The Reversibility of Adenosine Triphosphate Cleavage by Myosin

By C. R. BAGSHAW and D. R. TRENTHAM

Department of Biochemistry, University of Bristol Medical School, Bristol BS8 1TD, U.K.

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For the simplest kinetic model the reverse rate constants \(k_{-1}\) and \(k_{-2}\) associated with ATP binding and cleavage on purified heavy meromyosin and heavy meromyosin subfragment 1 from rabbit skeletal muscle in the presence of 5mm-MgCl\(_2\), 50mm-KCl and 20mm-Tris–HCl buffer at pH 8.0 and 22°C are: \(k_{-1}<0.02s^{-1}\) and \(k_{-2}=16s^{-1}\). Apparently, higher values of \(k_{-1}\) and \(k_{-2}\) are found with less-purified protein preparations. The values of \(k_{-1}\) and \(k_{-2}\) satisfy conditions required by previous \(^{18}\)O-incorporation studies of \(H_2^{18}O\) into the \(P_i\) moiety on ATP hydrolysis and suggest that the cleavage step does involve hydrolysis of ATP or formation of an adduct between ATP and water. The equilibrium constant for the cleavage step at the myosin active site is 9. If the cycle of events during muscle contraction is described by the model proposed by Lynn & Taylor (1971), the fact that there is only a small negative standard free-energy change for the cleavage step is advantageous for efficient chemical to mechanical energy exchange during muscle contraction.

The elucidation of the kinetic pathway of the Mg\(^{2+}\)-dependent ATPase* of myosin is important on account of its relevance to those states of muscle where the myosin heads are dissociated from the actin filaments. Such states exist in relaxed muscle and also occur transiently during contraction. Previous kinetic studies on the myosin ATPase (Trentham et al., 1972) have resulted in the proposal of the following scheme (eqn. 1). \(M\) represents myosin or its proteolytic subfragments, and the starred forms are intermediates with increased protein fluorescence compared with free myosin (Bagshaw et al., 1972). The following values have been ascribed to the forward rate constants: \(k_{+1}=2.4\times10^{6}m^{-1}s^{-1}\); \(k_{+2}=160s^{-1}\) (Lynn & Taylor, 1970, 1971); \(k_{+3}=0.04s^{-1}\); \(k_{+4}\), probably rapid (>10s\(^{-1}\)); \(k_{+5}=2.3s^{-1}\) (Trentham et al., 1972). The measurements to determine these rate constants were made at pH8 and room temperature which are the conditions used in the experiments described here, although some differences are to be expected because of the different KCl and buffer concentrations. The values of \(k_{+2}\) and \(k_{+3}\) were deduced from a kinetic model which assumed \(k_{-2}<k_{+2}\). It is important to evaluate the reverse rate constants because they have bearing on the steady-state concentration of each intermediate during ATPase activity and these concentrations need to be known for a wide range of studies on relaxed muscle. Further the more complete our understanding of the elementary steps of the Mg\(^{2+}\)-dependent ATPase, the more specifically the effects of actin and other muscle proteins on myosin can be determined.

The experiments described below estimate, or at least set limits for the reverse rate constants \(k_{-1}\) and \(k_{-2}\). The value of \(k_{-1}\) can be evaluated since \(k_{+2}+k_{-2}\)

\[
\begin{align*}
M + ATP & \xrightleftharpoons[k_{-1}]{k_{+1}} M^*ATP & \xrightleftharpoons[k_{+2}]{k_{-2}} M^*ADP \cdot P_i & \xrightleftharpoons[k_{+3}]{k_{-3}} MADP \cdot P_i & \xrightleftharpoons[k_{+4}]{k_{-4}} MADP \cdot P_i & \xrightleftharpoons[k_{+5}]{k_{-5}} M \quad (1)
\end{align*}
\]

is known from the observed rate of transient ADP or \(P_i\) formation at high ATP concentration (Lynn & Taylor, 1971; Bagshaw et al., 1972) and \(k_{-2}/k_{+2}\), the equilibrium constant of the transformation

\[M^*ATP \rightleftharpoons M^*ADP \cdot P_i\]

can be measured as follows. If \([\gamma^3\text{P}]ATP\) or \([2^3\text{H}]ATP\) is mixed with a large molar excess of myosin and the reaction is quenched at a time \(\tau\) when the initial binding is complete (controlled by \(k_{+1}\)) but before product release occurs (controlled by \(k_{+3}\)), ATP will be predominantly equilibrated between \(M^*ATP\) and \(M^*ADP \cdot P_i\), so that product analysis after quenching will give the ratio \(k_{-2}/k_{+2}\). The value of \(k_{-1}\) can be evaluated by treating the equilibrium mixture of radioactive \(M^*ATP\) and \(M^*ADP \cdot P_i\) with a large excess of non-radioactive ATP and allowing sufficient time for the radioactive substrate

