P_i exchange and tension versus 1/temperature at 10 mM P_i. Within the limits of experimental error, there are no obvious changes in slope, and the activation energies characterizing exchange rate and tension are 86 and 29 kJ/mol, respectively.

The ATP $\rightleftharpoons$ P_i exchange of acto-S1 and EDC-cross-linked acto-S1 was measured at 8 and 18 °C, and it was found that the temperature dependence of the two was similar and characterized by a much higher activation energy (180 kJ/mol; $Q_{10} \approx 15$) than the ATP $\rightleftharpoons$ P_i exchange of fibers so that the enhancement of ATP $\rightleftharpoons$ P_i exchange in fibers over acto-S1 is considerably greater at 8 °C than it is at room temperature. Because fiber performance was better and the results correspondingly more reliable at lower temperatures, attention will be focused in the Discussion on the relative rates at 8 °C. The activation energy of acto-S1 ATP $\rightleftharpoons$ P_i exchange is extremely high, but even in a simple model, the observed rate is a combination of a P_i binding constant and a rate of ATP release. The rate of product release (=steady-state ATPase rate at low [actin]) is very temperature dependent (Q_{10} = 5), and the unreported observation of Sleep and Hutton (1978) that the proportion of bound nucleotide, released as ATP, decreases at lower temperatures means that the rate of ATP release must be more temperature dependent than the ATPase. If P_i binds more tightly at low temperature, as suggested by the greater effect of P_i on tension at low temperature, the combination of factors would account for the extremely high overall temperature dependence of ATP $\rightleftharpoons$ P_i exchange.

The ATP $\rightleftharpoons$ P_i exchange of acto-S1 and EDC-cross-linked acto-S1 was compared at relatively low ionic strength (30 mM), conditions under which S1 could be maximally actin activated. The rates were found to be very similar which suggests that EDC does not cross-link S1 to actin in a manner which mimics the constraint imposed during an isometric contraction. This is consistent with the observations that the rate in the forward direction of EDC-cross-linked acto-S1 is similar to that of acto-S1 and not to the reduced rate observed in fibers (Mornet et al., 1981) and with the electron micrographs showing a random orientation of cross-linked heads in the presence of ATP (Craig et al., 1985).

Discussion

Comparison with Previous Measurements of Fiber ATP $\rightleftharpoons$ P_i Exchange. Although the effect of P_i concentration on ATP $\rightleftharpoons$ P_i exchange has not been reported before, investigations on the ATP $\rightleftharpoons$ P_i exchange of fibers have been made by Gillis and Marechal (1974) and by Ulbrich and Ruegg (1977). The former group measured the exchange of rabbit psoas muscles at 20 °C, and the rate is about one-fifth of our rate under these conditions obtained by interpolation of Figures 2 and 3. The most likely explanation of their lower rate is loss of exchanged ATP due to hydrolysis. There are three factors suggesting this: at 20 °C, the ATPase rate is about 6 times that at 8 °C; they used 5 mM ATP rather than 15 mM; they used bundles of 10 rather than 2 fibers. Ulbrich and Ruegg found that for insect fibers the ratio of reverse to forward rates at 1 mM P_i and 20 °C was 0.002, and our ratio under these conditions would be about 0.008. They carried out controls for loss of ATP through hydrolysis, and this difference is probably because of the different muscle types.

Comparison of the Exchange of Muscle Fibers and Acto-S1. The K_m for P_i describing fiber ATP $\rightleftharpoons$ P_i exchange is 3 mM (Figure 2) whereas that for acto-S1 is greater than 50 mM (Figure 4). Because of this high value for acto-S1, it is only possible to compare the second-order rates of exchange. For EDC-cross-linked acto-S1 at 8 °C, I = 0.2 M, the exchange rate was 4×10^{-5} s^{-1} at 2 mM P_i which gives a second-order rate constant of $4 \times 10^{-5}/2 \times 10^{-3} = 0.02$ M^{-1} s^{-1}. For fibers at 8 °C, the second-order rate constant is 10 M^{-1} s^{-1} (Figure 2). Thus, fibers catalyze exchange about 500 times (10/0.02) faster than acto-S1.

