Oxygen Exchange between P, in the Medium and Water during ATP Hydrolysis Mediated by Skinned Fibers from Rabbit Skeletal Muscle

EVIDENCE FOR P, BINDING TO A FORCE-GENERATING STATE*

(Received for publication, April 29, 1986)

Martin R. Webb‡, Mark G. Hibberd§, Yale E. Goldman§, and David R. Trentham‡

From the ²Division of Physical Biochemistry, National Institute for Medical Research, Mill Hill, London, NW7 1AA, United Kingdom and the §Department of Physiology, School of Medicine 64, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Oxygen exchange between (18O)P, in the medium and water accompanies ATP hydrolysis catalyzed by the calcium-regulated MgATPase of vertebrate skeletal muscle. Exchange was observed in chemically skinned fibers from rabbit psoas muscle held isometrically and activated by 30 μM free Ca2+. The rate of exchange was approximately proportional to P, concentration (up to 10 mM) and was characterized by an apparent second order rate constant >475 m⁻¹ s⁻¹ (pH 7.1, ionic strength 0.2 M, 22 °C). Much less exchange occurred in the absence of Ca2+ or when ATP was replaced by ADP. It has been inferred from mechanical experiments that P, can bind to a force-generating ADP-bound state of actomyosin with resultant suppression of force (Hibberd, M. G., Dantzig, J. A., Trentham, D. R., and Goldman, Y. E. (1985) Science 228, 1317-1319). The oxygen exchange results support this inference by providing direct evidence that P, in the medium binds at the ATPase catalytic site in activated isometric fibers. The inter-relationship of these two effects involving P, on mechanochemical coupling in muscle is discussed.

Oxygen isotope exchange measurements are an established method to probe elementary steps of the myosin and actomyosin ATPase mechanisms (Webb and Trentham, 1983, and references therein). In the present experiments we studied the MgATPase mechanism of Ca2+-activated skinned fibers prepared from vertebrate skeletal muscle through an investigation of oxygen exchange between P, in the medium and water during ATP hydrolysis.

A mechanism for ATP hydrolysis in a muscle fiber during contraction sufficient for our purpose is described in Scheme 1, where A and M represent actin and myosin. This scheme is based on solution studies of actomyosin (Taylor, 1979; Eisenberg and Greene, 1980; Sleep and Hutton, 1980; Hibberd and Trentham, 1986). K1 (k+ / k−), k+, and k−, are equilibrium and forward and reverse rate constants, respectively, of the ith step. ATP binding (Step 1) is followed by rapid dissociation of M·ATP from actin (Step 2). ATP hydrolysis and actin binding (Steps 2, 3, 3a, and 4) are readily reversible. Inclusion of two bound ADP states was proposed by Sleep and Hutton (1980), who inferred that P, can bind to AM·ADP during ATP hydrolysis by actomyosin, and that this state appears to be different from the equilibrium state, AM·ADP, formed when ADP is added to actomyosin. It is not clear whether AM·ADP is on the ATPase pathway (Sleep and Hutton, 1980). It is probable that the rate-determining step for the ATPase in Ca2+-activated muscle fibers held isometrically is associated with Steps 5 and/or 6 (Perenczi et al., 1984; Goldman et al., 1984b).

“Intermediate” exchange, arising from oxygen exchange between P, formed during ATP hydrolysis and the solvent water, has been demonstrated with Ca2+-activated skinned fibers (Hibberd et al., 1985b). This is evidence that ATP hydrolysis Steps 3 and/or 5a are readily reversible during fiber ATPase activity as in isolated actomyosin (Rosenfeld and Taylor, 1984; Biosca et al., 1985). Relaxed fibers show much more extensive intermediate exchange, comparable to that of isolated myosin.

Whether the binding of P, (Step 5) is reversible may be tested by studying oxygen exchange between P, in the medium and water during ATPase activity. If oxygen exchange occurs, it implies protein-bound ATP formation through reversal of either Step 3a or Steps 4 and 3. Scheme 2 shows how this could occur via reversal of Steps 5, 4, and 3, with filled circles representing 32O. Exchange depends on the water molecule formed during the condensation of ADP and P, in Step 3 being able to escape from the ATPase catalytic site. Multiple reversals of Step 3, together with rotation of P, in the catalytic site, allow more than one of the labeled phosphate oxygens to undergo exchange.

The reaction conditions used here resulted in low levels of oxygen exchange so that we may neglect multiple oxygen exchange arising due to an exchanged P, molecule in the solvent rebinding to AM·ADP. This is indicated in Scheme 2 by irreversible release of exchanged P,.

The presence of P, in the medium accelerates the rate of ATP-induced activation and relaxation of vertebrate muscle fibers from rigor in the presence and absence of Ca2+, respectively (Hibberd et al., 1985a). P, also attenuates the tension developed by an activated isometric muscle (Hibberd and Trentham, 1986, and references therein). These observations can be explained if P, release during the ATP hydrolysis cycle is reversible and coupled to formation of a dominant force-generating cross-bridge state (Hibberd et al., 1985a). The oxygen exchange experiments described here enable the extent and a lower limit on the rate constant of P, binding to...
Medium \( P_i \) Water Oxygen Exchange within Muscle Fibers

**Scheme 1**

\[
AM + ATP \rightleftharpoons AM.ADP + H_2O \rightleftharpoons AM.ADP + P_i
\]

Further Exchange

**Scheme 2**

\[
M.ATP + H_2O \rightleftharpoons M.ADP + \text{ATPase}
\]