* Abbreviation: ATPase, adenosine triphosphatase.

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and products to dissociate from the protein. The ratio of radioactive substrate to products is then \( k_{-1} k_{-2} / k_{+2} k_{+3} \), from which \( k_{-1} \) can be calculated. In practice the design of our quenched flow apparatus limits the time-ranges in which we can quench solutions with adequate mixing to 3–50 ms and greater than 2 s. This means \( M^* \text{ATP} \) concentration has to be measured when the step controlled by \( k_{+3} \) has progressed significantly and then the concentration at \( \tau \) has to be calculated by extrapolation.

Both measurements can be carried out with simple apparatus on a variety of myosin systems, although rapid-reaction equipment is necessary for control experiments and estimating the forward rate constants.

However, the evaluation of \( k_{-1} \) and \( k_{-2} \) from the experimental data presumes a specific model (eqn. 1). Recent experiments (C. R. Bagshaw & D. R. Trentham, unpublished work) suggest that a second binary complex of myosin and ATP can be characterized. In any new scheme the conclusions about reversibility presented here will still hold, but the values for \( k_{+1} \), \( k_{+2} \) and \( k_{-2} \) will have to be equated with more complex functions of rate constants because of the extra step involved.

**Experimental**

**Proteins**

Subfragment 1 and heavy meromyosin were prepared from myosin extracted from rabbit skeletal muscle essentially as described by Lowey et al. (1969). Both proteins were purified by ion-exchange chromatography on DEAE-cellulose. Concentrations of subfragment 1 are quoted as \( \mu \text{m}-\text{sites} \) on the basis of molecular weight 115 000 and \( E_{280}^{1%} = 7.9 \text{cm}^{-1} \). Concentrations of heavy meromyosin are quoted as \( \mu \text{m}-\text{solute} \) heads on the basis of molecular weight 340 000 (equiv. wt. 170 000) and \( E_{280}^{1%} = 6.47 \text{cm}^{-1} \) (Young et al., 1965). Small corrections for light-scattering were made by measuring the extinction between 320 and 400 nm and extrapolating back to 280 nm. The proteins were used within 48 h of column elution, and contained 0.1 mm-dithiothreitol.

**Reagents**

\( [\gamma-^{32}\text{P}] \text{ATP} \) was prepared by the method of Glynn & Chappell (1964) and \( [2-^{3}\text{H}] \text{ATP} \) was obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). Unlabelled ATP was purchased from C. F. Boehringer und Soehne, G.m.b.H., Mannheim, Germany. ATP concentrations were determined either spectrophotometrically from \( \epsilon = 1.54 \times 10^4 \) litre•mol\(^{-1} \)•cm\(^{-1} \) at 259 nm, or, for very dilute \( [2-^{3}\text{H}] \text{ATP} \) solutions, from the manufacturer’s specifications. All other reagents were A.R. grade where possible. Double-glass-distilled water was used throughout.

**Spectroscopic and rapid-reaction equipment**

The extinctions of protein and ATP solutions were measured with a Zeiss PMQII spectrophotometer and the u.v. spectra checked with a Perkin–Elmer 402 spectrophotometer. The fluorescence stopped-flow apparatus, built by Professor H. Gutfreund and Dr. D. W. Yates, has been described previously (Bagshaw et al., 1972). The protein was excited by light at 300 nm and the emitted light between 335 and 375 nm analysed. The quench flow apparatus has been described by Gutfreund (1969), although it was used mainly as a rapid-mixing device, the quenching solution being added manually from a syringe.