What Controls the Rate of ATP $\rightleftharpoons$ P_i Exchange? ATP $\rightleftharpoons$ P_i exchange can be characterized by the K_m for P_i and the rate at saturating [P_i]. It is convenient to lump the states that

$$\mathrm{AM + ATP} \overset{1}{\rightleftharpoons} \begin{pmatrix} \mathrm{A + M{\cdot}ATP} \\ \downarrow\uparrow a \\ \mathrm{AM{\cdot}ATP} \\ \mathrm{(E{\cdot}ATP)} \end{pmatrix} \overset{2}{\rightleftharpoons} \begin{pmatrix} \mathrm{A + M{\cdot}ADP{\cdot}P_i} \\ \downarrow\uparrow b \\ \mathrm{AM{\cdot}ADP{\cdot}P_i} \\ \mathrm{(E{\cdot}ADP{\cdot}P_i)} \end{pmatrix} \overset{3}{\rightleftharpoons}$$

$$\mathrm{AM'{\cdot}ADP} \overset{4}{\rightleftharpoons} \mathrm{AM{\cdot}ADP} \rightleftharpoons \mathrm{AM} \quad (1)$$

are in fast equilibrium, that is, A + M·ATP $\rightleftharpoons$ A·M·ATP and A + M·ADP·P_i $\rightleftharpoons$ A·M·ADP·P_i, and these lumped states will be referred to as E·ATP and E·ADP·P_i. It is also convenient

to underline species (e.g., $\underline{E{\cdot}ATP}$) for which the bound P_i or the γ-phosphoryl of bound ATP comes from medium P_i. It can readily be shown that at low P_i concentrations

$$[\underline{E{\cdot}ATP}] = k_{-2}k_{-3}[P_i][AM'{\cdot}ADP]/[(k_{-2} + k_3)(k_{-1} + k_2) - k_{-2}k_2]$$

Provided $k_{-1} \ll k_2$ and $k_{-2} \ll k_3$, this expression reduces to $[P_i][AM'{\cdot}ADP]/K_2K_3$; that is, the concentration of $\underline{E{\cdot}ATP}$ is simply that in equilibrium with medium P_i. The rate of ATP ⇌ P_i exchange is k_{-1} times this concentration. The results

$$\text{rate of ATP} \rightleftharpoons P_i = k_{-1}[P_i][AM'{\cdot}ADP]/K_2K_3 \quad (2)$$

of the present work and that on medium ATP ⇌ HOH exchange (Bowater et al., 1988) suggest that the condition $k_{-1} \ll k_2$ is met, and the results of the intermediate P_i ⇌ HOH exchange measurements of Hibberd et al. (1985b) support the case for $k_{-2} \ll k_3$. To include the behavior at high P_i concentrations, the term $[P_i]/K_3$ becomes $[P_i]/([P_i] + K_3)$. Consequently, the observed phosphate dissociation constant (K_m) is equal to the dissociation constant K_3 divided by $[AM'{\cdot}ADP/AM_0]$, the fraction of enzyme in the form to which P_i binds.

In the case of acto-S1 ATPase, the P_i dissociation constant from M·ADP·P_i to M·ADP is about 0.3 M (I = 0.2 M, pH 7; Cardon & Boyer, 1978), and the association constant of actin to M·ADP is about 10^6 M^{-1} and to M·ADP·P_i about 10^3 M^{-1}. Thus, K_3K_4 (see eq 1), the P_i dissociation constant from AM·ADP·P_i to give AM·ADP (the dominant ternary complex formed by mixing actin, S1, and ADP), is 300 M, a value consistent with the lack of a sign of saturation in the rate of acto-S1 ATP ⇌ P_i exchange at accessible P_i concentrations, and very much greater than the 3 mM K_m for P_i found for fibers.

The existence of the state AM′·ADP was inferred from the observation that the ATP ⇌ P_i exchange rate of acto-S1 was much higher than predicted if exchange started from AM·ADP. Moreover, the exchange rate was not enhanced by the addition of ADP (Sleep & Hutton, 1980). It was deduced that K_4 was at least 10, so that K_3 must be less than 30 M. A larger value for K_4 with a corresponding further reduction in K_3 would also be consistent with the data.

Why Do Fibers Have a Lower K_m for P_i and a Higher Exchange Rate than Acto-S1? The rate of ATP ⇌ P_i exchange is controlled by k_{-1}, $1/K_2$, $1/K_3$, and $[AM'{\cdot}ADP]/[AM_0]$ (eq 2). Step 2 occurs between states that are dissociated most of the time, and the energy change should be similar in solution and in fibers, an expectation supported by measurements of the equilibrium constant in fibers and myofibrils. Changes in $1/K_2$ do not account for the difference, and so it must result from a combination of the three remaining terms: $[AM'{\cdot}ADP]/[AM_0]$, $1/K_3$, and k_{-1}. The first factor is the steady-state concentration of AM′·ADP. This state is not a dominant intermediate during acto-S1 ATPase whereas, particularly if it is to be the force-generating state in isometric fibers, it would be expected to be highly populated. The K_m for P_i will be reduced, and the second-order rate of exchange (that is, the rate at nonsaturating P_i concentrations) will be increased by $[AM'{\cdot}ADP_{fib}]/[AM'{\cdot}ADP_{sol}]$.