Experimental Procedures

Skinned fibers from rabbit psoas muscle were prepared as described by Goldman et al. (1984a). Fibers were mounted isometrically in small troughs containing 30-45 \( \mu \)l of incubation solution covered with silicone oil to prevent evaporation (Hibberd et al., 1985b). Fibers were relaxed in a solution of 15 mM ATP, 15 mM \( \text{MgCl}_2 \), 10 mM EGTA,\(^1\) 10 mM reduced glutathione, 100 mM TES, and 1-10 mM \( (^{18} \text{O})_2 \text{Pi} \) (98\% enriched, prepared by the method of Hackney et al., 1980). The activating solution was the same except that the EGTA was replaced by \( \text{CaEGTA} \) resulting in approximately 30 \( \mu \text{M} \) free \( \text{Ca}^{2+} \). Rigor solution, + or − \( \text{Ca}^{2+} \), was the same as the activating or relaxing solution, respectively, except that ATP was not present, \( \text{MgCl}_2 \) was reduced to 2 mM, and ADP, when present, was added to 0.5 mM. Oligomycin and quercetin, when present, were at 1 \( \mu \text{g/m}l \) and 0.25 mM, respectively. Each solution had an ionic strength of 200 mM, 1 mM free \( \text{Mg}^{2+} \), and was adjusted to pH 7.1 at 20-22 °C with KOH.

After fiber mounting, a short active contraction was elicited in a solution not containing \( P_i \), fiber dimensions were measured (Goldman and Simmons, 1984), and then the fiber was transferred to a stirred incubation solution containing \( (^{18} \text{O})_2 \text{Pi} \) for 1-20 h. In experiments with ATP present, the fiber was removed from the medium before 20% ATP was hydrolyzed. For each incubation a control solution was maintained on the experimental apparatus in the absence of fibers for an equal time to provide a measure of spontaneous ATP hydrolysis and oxygen exchange. The extent of fiber-catalyzed ATP hydrolysis was determined by high performance liquid chromatography as described previously (Hibberd et al., 1985b). ATPase activities were calculated assuming that the myosin head concentration was 154 \( \mu \text{mol/liter of fiber volume} \) (Ferenczi et al., 1984). In some activating incubations the fibers broke after 5-30 min of contraction. Another fiber was then incubated in the same solution to catalyze an accumulated 3-15% ATP hydrolysis. The sum of \( \left( \text{fiber volume} \times \text{incubation time} \right) \) for each of the fibers in an experiment was used for calculation of \( k_{cat} \) of the ATPase.

For each incubation the number of solvent oxygens exchanged into the \( \text{Pi} \) was measured using a mass spectrometer (Hibberd et al., 1985b). The steps in this determination are outlined for one experiment in Table 1. \( \text{Pi} \) was converted to triethyl phosphate for the analysis and the intensities of mass spectral peaks were integrated in the region \( m/e \) 153-163. For the unlabeled molecule, this range contains a fragment of major intensity at \( m/e \) 155, the molecular ion minus \( C_2H_4 \), and a minor fragment at \( m/e \) 153, the molecular ion minus \( C_2H_2 \), with intensity approximately 6% of the major fragment. For the molecules containing \( ^{18} \text{O} \) there are equivalent isotopically enriched fragments in identical ratios. The exact ratio of the major to minor fragments was determined by obtaining the ratio of intensities at \( m/e \) 153:155 for unlabeled triethyl phosphate. This ratio then applies to each isotopically enriched species. Three mass spectral

---

\(^1\)The abbreviations used are: EGTA, \( \text{[ethylenebis(oxyethyl-}
\text{enitri1o)Jtetraacetic acid}; TES, \( N\)-\( \text{tris[hydroxymethyl]methyl-}
\text{2-aminoethanenesulfonic acid.} \)
Medium Pi-water oxygen exchange within muscle fibers

**Table I**

<table>
<thead>
<tr>
<th>Relative composition of fragments at m/e</th>
<th>153</th>
<th>155</th>
<th>157</th>
<th>159</th>
<th>161</th>
<th>163</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Injection 1</td>
<td>0.9</td>
<td>17.3</td>
<td>0.9</td>
<td>1.3</td>
<td>10.6</td>
<td>69.0</td>
</tr>
<tr>
<td>B. Average</td>
<td>0.9</td>
<td>17.2</td>
<td>0.9</td>
<td>1.2</td>
<td>10.6</td>
<td>69.2</td>
</tr>
<tr>
<td>C. Correction for fragment at 153</td>
<td>0</td>
<td>18.0</td>
<td>0.9</td>
<td>0.9</td>
<td>7.5</td>
<td>72.7</td>
</tr>
<tr>
<td>D. Correction for 98.32% Pi enrichment</td>
<td>18.0</td>
<td>0.9</td>
<td>0.7</td>
<td>2.6</td>
<td>77.8</td>
<td></td>
</tr>
<tr>
<td>E. Subtraction of unlabeled phosphate</td>
<td>0</td>
<td>0.7</td>
<td>0.7</td>
<td>2.6</td>
<td>77.8</td>
<td></td>
</tr>
<tr>
<td>F. Normalized percentage</td>
<td>0</td>
<td>0.9</td>
<td>0.8</td>
<td>3.2</td>
<td>95.1</td>
<td></td>
</tr>
</tbody>
</table>

*All the intensity at m/e 155 is subtracted together with the contribution at m/e 157 due to natural abundance of isotopes (see "Experimental Procedures").