**Estimation of \( k_{-2} \) and \( k_{-1} \)**

Experiments were carried out at room temperature (21\( ^\circ \)±2\( ^\circ \)C). Subfragment 1 or heavy meromyosin was made up to a concentration of 20–50 \( \mu \text{m} \)-sites in 5 mm-MgCl\(_2\)–50 mm-KCl–20 mm-Tris buffer adjusted to pH 8.0 with HCl. A single turnover of ATP hydrolysis was examined in the fluorescence stopped-flow machine by pushing against 5 \( \mu \text{m} \)-ATP, and from the trace the values of \( \tau \) and the turnover rate were ascertained.

The same stock protein solution was then mixed with \( [\gamma-^{32}\text{P}] \text{ATP} \) or \( [2-^{3}\text{H}] \text{ATP} \) (0.05–5 \( \mu \text{m} \) syringe concentration) in the quench flow apparatus, and ejected into a glass vial. After intervals between 2 and 60 s, timed with a stop watch, an equal volume (3.2 ml) of quenching solution, containing 7\( % \) (w/v) perchloric acid, 1 mm-carrier ATP and 1 mm-carrier Pi at 0\( ^\circ \)C, was added manually from a syringe. The mixture was immediately transferred to a bench-centrifuge tube containing 1.6 ml of cold 4 m-sodium acetate buffer to raise the pH to 3.4, and the protein precipitate was spun down for 1 min. Portions (20 \( \mu \)l) of the supernatant were applied in a polyethylene-imine-cellulose thin-layer chromatogram and developed in 0.75 m-KH\(_2\)PO\(_4\) buffer adjusted to pH 3.4 with HCl. The position of the labelled ATP and ADP (where applicable) spots were determined by inclusion of marker ATP and ADP. \( ^{32}\text{P} \) runs at the salt front as is readily seen under u.v. light (it also runs coincident with marker AMP). The radioactive bands were cut out and eluted for several hours with 1 ml of 1 m-HCl, and 0.5 ml of the eluates were mixed with 15 ml of scintillation fluid (containing 3 litres of toluene, 2 litres of 2-methoxyethanol, 400 g of naphthalene and 30 g of 5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxo-3,4-diazole (Koch–Light, Cockbrook, Bucks., U.K.) and counted for radioactivity in a Nuclear–Chicago Unilux II counter. The ATP and product counts were corrected for background, then the labelled ATP was expressed as a percentage of the total isotope content for each assay. Time-zero points were obtained during the course of the experi-
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ment by mixing the protein with the quenching solution before addition of labelled ATP. The assay counts could then be corrected for $^{32}$P$_i$ or [2-3H]ADP contamination originally present in the stock labelled ATP and that, which arose during HClO$_4$ treatment.

A similar technique was employed for estimating $k_{-1}$. Here 0.5ml of 10mm-non-radioactive ATP was added after 2s to the stirred mixture of labelled ATP and protein, which had been ejected from the quench flow apparatus. After quenching with HClO$_4$ the products were analysed as above. As a check on the efficiency of the non-radioactive 'chase' a control experiment was carried out by adding the unlabelled ATP before the labelled ATP. After a single turnover very little of the labelled ATP should be hydrolysed.

Results

Fluorescence stopped-flow experiments

When excess of subfragment 1 was mixed with ATP the reaction profile showed two distinct exponential phases (Fig. 1) as noted previously (Trentham et al., 1972; Bagshaw et al., 1972). The maximum protein fluorescence is reached at 300ms after mixing reactants and this gives a value for $\tau$, the time at which the binding equilibrium controlled by $k_{+1}$ is established. The rate of the slow phase was not affected by increasing subfragment 1 concentration supporting previous findings that the experiments were carried out at a subfragment concentration well above the $K_m$ value for ATP ($<10^{-7} M$; Lymn & Taylor, 1970). Therefore according to eqn. (1) the observed rate of decomposition of the high-fluorescent forms of the protein (0.10s$^{-1}$) equals $k_{+3} k_{-3}/(k_{+3}+k_{-3})$. It is conceivable this should be replaced by a more complex function involving $k_{-3}$ and $k_{+4}$ as well. However, this possibility would not affect subsequent arguments about the extent of reversibility of the cleavage step and is unlikely in view of the lack of effect of $P_i$ on myosin ATPase kinetics (Trentham et al., 1972). A single turnover is more than 99% complete in 60s. Similar traces were obtained with heavy meromyosin.