The second factor is the value of K_3, the dissociation constant of P_i. If AM′·ADP is a force-generating state, it must exist in a higher energy conformation or orientation than that which it would adopt in mechanical equilibrium (the situation in solution). In this case, the ratio of forward to reverse fluxes from force-generating to non-force-generating states (weakly bound states) will be less in a fiber. It should be noted that this effect is not due to the production of external work but to tension generation in the isometric state. An idea of the order of magnitude of the possible effect of force generation on K_3 is given by the following argument. For acto-S1, the K_3 is <30 M. The K_m for P_i in fibers is 3 mM, and K_3 will be less than this if AM′·ADP is not the dominant intermediate. The average increase in energy of the AM′·ADP state in fibers relative to acto-S1 might thus be of the order of 22 kJ [RT ln (30/0.003)]. Figure 6 is a comparison of the average energy of the different states in fibers (right scale) and in solution (left scale) based on this very simple model. The energy levels of weakly bound states are the same in both systems, but those of strongly bound states are higher in fibers than in solution due to storage of elastic energy. A more detailed consideration of this problem is given in the Appendix.

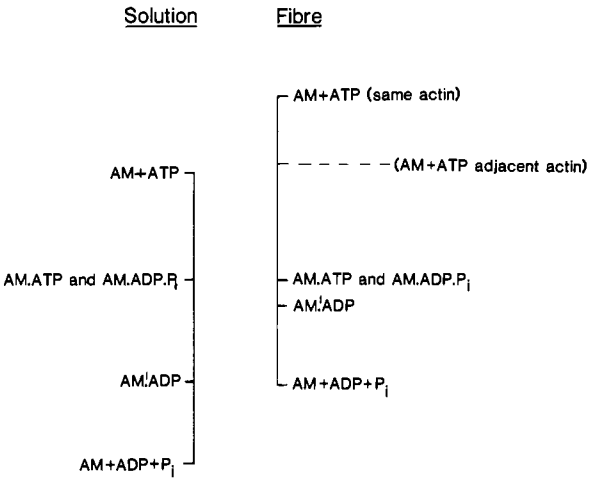

FIGURE 6: Diagram indicating the difference in free energy between myosin states in isometric fibers relative to those in solution.

The final factor is the rate of ATP release, k_{-1}. It can be seen from Figure 6 that if the energy levels for the weakly binding states, AM·ATP and AM·ADP·P_i, are the same in a fiber and in solution and if the energy of ADP release is the same, then if the energy of P_i release is 22 kJ less in a fiber the energy of ATP binding must correspondingly be 22 kJ more. This change in K_1 needs to be split into changes in k_{+1} and k_{-1}. The results of Goldman et al. (1984) suggest that the rate of ATP binding has only a slight dependence on cross-bridge strain, and this helps justify the initial simplifying assumption we will use that the larger free energy change on ATP binding in fibers is due to a reduction in the rate of ATP release (k_{-1}). In this model in which the energy of all the strongly bound cross bridges is increased to the same extent in fibers relative to acto-S1, the effect of tighter P_i binding tending to increase the rate of ATP ⇌ P_i exchange is exactly compensated by the slower rate of ATP release, and thus the higher energy levels of tightly bound states in fibers would not lead to a higher rate of ATP ⇌ P_i exchange. If Goldman's conclusions were not correct and k_{+1} showed a moderate dependence on strain, the reduction of k_{-1} in fibers would be correspondingly less significant.

Could the 500× increase in exchange rate be explained without invoking strain dependence? In itself, an increase in $[AM'{\cdot}ADP]/[AM_0]$ might be numerically adequate, but as the equilibrium between force-producing and non-force-producing states must be strain dependent, can a scheme be set up in which K_1 and K_3 are not strain dependent? The two cases are somewhat different, and we will begin with K_3. There are two requirements: first, AM′·ADP must be a weakly bound state; second, an irreversible step must separate AM′·ADP from the force-generating state, which would be

AM·ADP in this model. If there is a reversible equilibrium between AM′·ADP and AM·ADP, then strain on AM·ADP will result in a higher population of AM′·ADP and a higher rate of ATP $\rightleftharpoons$ P_i exchange. A model in which AM′·ADP is a weak binding state could be made to account for most observations, but if the second requirement of step 4 being irreversible were also included, then the mechanical effects of P_i could only be accounted for by action at a second site. Thus, it is probable that K_3 is strain dependent.

The case of K_1 is potentially different because the final state, AM, is definitively strong binding and the transition is from weak to strong rather than vice versa. The initial state E·ATP represents an equilibrium between states free and bound to actin which are established rapidly relative to neighboring steps, and, moreover, at physiological ionic strength, it is the dissociated form that dominates. Cross bridges are thus free to form a tight complex with the most suitable actin, and the rate of actin binding and forming the tightly bound AM state will be higher if the resultant complex is in an unstrained orientation. In the Appendix, it is shown using a simple model that if the head rebinds to the adjacent actin on the same filament, the strain dependence of the average value of k_{-1} could be dramatically reduced. Thus, K_1 for cross bridges undergoing ATP $\rightleftharpoons$ P_i exchange may show only a limited strain dependence. This would be further reduced if k_1 were also strain dependent.