The data are for the first experiment listed in Table II.

**RESULTS**

ATP was hydrolyzed by fibers in solutions containing 10 mM $^{32}$O-Pi, and the resulting Pi was analyzed for amount and distribution of $^{18}$O using a mass spectrometer. The results, after correction for isotopic enrichment and removal of contributions due to unlabeled P, as outlined under "Experimental Procedures," are given in Table II. The data show the presence of P containing 3, 2, and 1 solvent oxygens per molecule, whereas the control solutions, incubated in the absence of fibers, show little exchange. The fibers have caused the $^{18}$O-Pi, to undergo oxygen exchange with water.

Several general features of this phenomenon are apparent. First, for all conditions studied, less than 10% of the P has undergone exchange with loss of 1, 2, or 3 oxygen atoms. Because no exchange information is obtained from the unlabeled P, the total amount of exchange may be higher than that shown in Table II. The average rate of exchange (k') in Table II is 144 (±11 S.E.) m⁻¹ s⁻¹, and the average kcat for the ATPase is 3.5 (±0.1 S.E.) s⁻¹.

If the distributions in Table II are averaged after normalization to 100%, their ratio is 63.9 (±2.6 S.E.):21.7 (±2.0):14.4 (±1.2) for 1:2:3 solvent oxygens incorporated into P. We return to the implications of this distribution under "Discussion." The data in the table arises chiefly from two factors. First, the corrections to the data, as outlined in Table I, are relative to the extents of exchange (compare lines B and D in Table I), so that the distributions of exchanged oxygens are not precise. Second, there is variability in performance of different fibers that may be due in part to damage during preparation or storage.

The results of control experiments (Table III, lines A and B) show that there is little exchange in incubations in which ADP replaced ATP or nucleotide was absent.

Several further tests are shown in Table III to determine whether any of the observed exchange was due to other ATPases, in particular the Ca⁺⁺-pump ATPase of sarcoplasmic reticulum and the mitochondrial ATPase, both of which are known to catalyze medium Pi-water oxygen exchange in the presence of ADP (Hill and Boyer, 1967; Mc-

**Fig. 1** Effect of 10 mM Pi on steady-state tension. After two washes in activating solutions (Pre), the fiber was transferred to an activating solution for a brief wash (W), followed by incubation in an activated solution (S) while held isometrically. The fiber was then transferred via a wash solution between activating solutions with or without 10 mM Pi.
Medium P<sub>i</sub>-Water Oxygen Exchange within Muscle Fibers

**Table II**

<table>
<thead>
<tr>
<th>Number of solvent oxygen exchanged</th>
<th>Exchange in control</th>
<th>Myosin heads x time</th>
<th>Exchange rate constant, k&lt;sup&gt;†&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.9</td>
<td>0.8</td>
<td>3.2</td>
</tr>
<tr>
<td>3.3</td>
<td>1.0</td>
<td>1.0</td>
<td>3.2</td>
</tr>
<tr>
<td>3.5</td>
<td>0.9</td>
<td>3.1</td>
<td>1.2</td>
</tr>
<tr>
<td>3.4</td>
<td>0.6</td>
<td>1.2</td>
<td>0.1</td>
</tr>
<tr>
<td>3.5</td>
<td>0.5</td>
<td>1.5</td>
<td>0.1</td>
</tr>
<tr>
<td>3.2</td>
<td>0.4</td>
<td>1.3</td>
<td>0.2</td>
</tr>
<tr>
<td>3.6</td>
<td>0.4</td>
<td>2.7</td>
<td>0.1</td>
</tr>
</tbody>
</table>

The number of heads in the fibers was calculated from measurement of the fiber volume and assuming the head concentration within the fiber is 154 μM (Ferruccio et al., 1984).

The exchange rate constant, k<sup>†</sup>, defined as (P<sup>-1</sup>)M<sup>-1</sup>d[P<sup>-1</sup>]/dt, is the fraction of labeled P<sub>i</sub> undergoing exchange per second, divided by [M<sub>i</sub>]. P<sub>i</sub> indicates P<sub>i</sub> with solvent oxygens. This is calculated as follows, taking the first row as an example. The percent exchange is taken as the sum of P<sub>i</sub> with 3, 2, and 1 solvent oxygens (0.9 + 0.8 + 3.2), corrected for the percent exchange in the control (0.3). This ignores the small amount of exchanged P<sub>i</sub> with 4 solvent oxygens as explained in the text. The exchange due to the fiber, as a fraction of total P<sub>i</sub>, is 0.046. The total myosin head concentration x time is equal to myosin heads x time divided by the incubation solution volume (35 μl for this experiment). k<sup>†</sup> is 0.046 x (35 x 10<sup>-4</sup>) + (14.9 x 10<sup>-4</sup>) M<sup>-1</sup>s<sup>-1</sup>. Because the amount of P<sub>i</sub> present due to ATP hydrolysis is average less than 10% of the labeled P<sub>i</sub>, no correction is made for inhibition of exchange by this P<sub>i</sub>.