Measurement of $k_{-2}$

$k_{+2}+k_{-2}$ (160s$^{-1}$; Lymn & Taylor, 1971, Fig. 3) is rapid compared with the enhanced fluorescence decay rate, $k_{+2} k_{+3}/(k_{+2}+k_{+3})$ (0.10s$^{-1}$), so that the equilibrium between M$^*$ATP and M$^*$ADP$\cdot$Pi will be maintained throughout their decay to the low-fluorescent forms of myosin. This means that both M$^*$ATP and M$^*$ADP$\cdot$Pi will decay exponentially at a rate of 0.10s$^{-1}$. The results of Table I show first that there is a significant amount of ATP present when all the nucleotide is bound (at time $\tau$, 300ms) and that it decays at a rate comparable with the fluorescence decay rate (Fig. 1). By using this

Table 1. Concentration of free ATP$+M^*$ATP during a single turnover of subfragment 1 ATPase

The experiment was carried out with 15$\mu$M-subfragment 1 (reaction-chamber concentrations) and 2.5$\mu$M-[y-$^{32}$P]ATP in 5mm-MgCl$_2$-50mm-KCl-20mm-Tris buffer adjusted to pH8.0 with HCl as described in the Experimental section. The 37ms time-point was obtained by using the quench flow apparatus in the normal manner. Each value is an average of duplicate assays which agreed within 2% and has been corrected for the decomposition of ATP during the quenching procedure which was 4% of the ATP present at the time of quenching. The subfragment 1 preparation was the same as used in the fluorescence stopped-flow experiment (Fig. 1) and this quenching experiment was completed within 5h of obtaining the stopped-flow traces.

<table>
<thead>
<tr>
<th>Quenching time (s)</th>
<th>% labelled ATP present</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>0.037</td>
<td>51</td>
</tr>
<tr>
<td>2.0</td>
<td>7</td>
</tr>
<tr>
<td>5.0</td>
<td>4</td>
</tr>
<tr>
<td>60</td>
<td>0.3</td>
</tr>
</tbody>
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exponential decay rate (0.10s\(^{-1}\)) and the ATP concentration at 2s, the ATP concentration can be calculated at time \(\tau\) by extrapolation and equals 10% of the total nucleotide. It follows that \(k_{-3}/k_{+2} = 10/90 = 0.11\), \(k_{+2} = 144\text{s}^{-1}\) and \(k_{-2} = 16\text{s}^{-1}\).

As a control that the rapid cleavage step occurred almost concomitant with the fluorescent change, the ATP concentration was measured after 37ms and found to be 51% of the total nucleotide, so that about 50% of the myosin should be in its high-fluorescence form at 37ms, which is consistent with the result shown by the amplitude of rapid phase of Fig. 1.

**Measurement of \(k_{-1}\)**

Eqn. (2) represents the kinetics of the dissociation of radioactive nucleotide from myosin after mixing the protein with unlabelled ATP. In the scheme, \(\text{M*ATP} + \text{M*ADP}\cdot P_1\) decays exponentially to ATP, ADP and \(P_1\) with a rate constant of \(k_{+2}k_{+3}/(k_{+2} + k_{-2}) + k_{-1}k_{-3}/(k_{+2} + k_{-2})\) and the product ratio of ATP to ADP or \(P_1\) is \(k_{-1}k_{-3}/k_{+2}k_{+3}\).

Comparison of the results in Tables 1 and 2 shows that after mixing \(\text{M*ATP}\) with unlabelled ATP at 2s the product distribution at both 5s and 60s is the same as if no ATP had been added. This shows that \(k_{-1} = 0\) because the product ratio of ATP to \(P_1\) is very small and the rate of decay of ATP is the same as in Table 1 and therefore equals \(k_{+3}k_{-3}/(k_{+2} + k_{-2})\). In practice we conclude that \(k_{-1} < 0.02\text{s}^{-1}\) since this is the limit set by the experimental sensitivity.

Table 2 also shows in a control experiment that if ATP is mixed with protein before addition of radioactive ATP, then no radioactive ATP can bind. The fact that 96% rather than 99% of ATP remains (the expected efficiency of blocking the myosin site to [\(y-32\text{P}\)]ATP) is probably within the limits of experimental error since the same result was obtained if the concentration of unlabelled ATP was increased tenfold.