Comparison with Medium P_i $\rightleftharpoons$ HOH Exchange of Fibers. The results of Webb et al. (1986) indicated that the rate k_{-3} for isometric fibers at 23 °C was $500[AM_0]/[AM'\cdot ADP]$ M^{-1} s^{-1}. An estimate of the rate of P_i release for fibers comes from the intermediate P_i $\rightleftharpoons$ HOH exchange measurements of Hibberd et al. (1985b). Using the simplest possible model in which P_i is released directly from AM·ADP·P_i, they deduced that k_3, the rate of P_i release, is about 50 s^{-1}, a value roughly consistent with the rate of force recovery under these conditions (Webb et al., 1986; Brenner & Eisenberg, 1986). The estimate of the apparent P_i dissociation constant (the K_m for P_i) is thus (50 s^{-1}/500 M^{-1} s^{-1}) 0.1 M. This is not in good agreement with our directly measured K_m of 3 mM at 8 °C. Part of the discrepancy may be due to the difference in temperature.

Rate of ATP Release. If, as seems very likely, the step of P_i binding in fibers is markedly strain dependent, then only the most strained AM′·ADP states are likely to reverse to E·ADP·P_i and on to E·ATP. Is the E·ATP state formed in this manner the same as the typical E·ATP formed by AM binding ATP? In principle, this question can be answered by determining whether the rate of ATP release is the same in both cases.

An estimate of the rate of ATP release from E·ATP can be made by combining the P_i dissociation constant and the maximum rate of exchange at saturating [P_i]. The rate of ATP $\rightleftharpoons$ P_i exchange is about 0.2 s^{-1} at 10 mM P_i (room temperature), and as the K_m for P_i is 3 mM, [AM·ADP·P_i]/[AM_0] is equal to 10/(10 + 3). The concentration of AM·ATP is equal to [AM·ADP·P_i]/K_2, and as K_2 is about 3, the rate of ATP release is about $0.2 \times 3 \times 13/10 = 0.8$ s^{-1}.

The rate of ATP release from myofibrils at 0 °C was investigated by Sleep (1981) using single turnover methods which should give the rate of release from E·ATP as opposed to E·ATP. It was found that at low ionic strength the rate of ATP release was comparable to that of P_i release. However, the rate was very ionic strength dependent so that upon extrapolation to physiological ionic strength the rate would be an order of magnitude less than that of P_i release, which is about 60 s^{-1} at 20 °C, so that 6 s^{-1} would be the best estimate

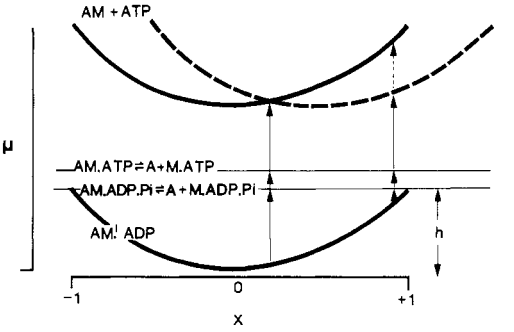

FIGURE A1: Dependence of the chemical potential (μ) of cross-bridge states on displacement relative to an actin binding site. This diagram follows the notation and restrictions of Hibberd and Trentham (1986) on the basic Eisenberg and Hill (1978) formulation of the cross-bridge cycle. The dashed line represents the AM′·ADP curve displaced by 0.5 unit along the x axis.

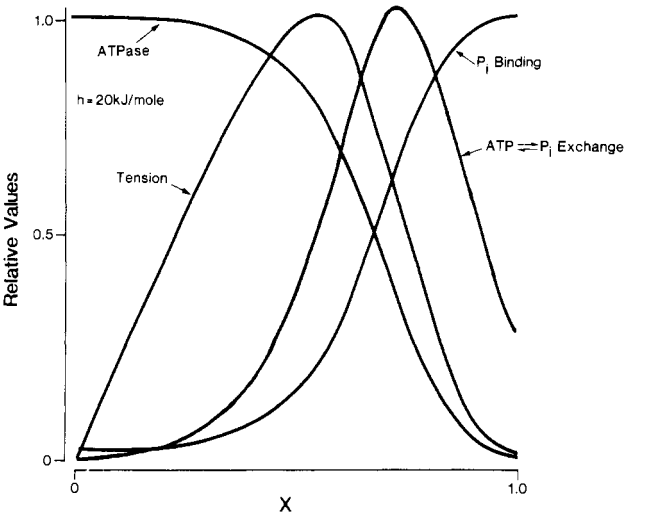

FIGURE A2: Dependence of ATPase, tension, P_i binding, and ATP $\rightleftharpoons$ P_i exchange on x. The ATP $\rightleftharpoons$ P_i exchange curve is calculated with x = 0.5 unit displacement for the ATP release step. All curves were calculated with h = 20 kJ/mol at 10 mM P_i.