**Table III**

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>k&lt;sub&gt;ex&lt;/sub&gt; of ATPase</th>
<th>% exchange</th>
<th>Myosin heads x time</th>
<th>Exchange rate constant, k&lt;sup&gt;†&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Rigor (+Ca&lt;sup&gt;2+&lt;/sup&gt;, 0.5 mM ADP)</td>
<td>0.4</td>
<td>0.2</td>
<td>21.7</td>
<td>0</td>
</tr>
<tr>
<td>B. Rigor (+Ca&lt;sup&gt;2+&lt;/sup&gt;, zero ADP)</td>
<td>0.2</td>
<td>0.1</td>
<td>34.6</td>
<td>1</td>
</tr>
<tr>
<td>C. Active (plus oligomycin + quercitin)</td>
<td>3.7</td>
<td>0.1</td>
<td>88</td>
<td>18</td>
</tr>
<tr>
<td>D. Relaxed</td>
<td>2.7</td>
<td>0.2</td>
<td>11.8</td>
<td>1</td>
</tr>
<tr>
<td>E. Rigor (-Ca&lt;sup&gt;2+&lt;/sup&gt;, zero ADP)</td>
<td>0.97</td>
<td>0.3</td>
<td>170.9</td>
<td>9</td>
</tr>
</tbody>
</table>

The concentration of P<sub>i</sub> is 10 mM and of Ca<sup>2+</sup> is 50 μM for "+Ca<sup>2+</sup>" and "-Ca<sup>2+</sup>.", "Other components of the solutions are described under "Experimental Procedures."

Intosh and Boyer, 1983). Oligomycin inhibits the P<sub>i</sub>-water oxygen exchange mediated by the subunitochondrial particle ATPase (Crosa and Boyer, 1975). Quercetin inhibits several partial reactions and the overall ATPase activities of sarcoplasmic reticulum membranes and purified Ca<sup>2+</sup>-pump ATPase. In particular, ATP-P<sub>i</sub> phosphor exchange and phosphorylation of the enzyme by P<sub>i</sub> are inhibited, suggesting that quercetin would inhibit P<sub>i</sub>-water oxygen exchange during Ca<sup>2+</sup>-pump ATPase activity (Shoshan and MacLennan, 1981). Table III, line C shows that these inhibitors have little effect on the rates of oxygen exchange observed in Table II.

In contrast to the relatively fast exchange during ATP hydrolysis in the presence of Ca<sup>2+</sup>, there is only slow exchange in relaxed fibers in the absence of Ca<sup>2+</sup> (cf. Tables II and III, line D).

A further control experiment was the exclusion of nucleotide from the incubation medium also in the absence of Ca<sup>2+</sup>. The actomyosin system is unlikely to catalyze exchange in this case, because the mechanism for exchange presumes the synthesis of protein-bound ATP (Scheme 2 and Webb and Trentham, 1983). However, sarcoplasmic reticulum Ca<sup>2+</sup>-pump ATPase catalyzes Pi-water oxygen exchange by a mechanism involving the formation of an aspartyl phosphate intermediate (Dahms et al., 1973; Degani and Boyer, 1973). In the absence of both ATP and Ca<sup>2+</sup>, the rate of exchange is at least 4-fold faster than in their presence (McIntosh and Boyer, 1983). It follows from the average exchange rate of 65 M<sup>-1</sup>s<sup>-1</sup> observed in this test (Table III, line E) that 16 M<sup>-1</sup>s<sup>-1</sup> is an upper limit for the contribution of the Ca<sup>2+</sup>-pump ATPase toward k<sup>†</sup>(=144 M<sup>-1</sup>s<sup>-1</sup>) measured in Table II. The fact that the test incubation (Table III, line B) with no nucleotide but +Ca<sup>2+</sup> has essentially no exchange is consistent with exchange in the absence of nucleotide and Ca<sup>2+</sup> being due to the Ca<sup>2+</sup>-pump ATPase (McIntosh and Boyer, 1983).

Exchange promoted by the Ca<sup>2+</sup>-pump ATPase in the presence of ATP but absence of Ca<sup>2+</sup> is 15% of that when neither ATP nor Ca<sup>2+</sup> is present (McIntosh and Boyer, 1983). So it is possible that most or all the exchange observed in Table III, line D is due to Ca<sup>2+</sup>-pump ATPase, since its rate equals 15% of the 65 M<sup>-1</sup>s<sup>-1</sup> recorded in Table III, line E. We conclude that the rate of P<sub>i</sub>-water oxygen exchange due to the myofibrillar ATPase of relaxed fibers is very low compared to that observed with Ca<sup>2+</sup>-activated fibers.

Overall, the results from the control experiments listed in Table III strongly suggest that the observed exchange in Table II is almost entirely mediated by the Ca<sup>2+</sup>-regulated ATPase of myofibrillar proteins.

Whether second order binding of P<sub>i</sub> or a subsequent first order transition limits the oxygen exchange rate might be deduced by determining whether the rate is saturated at 10 mM P<sub>i</sub>. Therefore, the rates of exchange in the presence of Ca<sup>2+</sup> were studied at lower concentrations of (180)<sub>4</sub>P<sub>i</sub> (Table IV). The rates show considerable scatter. In addition to variable performance of the individual fibers as described above, with low concentrations of P<sub>i</sub> there are only small amounts of P<sub>i</sub> that have undergone exchange, leading to relatively large errors in the mass spectrometer data. The calculated second order rate constants of exchange increased 2-fold over a 10-fold decrease in P<sub>i</sub> concentration. (The increase would have been 10-fold if 1 mM P<sub>i</sub> was saturating.) Although the data are compatible with a P<sub>i</sub> dissociation constant of about 10
mm, the imprecision of the data precludes any firm conclusion. In the calculations that follow, we make the simplifying assumption that $P_i$ at 10 mM is only weakly associated to the protein. If the protein is half-saturated by 10 mM $P_i$, the analysis would be more complex, although the general conclusions are unaltered.