Identical results yielding \(k_{-2} = 16\text{s}^{-1}\) and \(k_{-1} < 0.02\text{s}^{-1}\) were found with heavy meromyosin prepared by a trypptic digestion and purified on DEAE-cellulose.

These experiments were repeated about 20 times with a variety of modifications and controls and also working in 0.5M-KCl and by using myosin. In particular we have looked for possible artifacts. The most likely cause of an artifact would be due to there being a heterogeneous population of protein molecules, as might arise for example because of thiol oxidation. In control experiments no significant difference in rate constants was found in myosin solutions prepared at the same time under identical conditions except for the presence of protecting EDTA and thiol reagent. Changing the quenching reagent to KOH and neutralizing with acetic acid to pH3.4 for product chromatography gave the same values of \(k_{-2}\) and \(k_{-1}\). However, the ratio of \(\text{M*ATP}\) to \(\text{M*ADP}\cdot P_1\) rose significantly to 0.2 in a number of cases and this was generally paralleled by an increase in the apparent value of \(k_{-1}\) up to 0.05s\(^{-1}\). These anomalous results were associated with aged or less-purified protein solutions. In no case did the ratio of \(\text{M*ATP}\) to \(\text{M*ADP}\cdot P_1\) drop below 0.09 and this ratio was consistently reproduced by the purest protein samples.

In further control experiments \(k_{-2}/k_{+2}\) was measured and found to be 0.09 in each case when the subfragment concentration was 37 or 12\(\mu\)M and the ATP concentration was 0.07\(\mu\)M. This shows, as was indicated above, that the protein concentration was well above the \(K_m\) value for ATP and so all the nucleotide is bound at time \(\tau\). The ratio \(k_{-2}/k_{+2}\) was also unaltered if unlabelled nucleotide was omitted from the quenching solution, eliminating artifacts arising from binding of nucleotide to denatured protein.

**Discussion**

Although a significant value for \(k_{-2}\) can account for our results, other possibilities that would indicate

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**Table 2. Effect of 'chasing' labelled M*ATP and M*ADP·P1, intermediates with non-radioactive ATP**

The conditions were as reported in Table 1. ‘Chasing’ involved the addition of unlabelled ATP to a concentration of 1.3mM.

<table>
<thead>
<tr>
<th>Quenching time (s)</th>
<th>'Chasing' time (s)</th>
<th>% labelled ATP present</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>2.0</td>
<td>4.5</td>
</tr>
<tr>
<td>60</td>
<td>2.0</td>
<td>0.3</td>
</tr>
<tr>
<td>60</td>
<td>0</td>
<td>96</td>
</tr>
</tbody>
</table>
apparent reversibility of the cleavage step should be considered. The presence of a dead-end complex of ATP and myosin is ruled out because in the experiment to measure $k_{-1}$ the radioactive ATP decays at about 0.10s$^{-1}$ and yet does not dissociate into the ATP pool (Table 2). The possibility that any ATP is not bound to myosin at 2s after mixing is ruled out by the control experiments described. The myosin preparations used contain hardly any slow muscle as characterized by the light chains (A. G. Weeds, personal communication) and the association rate constants of ATP to fast and slow cat muscle myosin only differ by a factor of 3 (D. R. Trentham, unpublished results). A third possibility that the protein exists as a heterogeneous population containing 10% of a modified form is difficult to rule out absolutely. However, our experiments indicate that such a modified form, if it exists, does not arise from thiol oxidation or metal-ion inactivation. Moreover, the cleavage rate of this hypothetical modified form would equal the turnover rate of normal subfragment 1 which would be fortuitous (Fig. 1 and Table 1). In the experiments with aged or less-purified protein the increase in $k_{-2}$ is generally paralleled by a rise in $k_{+1}$ indicating reversible dead-end complex formation and/or heterogeneous protein populations in these samples.

In any event the results show that in steady-state kinetic studies of the myosin ATPase a significant fraction of the steady-state complex is the binary complex of myosin and ATP. This suggests that at least a fraction of the refractory state described by Eisenberg et al. (1972) is this binary complex. It also may account in part for the 0.8:1 rather than 1:1 stoichiometry between the transient phosphate and heavy meromyosin subfragment 1 site concentrations in the transient kinetic studies of ATP cleavage of Lymn & Taylor (1970) (Bagshaw et al., 1972).