of the rate of ATP release, a value quite similar to that deduced from ATP $\rightleftharpoons$ P_i exchange. ATP $\rightleftharpoons$ HOH exchange also measures the rate of ATP release from E·ATP as opposed to E·ATP. Bowater et al. (1988) applied this method to fibers at room temperature and found a rate of about 2 s^{-1}, a value which is again similar to that inferred from ATP $\rightleftharpoons$ P_i exchange experiments. If E·ATP had to reverse to form AM with the same actin monomer, thus producing the same force and assuming the same high-energy orientation as before, the rate would be much slower than the average rate for E·ATP. The similarity of observed rates in the two experiments suggests that E·ATP states must be able to reverse to form AM states in which the actin monomer allows a less strained orientation than that to which the head was bound in the starting AM′·ADP state.

In summary, the apparent dissociation constant of P_i at the active site of fibers is about 3 mM, and this is comparable to the P_i concentrations which have an effect on the mechanical properties of fibers. This dissociation constant is much lower than for actomyosin subfragment 1 in solution, but this difference is a thermodynamically necessary consequence of AM′·ADP being a force-generating state. The 500 times increase in the ATP $\rightleftharpoons$ P_i exchange rate of fibers relative to acto-S1 at 8 °C implies that at any fixed position of a myosin filament relative to the surrounding actin filaments a myosin head can bind to more than one actin subunit.

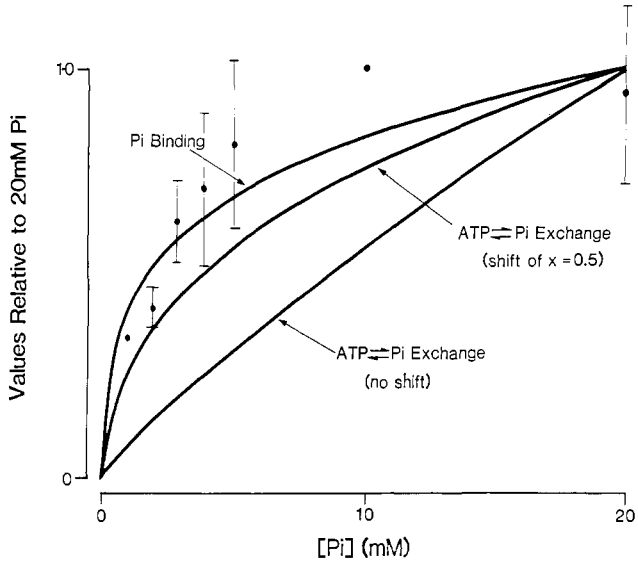

FIGURE A3: Dependence upon $[P_i]$ of P_i binding and ATP ⇌ P_i exchange if x remains the same throughout the reaction and ATP ⇌ P_i exchange if there is a half unit shift in x in the ATP release step relative to the P_i binding step. In each case, the y-axis values are relative to those at 20 mM. The normalized experimental results are superimposed on the theoretical plots.

ACKNOWLEDGMENTS

We thank Professor R. M. Simmons and Dr. D. R. Trentham for help during this project.

APPENDIX

As already discussed, the 500× enhancement of ATP ⇌ P_i exchange in fibers could superficially not be due to the strain dependence of equilibrium constants but be entirely due to a correspondingly high ratio of $[AM'\cdot ADP]/[AM_0]$ in fibers relative to acto-S1. However, in this case, AM′·ADP would have to be a weakly bound state, and the transition to the force-generating AM·ADP state would have to be irreversible. The latter constraint would necessitate explaining the effect of P_i on the mechanical properties of muscle in terms of it binding to a second site. It seems very much more likely that ATP ⇌ P_i exchange in fibers is affected by strain dependence, and the simplest model of this type which will exemplify the effect will be developed.