The data in Tables II and IV show a small (although not statistically significant) decrease in fiber ATPase activity as the concentration of $P_i$ is increased. This inhibition by $P_i$ of ATPase activity in the isometric state was tested more precisely by monitoring ADP formation during incubations in activating solution with or without $P_i$, as described under “Experimental Procedures.” The ATPase rate per myosin head in the presence of 10 mM $P_i$ was 2.9 $(\pm 0.1$ S.E., $n = 10)$ s$^{-1}$. The rate was 91% $(\pm 4$% S.E., $n = 10)$ of that at zero $P_i$. Tension at 10 mM $P_i$ was measured as shown in Fig. 1 and was 78% $(\pm 2$% S.E., $n = 10)$ of that at zero $P_i$. $P_i$ caused a significantly ($p < 0.001$) greater reduction of tension than of steady-state ATPase activity, a result qualitatively similar to that of Kawai and Guth (1986).

**DISCUSSION**

The above results are evidence that $P_i$ in the medium binds at the ATPase active site to an actomyosin-ADP state during fiber-mediated ATP hydrolysis and that reactions back to protein-bound ATP formation, the processes giving rise to oxygen exchange, are readily reversible. The rates of exchange in the presence of ATP compared to ADP show that this actomyosin-ADP state (AM'ADP, Scheme 1) is not accessible via ADP binding to AM, consistent with the results of Sleep and Hutton (1980) for the isolated proteins. Evidence for AM'ADP in skinned fibers has been also obtained by Dantzig and Goldman (1985) from studies of vanadate binding.

Because the exchange depends on formation of protein-bound ATP, the kinetics of processes associated with ATP hydrolysis can be inferred from the distribution of solvent oxygens in the exchanged $P_i$. The distributions in Table II show that $P_i$, once condensed with ADP to form ATP and so losing 1$^{18}$O, has an 0.36 probability of undergoing further exchange. The average distribution of 1:2:3 solvent oxygens in $P_i$ is 63.9:21.7:14.4 and can be compared to the corresponding ratio obtained from intermediate exchange experiments in activated fibers in which oxygen exchange occurs between water and product $P_i$ formed during ATP hydrolysis (Hibberd et al., 1985b). The ratio in those experiments averaged 62.5:24.2:13.3, well within the standard errors of the results reported here. It is probable that these are the same distributions, and so we may apply the information obtained from the intermediate exchange experiments to the experimental data described in this paper.

In the following calculations we assume that equilibration of Steps 2 and 4 is rapid relative to the rate of Steps 3, 6, 7, and 5 in fibers, as found with isolated actomyosin subfragment 1 (Eisenberg and Greene, 1980; Sleep and Hutton, 1978). The distribution of exchanged oxygens is determined by the ratio of the rate constant controlling ATP formation, $(k_{-3}K_i + k_{-5})/(K_i + 1)$, to that controlling $P_i$, release from AM'ADP, $k_{-5}K_i/(K_i + 1)$. The equilibrium constant $K_i$ is taken as dimensionless, in view of the probable concentration independence of actin-myosin interactions in fibers. The ratio $R = k_{-3}K_i/(k_{-3}K_i + k_{-5})$ is calculated from the distribution as described by Webb and Trentham (1981). The data from Hibberd et al. (1985b) are most simply described by two pathways of ATP hydrolysis differing in their value of $R$. The major flux (63%) leading to product $P_i$ has $R = 2.3$. Since the ATPase rates associated with the different fluxes are unknown, we are unable to quantify what fraction of ATPase sites function with $R = 2.3$. We make the simplifying assumptions that all the sites do and, based on the similarity of the oxygen exchange distributions described above, that the value of $R = 2.3$ can be applied to the medium exchange data of this paper.

A lower limit can be measured for the rate constant of $P_i$ binding to AM'ADP, $k_{-3}$, in Scheme 1, from the rate of solvent oxygen incorporation into $P_i$, $(d[P_i]/dt$, where $P_i$ indicates $P_i$ with solvent oxygens). $d[P_i]/dt$ equals the product of the rate of $P_i$ binding (= $k_{-3}$[AM'ADP][P_i]) and the fraction of AM'ADP - $P_i$, that undergoes exchange by reforming $M$-ATP or AM-ATP, as in Scheme 1. This fraction equals $(k_{-3}K_i + k_{-5})/(k_{-3}K_i + k_{-5}K_i + k_{-5})$, which simplifies to $(R + 1)^{-1}$. It follows that

$$d[P_i]/dt = (R + 1)^{-1}k_{-3}[AM'ADP][P_i].$$

$[AM'ADP]$ must be less than or equal to the total concentration in the trough ([$M_{j}$]). Exchange rate constants, $k'$, are calculated as described under Table II where

$$k' = [P_i]^{-1}[M_{j}]^{-1}d[P_i]/dt = (R + 1)^{-1}k_{-3}[AM'ADP][M_{j}]^{-1}.$$  

Taking $k' = 144$ M$^{-1}$ s$^{-1}$ from Table III and $R = 2.3$, $(R + 1)^{-1}k' = 475$ M$^{-1}$ s$^{-1}$, and so

$$k_{-3} = 475[M_{j}]/[AM'ADP]M^{-1}s^{-1}.$$  

Thus $k_{-3} \approx 475$ M$^{-1}$ s$^{-1}$ and, if $[AM'ADP]$ is the major steady-state intermediate, then $k_{-5} \approx 475$ M$^{-1}$ s$^{-1}$.