The reversible cleavage step indicated by our results is particularly interesting when taken in conjunction with the work of Sartorelli et al. (1966). They showed that after mixing myosin with excess of ATP at 0°C and quenching after 30s into HClO$_4$ containing H$_2$O, there was no incorporation of $^{18}$O into Pi. The major species leading to Pi production at the time of quenching would be M*ADP-Pi according to eqn. (1) indicating the intermediate is hydrolysed ATP or an adduct of ATP and water such as might exist with a pentacoordinate $\gamma$-P atom and not some form of anhydride.

They further showed that if the experiment was repeated except that H$_2$O was in the original myosin solution but not in the quenching solution, then approximately three water oxygen atoms were introduced into the Pi. This has two implications. First, the step in which $^{18}$O is introduced into ATP must be rapidly reversible relative to the rate at which P$_i$ is released into the medium, which is controlled by the step M*ADP-P$_i$ $\rightarrow$ MADP-P$_i$, which is rate limiting (Trentham et al., 1972) and secondly, there must be a rearrangement of the moiety which contains the incorporated $^{18}$O on the protein otherwise the same $^{18}$O would be abstracted in the reversal of the $^{18}$O-incorporation step by the principle of microscopic reversibility. The rapid reversibility condition is satisfied by the rate constants reported here for $k_{+2}$ and $k_{-2}$ and the species undergoing the rearrangement can be identified as M*ADP-P$_i$. (The rearrangement should not be confused with the transition to MADP-P$_i$ controlled by $k_{+3}$.)

If the proposed rearrangement of the protein-bound moiety is also rapid compared with the cleavage step, then one might expect $^{18}$O exchange on all four oxygen atoms of P$_i$ both in the experiments of Sartorelli et al. (1966) and in other experiments in which a large concentration of ATP was hydrolysed (Levy & Koshland, 1959). In practice the measured $^{18}$O incorporation is more nearly equal to three oxygen atoms per phosphorus atom in most cases for which this reaction has been analysed. If this value of three has mechanistic significance it implies that one oxygen atom of the protein-bound moiety is restrained from exchange. A simple postulate consistent with this restriction is that M*ADP-P$_i$ does represent hydrolysed ATP and that the phosphate moiety is bound to the protein through an oxygen atom leaving the other three oxygen atoms free to rotate round the fixed P-O bond. However, the value for $^{18}$O incorporation has been reported to be as high as four oxygen atoms incorporated per phosphorus atom (Yount & Koshland, 1963; Swanson & Yount, 1966) so that an unequivocal determination of the extent of oxygen exchange is required before any models on the structural nature of M*ADP-P$_i$ can be substantiated.

It is interesting that when Levy & Koshland (1959) investigated $^{18}$O exchange in P$_i$ produced from actomyosin-catalysed ATP hydrolysis, the total water oxygen incorporation dropped to about two oxygen atoms per ATP hydrolysed. Actomyosin ATPase has a turnover rate (~10s$^{-1}$) (Lowey et al., 1969) comparable with $k_{-2}$ so that the decreased oxygen exchange is to be expected provided actin does not greatly modify the kinetics of the cleavage step as has been indicated by the results of Lynn & Taylor (1971), whose scheme (eqns. 3, 4 and 5) is described below. Although Levy & Koshland's (1959) results did indicate that actin influences the rate of formation of the intermediate capable of $^{18}$O exchange if four oxygen atoms are exchangeable, their results were consistent with no influence of actin on the cleavage step if only three oxygen atoms are exchangeable (or if their measured value of three was low). A kinetic analysis of the influence of actin on the $^{18}$O-exchange reaction is given in their paper.
In further experiments in which myosin was mixed with ATP in the presence of $\text{H}_2\text{O}^\text{18}$O no $\text{H}^\text{18}$O was found in the ATP (Sartorelli et al., 1966). This is expected in view of the small value found for $k_{-1}$. (The concentration of $\text{M}^\text{*ATP}$ that would have been expected to contain $\text{H}^\text{18}$O was less than 0.1% of the ATP concentration at the time of quenching.) The small value of $k_{-1}$ is consistent with our previous studies which showed that $k_{-1}<k_{2}+k_{3}$ for ATP and $k_{-1}<0.25^{-1}$ for ATP analogues modified in the triphosphate moiety (Bagshaw et al., 1972).