Acto-S1 ATP ⇌ P_i Exchange. The rate of ATP ⇌ P_i exchange of EDC-cross-linked acto-S1 at $I = 0.2$ M, 8 °C and at 2 mM P_i is 4×10^{-5} s^{-1}. As discussed above, the rate of ATP release from AM·ATP is 6 s^{-1} at 23 °C, and the ratio of the rate of ATP release to the steady-state ATPase rate is about 6:15. In a preliminary experiment (Sleep and Hutton, unpublished observations), this ratio was found to be less at 5 °C, and thus the best estimate of the rate of ATP release at 8 °C is 2.5 s^{-1} × (6/15) × (1/2) = 0.5 s^{-1} (2.5 s^{-1} is the ATPase rate at 8 °C and I = 0.2 M). Thus, $[\underline{AM\cdot ATP}]/[AM_0]$ must be $4 \times 10^{-5}/0.5 = 8 \times 10^{-5}$. If all the S1 were in the form AM′·ADP during ATP turnover, then this rate would be accounted for by a P_i dissociation constant between AM·ADP·P_i and AM′·ADP of 25 M ($K = [AM'\cdot ADP][P_i]/[AM\cdot ADP\cdot P_i]$, that is, $2 \times 10^{-3}/8 \times 10^{-5}$, using $[AM\cdot ADP\cdot P_i] \approx [AM\cdot ATP]$). As already discussed, less than one-tenth of S1 is in this form, and for the present purposes, we will assume this fraction is $^1/_{50}$, in which case the P_i dissociation constant would be 0.5 M and the free energy change in this step at a standard $[P_i]$ of 0.1 mM would be 20 kJ. As already discussed, the P_i dissociation constant between AM·ADP·P_i and AM·ADP is about 300 M, corresponding to a free energy change of 36 kJ/mol in this span of the reaction at the standard P_i concentration. The free energy change between AM′·ADP and AM·ADP is thus (36 − 20) 16 kJ in this model.

At low ionic strength, the second-order rate constant of ATP binding to acto-S1 is 4×10^6 M^{-1} s^{-1} (White & Taylor, 1976), and the rate of ATP release is 6 s^{-1} (see Discussion), and thus at 10 mM MgATP, the free energy change on ATP binding is $RT \ln [(0.01)(4 \times 10^6)/6] = 21$ kJ/mol. The equilibrium constant between AM·ATP and AM·ADP·P_i is about 1, and so there is no free energy change. The physiological ADP concentration of 0.2 mM is approximately equal to the ADP dissociation constant, and thus again there is no free energy change. The total free energy change deduced in this manner, 21 + 20 + 16 = 57 kJ/mol, corresponds reasonably to that of ATP hydrolysis under these conditions (Rosing & Slater, 1972).

$$\text{AM} + \text{ATP} \underset{21\text{ kJ}}{\overset{1}{\rightleftharpoons}} \text{AM}\cdot\text{ATP} \underset{0\text{ kJ}}{\overset{2}{\rightleftharpoons}} \text{AM}\cdot\text{ADP}\cdot P_i \underset{20\text{ kJ}}{\overset{3}{\rightleftharpoons}} \text{AM}'\cdot\text{ADP} \underset{16\text{ kJ}}{\overset{4}{\rightleftharpoons}} \text{AM}\cdot\text{ADP} \underset{0\text{ kJ}}{\overset{5}{\rightleftharpoons}} \text{AM} \quad \text{(A1)}$$

ATP ⇌ P_i Exchange in Fibers. The principle of introducing strain dependence of the transition rates between intermediates was introduced by Huxley (1957) and developed by Huxley and Simmons (1971). Strain is characterized by x, the position of the origin of the myosin molecule relative to the actin subunit with which it is interacting.

However, the aim of this appendix is to demonstrate that our results can adequately be explained in terms of the current view of the cross-bridge cycle, and for this reason, we will develop the Eisenberg and Hill (1978) formulation of the model as simplified by Hibberd and Trentham (1986). There are three states in the model: the first, AM′·ADP, binds actin strongly and generates force; the second includes all the states, such as E·ATP and E·ADP·P_i, which bind weakly to actin; the third is the rigor state AM + ATP. The first step in ATP ⇌ P_i exchange is the transition from AM′·ADP, the lower parabola, to $\underline{E\cdot ADP\cdot P_i}$, the lower horizontal line (Figure A1). The second step is the transition between the two weak states ($\underline{E\cdot ADP\cdot P_i}$ to $\underline{E\cdot ATP}$, the upper horizontal line). The final step is the transition to one of the upper parabolas, representing AM + ATP for different actin monomers. The transition from AM′·ADP to the weak states only occurs for AM′·ADP states that are strained; that is, at large values of x and for these myosin heads, the free energy change from the weak states to AM + $\underline{ATP}$ (using the same actin; the solid line) becomes correspondingly large and unfavorable. A value of x which favors the initial step of P_i binding has exactly the opposite effect on the subsequent step of ATP release if the latter step involves the same actin. However, if the myosin head could bind to another actin such that the resultant AM state was less strained, ATP release would be much faster. As the actin repeat is about 5 nm and the envisaged stroke length (which would approximately correspond to $x = 1$) about 10 nm, a shift in x of 0.5 could readily be envisaged, and this could influence the rate by 3 orders of magnitude. In models of this type, ATP ⇌ P_i exchange is catalyzed by the few cross bridges at favored values of x, and predictions become extremely sensitive to the model parameters.