A comparison can be made between this value of $k_{-3}$ derived from oxygen exchange data with that estimated from the reduction of mechanical force by $P_i$. If in Scheme 1 M-ADP - $P_i$, AM-ADP - $P_i$, and AM'ADP are the dominant intermediates during ATP hydrolysis in an active fiber, then

$$[AM'ADP] = k_2[M_{j}]/(k_2 + k_{-5} + [P_i]),$$

where $k_2 = k_{-3}K_i/(K_i + 1)$ and $k_{-5}$ is the rate constant limiting the turnover of the ATPase mechanism, probably controlling one or a combination of Steps 6, 7, and 8. If we assume that force is proportional to [AM'ADP], then the ratio of the forces $F_2$ and $F_3$ at two $P_i$ concentrations, [$P_i$]$_2$ and [$P_i$]$_3$, is:

$$F_2/F_3 = (k_2 + k_{-5} + [P_i]_2)/(k_2 + k_{-5} + [P_i]_3),$$

which on rearrangement gives

$$k_{-5} = (k_2 + k_3)(1 - F_3/F_2)/(F_2/F_3 - [P_i]_2).$$

To use this equation, values are needed for $k_2$, $k_3$, and [$P_i$]$_2$.

Cooke and Pate (1985) have estimated the average concentration of accumulated $P_i$ inside chemically skinned psoas fibers contracting in $P_i$-free solution to be 200 $\mu$M, which we define as [$P_i$]$_2$, and take as the standard state concentration of $P_i$. $k_2$ can be estimated as follows from intermediate oxygen exchange studies (Hibberd et al., 1985b) and from transient chemical changes in activated fibers. Ferenczi (1986) has measured the transient rate of ATP hydrolysis when MgATP is rapidly released in a fiber held isometrically in the presence of $Ca^{2+}$. The rate constant, $k_1$, for the hydrolysis transient calculated from the data is 60 s$^{-1}$ at 12 $^\circ$C, and is presumably about 120 s$^{-1}$ at 22 $^\circ$C. Also, $k_2 = [(k_2 + k_{-3})K_i + k_{-5} + k_{-3}]/(K_i + 1)$.

The equilibrium constants for the hydrolysis steps ($K_i$ and $K_o$) are in the range 5–10 in isolated proteins when extrapolated from low to physiological ionic strength (Bagshaw and Trentham, 1973; Rosenfeld and Taylor, 1984) and probably
have the same values in fibers. Combining these observations it follows that \((k_{s0}K_x + k_s)/(K_x + 1) = 10 - 20 \text{ s}^{-1} \) at 22 °C. As noted above, \(k_{s0}K_x/k_{s0}(K_x + 1) = R = 2.3\), so that \(k_s = k_{s0}K_x/(K_x + 1) = 25 - 50 \text{ s}^{-1} \) at 22 °C.

Taking \(k_s = 3.9 \text{ s}^{-1}\) (since \(k_sp/k_s + k_{s0}) = k_{s0} = 3.5 \text{ s}^{-1}\), Table II), and noting that in 10 mM P, tension is 75% of that without added P, \(k_{s0} = 1000 - 1800 \text{ M}^{-1} \text{ s}^{-1}\), from Equation 3. This value is approximately 3-fold greater than the value for \(k_s\) estimated from the medium exchange data, assuming AM'.ADP is the dominant intermediate of the ATPase mechanism, although the values agree if \([M_A]/[AM'.ADP] = 3\) (see Equation 1).

An alternate estimate of \(k_s\) comes from the effect of P, on isometric mechanical transients initiated by caged ATP within fibers in the presence of Ca++. In the absence of P, the rate constant of approach to the apparent steady tension level is approximately 100 s⁻¹, but in the presence of 10 mM P, this transient phase is accelerated typically to 150 s⁻¹. This suggests that the apparent rate constant of 10 mM P, rebinding is 50 s⁻¹, which corresponds to a second order rate constant value for \(k_{s0}\) of 5000 M⁻¹ s⁻¹.

The force reached after this rapid phase of tension development was reduced by inclusion of P, considerably more than the 25% reduction of steady tension in an activated isometric fiber. Further study of this effect has shown a slow phase of tension development (\(t_s = 0.5 \text{ s}\) following the initial rapid phase. The presence of this slow phase, representing approximately 25% of the total tension, means that the depression in tension measured from the steady and transient contraction experiments are now in close agreement. The events during this slow phase have not been clarified, so it is presently unclear which of the values for \(k_{s0}\), 1000-1800 M⁻¹ s⁻¹ (appropriate for a tension reduction of 25%) or 5000 M⁻¹ s⁻¹ (from the transient mechanical experiment), is valid.

These two mechanical estimates of \(k_{s0}\) are greater than that from the oxygen exchange data. An explanation of this difference in estimates for \(k_{s0}\) may lie in a hypothesis that presumes that a wide range of forces is exerted by cross-bridges in the AM'.ADP state, and that P, only binds readily to those exerting the greatest force. Thus P, might bind readily to AM'.ADP in only a small fraction of the cross-bentities, but those particular cross-bridges would exert a greater proportion of the force. The apparent second order rate constant for medium oxygen exchange (Equation 1) is derived from the total AM'.ADP concentration rather than the fraction of AM'.ADP that binds P, readily. So this rate constant would be expected to be less than that for P, association to AM'.ADP deduced from the mechanics in a calculation that is independent of AM'.ADP concentration. In addition, this hypothesis would predict that the decrease in steady-state tension of isometric activated fibers in the presence of 10 mM P, is greater than the decrease in steady-state ATPase activity.