It is interesting to relate these experimental results to the molecular model of muscular contraction outlined by Lynn & Taylor (1971) from the viewpoint of converting chemical free energy into mechanical work. Lynn & Taylor's (1971) scheme for actomyosin ATPase is summarized by eqns. (3), (4) and (5), where A represents actin and AM actomyosin:

\[
\text{AM} + \text{ATP} \rightleftharpoons \text{A} + \text{M}^\text{*ATP} \tag{3}
\]

\[
\text{M}^\text{*ATP} \rightleftharpoons \text{M}^\text{*ADP} + \text{P}_1 \tag{4}
\]

\[
\text{A} + \text{M}^\text{*ADP} + \text{P}_1 \rightleftharpoons \text{AM} + \text{ADP} + \text{P}_1 \tag{5}
\]

Their results indicated that the kinetics of ATP cleavage (eqn. 4) were similar whether or not actin was present, so for the purpose of this discussion they will be treated as common steps in both the myosin and actomyosin ATPases. In their model mechanical work is obtained from the processes involving actomyosin and not therefore from the cleavage step (eqn. 4). The free energy, $\Delta G_e$, liberated in the cleavage step either as heat or through an increase of entropy will not be available for conversion into mechanical work (temperature in muscle being effectively constant). It follows that for efficient conversion of chemical free energy into mechanical work the negative free-energy change of the cleavage step should be small relative to the total negative free-energy change derived from the chemical reactions. The standard free-energy change, $\Delta G^\circ_e$, of the cleavage step equals $-5.5\text{kJ} \cdot \text{mol}^{-1}$ calculated from the equilibrium constant ($=k_{+2}/k_{-2} = 9$) and

\[
\Delta G_e = \Delta G^\circ_e + RT\ln \frac{[\text{M}^\text{*ADP} + \text{P}_1]}{[\text{M}^\text{*ATP}]}
\]

The total free-energy change, $\Delta G_t$, derived from chemical reactions during muscle contraction is difficult to evaluate precisely, but in muscle that has been treated with fluorodinitrobenzene $\Delta G_t$ is essentially the free-energy change derived from ATP hydrolysis and equals $-45\text{kJ} \cdot \text{mol}^{-1}$ (Kushmerick, 1969). Present knowledge suggests that the steady-state concentration of $\text{M}^\text{*ATP}$ is larger than that of $\text{M}^\text{*ADP} + \text{P}_1$ during actomyosin ATPase activity (Lynn & Taylor, 1971), so the term $RT\ln [\text{M}^\text{*ADP} + \text{P}_1]/[\text{M}^\text{*ATP}]$ will be negative. The maximum efficiency (for negative values of $\Delta G_e$) that can be derived from the chemical reactions is given by:

\[
\frac{\Delta G_t - \Delta G_e}{\Delta G_t} = 1 - \frac{\Delta G^\circ_e + RT\ln \frac{[\text{M}^\text{*ADP} + \text{P}_1]}{[\text{M}^\text{*ATP}]}}{\Delta G_t}
\]

and the advantage in $\Delta G^\circ_e$ being much less negative than $\Delta G_t$ is apparent. This argument has a number of simplifications; in particular (1) the ratio of the concentrations of both free ADP and $\text{M}^\text{*ADP} + \text{P}_1$ relative to free ATP and $\text{M}^\text{*ATP}$ respectively can be expected to increase when mechanical work is done which would make both $\Delta G_t$ and $\Delta G_e$ less negative (Wilkie, 1970; Taylor, 1973), (2) it is assumed that there is no interaction between neighbouring crossbridges and (3) the value of $\Delta G^\circ_e$ for the situation in vivo has yet to be evaluated.

However, if the statement of the Lynn–Taylor (1971) model that actin is not associated with ATP cleavage is correct, it is desirable for there to be a relatively small negative standard free-energy change in this cleavage step. The fact that our results suggest this is so strengthens the case for this molecular model.

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