Hibberd and Trentham assumed that cross bridges were uniformly distributed along x at a concentration AM_0 and that the rate-limiting step was k_4 and thus

$$[\underline{AM\cdot ADP\cdot P_i}] = [AM_0]k_{-3}/(k_3 + k_{-3})$$

The chemical potential, μ, of the state AM′·ADP has a par-

abolic dependence on x (Figure A1) (Huxley & Simmons, 1971). The values of μ are equal for AM·ADP·P_i and AM′·ADP at $x = 1$ at the standard P_i concentration, 0.1 mM. Q is the ratio of the P_i concentration to the standard P_i concentration. h is the chemical potential difference between AM·ADP·P_i and AM′·ADP at $x = 0$ at the standard P_i concentration.

$$k_{-3} = k_3 Q \exp[h(x^2 - 1)/RT] = k_3 Z$$

where $Z = Q \exp[h(x^2 - 1)/RT]$.

$$[\underline{\text{AM·ADP·P}_i}] = [\text{AM}_0]Z/(1 + Z)$$

The dependence of P_i binding on $[P_i]$ is given by integrating this function over a range of x. For purposes of direct comparison, we will use the same range of x (0–1) as Hibberd and Trentham. A plot of this function is given in Figure A2 for $h = 20$ kJ/mol, a value which accounts reasonably for the dependence of tension on $[P_i]$. It might be noted that considering the maximum efficiency of muscle contraction, this is a low value compared to the total energy of ATP hydrolysis. It is of interest to compare which cross bridges give the ATPase activity, which give the tension, and which bind P_i. The ATPase is simply $k_4[\text{AM′·ADP}_x]$, and the tension is Kx-$[\text{AM′·ADP}_x]$ where K is the force constant describing the parabola of the state AM′·ADP: these functions are included in Figure A2, all for $h = 20$ kJ/mol.

The next step toward ATP ⇌ P_i exchange, the reversal to <u>AM·ATP</u>, is simple because it occurs between weakly bound states ($[\underline{\text{AM·ATP}}] = [\underline{\text{AM·ADP·P}_i}]/K_2$). A value of 3 rather than 1 will be used for K_2 because of the higher ionic strength (0.2 M) of the fiber experiments compared to the acto-S1 experiments (0.03 M). The most speculative part of the model is the ATP release step. The simplest assumptions are that the energy curve for AM + ATP has the same form, a minimum value at the same x as AM′·ADP and an energy minimum 21 kJ/mol above AM·ATP as suggested from the acto-S1 experiments. Further, we assume that when ATP is released the AM state is formed with the same actin as that which formed the AM′·ADP state; that is, it goes to the solid upper parabola.

$$\text{ATP} \rightleftharpoons P_i = k_{-1}[\underline{\text{AM·ATP}}] = (k_{+1}/K_1)[\underline{\text{AM·ADP·P}_i}]/3$$

$$K_1 = \exp[(21 + hx^2)/RT]$$

$$\text{ATP} \rightleftharpoons P_i = k_{+1} \exp[(-21 - hx^2)/RT][Z/(1 + Z)](1/3)$$

As x increases, P_i binding becomes progressively easier, but this does not result in an increased rate of ATP ⇌ P_i exchange because the effect is exactly compensated by the reduced rate of ATP release (assuming the x dependence of the free energy difference of step 1 is accounted for by the x dependence of ATP release rather than binding). At sufficiently large x, $[\text{AM·ADP·P}_i]/[\text{AM}_0]$ plateaus at 1, and the rate of ATP ⇌ P_i exchange falls due to the reduced rate of ATP release at large x. Overall, the rate is very slow, similar to that occurring with acto-S1 in solution. However, once P_i binds to a myosin head in the form AM′·ADP, it will dissociate and reassociate many times before either P_i or ATP is released and thus will have the opportunity to bind to a more favorable actin. The actin repeat is about 5 nm, and $x = 1$ corresponds to about 10 nm, and thus it is appropriate to shift x by 0.5 for a trial calculation. This is represented by the dashed upper curve in Figure A1.

rate of ATP ⇌ P_i =

$$k_{+1} \exp\{[-21 - h(x - 0.5)^2]/RT\}[Z/(1 + Z)](1/3) \quad \text{(A2)}$$

A plot of the function is included in Figure A2. For a cross bridge at $x = 1$ (a favored position for P_i binding), the step of ATP release is 5000 times slower than for acto-S1 ($x = 0$), but if <u>M·ATP</u> could bind to the adjacent actin at $x = 0.5$, the rate of ATP release would only be 8 times slower than for acto-S1. This extreme sensitivity of the model to parameters which are essentially unknown makes very clear the impossibility of producing a model which might in any way be thought to be a unique solution. However, the results do suggest that ATP ⇌ P_i exchange is much more likely to occur by reversal to an <u>M·ATP</u> with an actin at a different x from that to which it was bound when in the form AM′·ADP.