If the hypothesis is correct that AM'.ADP-P, and AM'.ADP support different amounts of mechanical stress, it follows from the formalism developed by Hill and Eisenberg (Hill 1974, 1975; Eisenberg and Hill, 1985) that the ratio \(k_{s0}/k_s\) will vary with cross-bridge strain. It is useful to analyze the data obtained within the framework of this formalism. This has been done in a general way by Hibberd and Trentham (1986). We now analyze the specific data obtained here in these terms. The available data are sufficiently sparse so that, of necessity, any model is over-restrictive, and so various constraints are made on the rate constants, for example by making \(k_{s0}\) independent of cross-bridge strain. We show that simple assumptions concerning strain dependence of the AM'.ADP-P, ↔ AM'.ADP + P, equilibrium predict different values for \(k_{s0}\) measured chemically and mechanically, and different degrees of ATPase inhibition and tension attenuation by P, The formalism is expressed by describing the ATPase in terms of three key states of Scheme 2 and the rate constants of their formation and decay as in Equation 4:

\[
\begin{align*}
&M-ADP-P, \quad &AM-ADP-P, &\quad \frac{k_{s0}}{k_{s0}} \quad AM'-ADP + P, &\quad K_s \quad (4)
\end{align*}
\]

In Equation 4 M-ADP-P, and AM-ADP-P, are presumed to be in rapid equilibrium and are represented as \([M-ADP-P, AM-ADP-P,]\). The total concentration of cross-bridges, \([M,]\) = \([M-ADP-P,] + \[AM-ADP-P,]\) + \([AM'-ADP-P,]\). Fig. 2 shows the chemical potential, \(\mu\), of \([M-ADP-P,]\) + AM-ADP-P, and AM'-ADP + P, as a function of \(x\), the normalized longitudinal strain of the cross-bridge. The potential of AM'-ADP + P, is drawn at two P, concentrations, 10 m and 200 \(\mu M\) (defined above as the standard state concentration).

Cross-bridges in the isometric fiber are assumed to be attached with a uniform distribution over values of \(x\) from 0 to 1 as in the model of Huxley (1957). The force exerted by a cross-bridge at a particular value of \(x\) is proportional to the slope of the curve. The curves are parabolas, since the force at any \(x\) is proportional to \(x\), as seems likely from mechanical studies (Ford et al., 1977). The vertical position of the curve for AM'-ADP + P, depends on P, concentration. For simplicity the \([M-ADP-P, AM-ADP-P,]\) state is assumed to exert no force.

Qualitatively, it can be seen from Fig. 2 that attenuation of tension by 10 mM P, occurs because at elevated P, concentrations the equilibrium between those AM'-ADP states generating the most force (i.e. those toward the right of the diagram) and the corresponding \([M-ADP-P, AM-ADP-P,]\) states favors the latter. Thus the AM'-ADP population is biochemically inhomogeneous with a range of P, affinities, and the AM'-ADP states exerting the most positive force are those that have the largest P, association constants.

The rate constants associated with transformation between \([M-ADP-P, AM-ADP-P,]\) and AM'-ADP in isometric fibers depend on \(x\) in a manner described by Hill's formalism (1974, 1975). We define \([AM'-ADP]\), as the concentration of AM'-ADP at a particular \(x\), with \(k_{s0}\), the rate constant for P, binding to AM'-ADP at \(x\) to give AM-ADP-P, The forward rate constant, \(k_{s0}\), of the reaction \([M-ADP-P, AM-ADP-P,]\) to AM'-ADP + P, is taken to be independent of \(x\) in this simple description. The breakdown of AM'-ADP, in
the forward direction, is assumed to be rate-limiting for the whole cycle. At \([P_1]_0\), the standard state concentration of free Pi, the difference in chemical potential between \([M\cdot ADP\cdot P_i; AM\cdot ADP\cdot P_i]\) and AM'.ADP at zero strain (i.e. \(x = 0\)) is \(h\). The potential for AM'.ADP at a particular \(x\) and \([P_1]_0\) equals \(hx^2\). At other \([P_1]_0\), \(Q = [P_1]/[P_1]_0\) and \(\mu = hx^2 + RT \ln Q\). It follows that

\[
k_{-5}[P_1] = k_{-5}'\exp[-(h - hx^2 - RT \ln Q)/RT]
\]

\[= k_{-5}'Q \exp[h(x^2 - 1)/RT].\tag{5}\]

In this analysis \(h\) is considered to be a constant but unknown quantity, and calculations are made for various values of \(h\). Values of \(h\) between 30 and 40 kJ/mol are in the range expected from solution biochemistry (White and Taylor, 1976) but are some 5-fold greater than might be expected from cross-bridge stiffness measurements (Huxley and Simmons, 1971; Hibberd and Trentham, 1986).