Figure A3 shows the dependence on $[P_i]$ of P_i binding, the ATP ⇌ P_i exchange rate if <u>E·ATP</u> forms AM with the same actin, and the ATP ⇌ P_i exchange rate if <u>E·ATP</u> forms AM with another actin shifted by 0.5 unit along the x axis (all for $h = 20$ kJ/mol). It can be seen that as expected, if x remains constant the rate of fiber ATP ⇌ P_i exchange is essentially linearly dependent on $[P_i]$ in the accessible range in a similar manner to acto-S1. The only difference is introduced by the difference in concentrations of AM′·ADP in the two situations. Shifting x by 0.5 unit makes the P_i dependence of ATP ⇌ P_i exchange much more similar to that of P_i binding and also more in agreement with the experimental data. This model with $h = 20$ kJ mol^{-1} accounts reasonably well for the loss of tension and ATPase activity at increasing concentrations of P_i.

Rate of ATP Release in Fibers Relative to Acto-S1. All myosins in the M·ATP state contribute to fiber ATP ⇌ HOH exchange, and thus if we again assume that cross bridges are uniformly distributed along x, it is appropriate to take the average rate of ATP release over the range $x = -1$ to $+1$. For $h = 20$ kJ/mol (the value used to define the characteristics of all the energy wells), the rate of ATP release averaged in this way is 3.3 times slower than the rate at $x = 0$, that is, the acto-S1 rate. The data are consistent with this model because the acto-S1 rate is 6 s^{-1} (low ionic strength, 22 °C) and the fiber rate is about 2 s^{-1} (higher ionic strength), so there is at least agreement on the moderate size of the effect.

Registry No. P_i, 14265-44-2; ATPase, 9000-83-3.

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Phospholipid and Guanine Nucleotide Sensitive Properties of the Smooth Muscle Adenylate Cyclase Catalytic Unit†

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ABSTRACT: The adenylate cyclase catalytic unit was partially purified from uterine smooth muscle by chromatography on columns of SM-2 Bio-Beads and Sepharose 6B. Stimulation of catalysis by forskolin was much greater in the presence of Mn^{2+} than in the presence of Mg^{2+}. Neither NaF nor guanine nucleotide stimulated catalysis in the presence of Mg^{2+} or Mn^{2+}. These properties indicated the catalytic unit was not sensitive to regulation by the G_S regulatory protein. Guanine nucleotide inhibited catalysis, however, and was a competitive inhibitor of the ATP substrate ($K_i \sim 50$ μM). Since inhibition affected K_m but not V_{max}, the catalytic unit also seemed insensitive to regulation by the G_i regulatory protein, which does not act like a competitive inhibitor in other enzyme systems. The catalytic unit was also phospholipid sensitive. Only phosphatidic acid (Pho-A) had a direct effect on catalysis and was a potent inhibitor. Its effects were antagonized by the concomitant addition of phosphatidylcholine (Pho-C) but not by phosphatidylethanolamine, phosphatidylserine, or phosphatidylinositol. Acyl chain composition had a marked effect on Pho-C binding when this was determined by antagonism of Pho-A-dependent inhibition. These properties suggest the catalytic unit has both polar head group and acyl chain requirements for phospholipid binding.

The components of adenylate cyclase have been purified to homogeneity, and the enzyme reconstituted from the purified subunits synthesizes cAMP in an agonist-dependent manner (Lefkowitz et al., 1985; May et al., 1985). The subunits are frequently purified from different enzyme sources, so the absence of species- or tissue-specific differences in functional reconstitution is testimony to the accuracy of the fundamental control mechanisms that have been proposed. Despite the functional homology of subunits obtained from different sources, however, there may be subtle differences in their properties that contribute to tissue-specific differences in the regulation of cAMP production.

cAMP synthesis mediates the relaxing effects that β-adrenergic catecholamines have on smooth muscle from the uterus and other organs (Krall et al., 1983). As in other target tissues, β-adrenergic receptor-dependent cAMP production is regulated by guanine nucleotide binding by the G_S regulatory protein in rat uterine smooth muscle (Krall et al., 1985). Characterization of the smooth muscle cell catalytic unit has been difficult, however, because it is not easily obtained in a state that is free of the functional influence of G_S (Frolich et al., 1983).

We previously characterized some guanine nucleotide sensitive properties of adenylate cyclase solubilized from rat uterine smooth muscle with Lubrol PX (Frolich et al., 1983). We now show that chromatographic removal of the detergent from unactivated smooth muscle adenylate cyclase produces

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