Considering first the chemical data, the rate constant for Pi binding, based on oxygen exchange measurements, represents the value obtained from the average flux of Pi exchange, \(k_{-5}[AM'\cdot ADP]/[P_i]\). Recalling that the total cross-bridge distribution along \(x\) is uniform,

\[
(AM'\cdot ADP) = k_{-5}[AM_2]/(k_{-5} + k_{-5}[P_1]).
\tag{6}\]

At each value of \(x\), using Equations 5 and 6, this flux is given by

\[
k_{-5}[AM'\cdot ADP],[P_1] = k_{-5}'[AM_2] \left(\frac{Q \exp[h(x^2 - 1)/RT]}{1 + k_{-5}'Q \exp[h(x^2 - 1)/RT]}\right).
\]

Taking \(k_{-5}' = 50 s^{-1}\) and \(k_{-5} = 3.9 s^{-1}\), an apparent rate constant for the chemical process \((k_{-5}\) of Equation 1) was calculated from the ratio of \(k_{-5}[AM'\cdot ADP]\), integrated over \(0 < x < 1\) divided by \([AM'\cdot ADP]\), also integrated over \(0 < x < 1\) by using Equations 5 and 6. \(k_{-5}\) is plotted against \(h\) in Fig. 3. \(k_{-5}\) decreases from 1150 to 800 M\(^{-1}\) s\(^{-1}\) over the range \(h = 30-40\) kJ/mol.

The elasticity of active cross-bridges is nearly linear (Ford et al., 1977), so the force generated per cross-bridge at a particular \(x\) is defined as \(K_x\), where \(K\) is the stiffness. Taking \(\alpha\) as a constant relating the number of cross-bridges to their concentration, the total force, \(F'\), produced in the range \(x\), to \(x_2\) is

\[
F' = \int_{x_1}^{x_2} \alpha[AM'\cdot ADP]\cdot K\cdot dx.\tag{7}\]

Combination of Equations 5, 6, and 7 gives

\[
F' = \int_{x_1}^{x_2} \alpha K[AM_2]\cdot dx/[1 + k_{-5}'Q + Q \exp[h(x^2 - 1)/RT]].
\]

From this it follows that

\[
F' = \frac{1}{2} \alpha K[AM_2] \left[1 - \frac{RT}{h} \ln \left(\frac{1 + k_{-5}'Q + Q \exp[-h/RT]}{1 + k_{-5}'Q + Q \exp[-h/RT]}\right)\right],
\]

and

\[
F' = \frac{1}{2} \alpha K[AM_2] \left[1 - \frac{RT}{h} \ln \left(\frac{1 + k_{-5}'Q + Q \exp[-h/RT]}{2 + k_{-5}'Q + Q \exp[-h/RT]}\right)\right].\tag{8}\]

where \(F'\) is the total force at \([P_1]_0\). The apparent rate constant, \(k_{-5}\), defined by the mechanical force reduction (combining Equations 3 and 8) is also plotted against \(h\), also taking \(k_{-5}' = 50 s^{-1}\) and \(k_{-5} = 3.9 s^{-1}\), \(k_{-5}\) decreases from 2100 to 1400 s\(^{-1}\) over the range \(h = 30-40\) kJ/mol.

The average chemical \(k_{-5}\) value is lower than the mechanical value because the AM'.ADP states that bind Pi toward the right of Fig. 2 produce force in greater proportion than their number due to their high strain.

The relative value of the ATPase rate constant, \(k_{cat}\), expected from the model is plotted over the same range of \(h\) in Fig. 3 with the assumption that \(k_\alpha\), the rate constant controlling AM'.ADP breakdown is rate-limiting and independent of \(x\), so that \(k_{cat} = k_{-5}[AM'\cdot ADP]/[AM_2]\). The value for [AM'.ADP]/[AM_2] averaged over \(x\) was calculated from Equations 5 and 6. For example at \(h = 35\) kJ/mol, we find at 10 mM Pi, 85% of attached cross-bridges exist as AM'.ADP and at 200 mM Pi, the figure is 97%. It follows that in the model the ATPase activity at 10 mM Pi is 87% of that in 200 mM Pi. On the other hand, the relative force, \(F'/F_\alpha\) (Equation 8), decreases to the 77% level of \(h = 35\) kJ/mol (Fig. 3). Thus the model predicts that ATPase activity is reduced less than force by increasing concentrations of Pi.

Two simplifying features of this model need further consideration. First, if AM·ADP·Pi were to have a chemical potential that varied with \(x\) then this state would contribute to the force. However, qualitatively, the above argument depends on the difference in chemical potential between AM·ADP·Pi and AM·ADP·Pi decreasing as \(x\) increases and can thus accommodate AM·ADP·Pi, contributing to tension.

Second, \(k_{-5}\) is assumed to be independent of \(x\). Taken together with other assumptions made above, this leads to \(R=[k_{-5}K_\alpha]/(k_{-5}K_\alpha + k_{-5})\) being independent of \(x\). It is inter-
testing that \( R \) is the same whether measured by intermediate or medium oxygen exchange. In this latter case, as we have seen, the exchanged P, is derived mostly from cross-bridges to the right of the diagram in Fig. 2. In the former case we can expect the flux of the ATPase to be either uniform or else biased toward cross-bridges to the left of the diagram, since cross-bridges in the AM'-ADP state with no strain may be expected to correspond to the actin-subfragment 1 interaction in solution that is associated with relatively high ATPase activity. Hence our assumptions made for simplicity that certain rates and equilibrium constants are independent of the rigor state have complex time courses, indicating a wide range of cross-bridge dissociation rate constants. We conclude that the oxygen exchange process observed here can be directly related to the chemical effect of P, on transient and steady-state tension of activated fibers. Oxygen exchange between solvent and medium P, implies that P, binds directly to the active site on myosin, and together with mechanical data, that P, release during ATPase activity is coupled to the formation of a force-generating state.

Acknowledgments—We thank Peter A. Fletcher and Mark Luttman for excellent technical assistance.

REFERENCES