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On the mechanism of actomyosin ATPase from fast muscle

(exchange kinetics/intermediate oxygen exchange/mass spectrometry/muscle contraction)

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ABSTRACT **The labeled inorganic phosphate formed by enzymatic hydrolysis of [γ-^{18}O]ATP in normal water was derivatized to trimethyl phosphate and analyzed for the proportions of [$^{18}O_3$]P_i, [$^{18}O_2$]P_i, [$^{18}O_1$]P_i, and [$^{18}O_0$]P_i. The proportions observed were correlated with the kinetics of intermediate exchange by using a kinetic relationship in which it is assumed that binding of ATP and subsequent release of products are irreversible. Actomyosin and acto-heavy meromyosin catalyze intermediate exchange at a mean rate that is more than 1 order of magnitude slower than that predicted by rapid kinetic studies or implied by the essentially complete intermediate exchange observed with myosin alone. The reason for the slow apparent exchange is that there are two ATPase activities with different exchange properties. The effect of varying heavy meromyosin concentrations at a constant actin concentration shows that the two activities are interrelated and suggests further that one is due to ATP hydrolysis by the undissociated actomyosin crossbridge.**

The mechanism of ATP hydrolysis by actomyosin is important to the problem of muscle contraction. From measurements of the pre-steady-state decrease in light scattering and of the rate of the early phosphate burst, Lymn and Taylor (1) proposed a simplified mechanism in which binding of ATP to the active site of myosin promotes dissociation of the actomyosin crossbridge, reversible ATP hydrolysis is catalyzed by dissociated myosin, and release of products takes place upon the rate-determining recombination with actin. Inoue *et al.* (2) found the recombination of the myosin–products complex (M·ADP·P_i) with actin to be slower than the observed rate of ATP hydrolysis at high actin concentrations. They proposed a "two-route" mechanism in which some ATP hydrolysis takes place without dissociation of the crossbridge complex. Similar observations led Eisenberg *et al.* (3) to propose a "refractory state" of M·ADP·P_i that had a decreased affinity for actin. However, the evidence for the two-route and refractory-state mechanisms was contested by White and Taylor (4).

Several experimental observations with actomyosin and muscle fibers are not readily explained by Lymn and Taylor's simplified hypothesis. One is the two phases observed upon addition of MgATP to an actomyosin gel (5); a second is the conservation of energy observed with muscle fibers undergoing isometric contraction against a heavy load (6); and a third is the limited intermediate oxygen exchange during the hydrolysis of ATP (7, 8).

When [γ-^{18}O]ATP is hydrolyzed to ADP + P_i by purified myosin or its soluble proteolytic fragments heavy meromyosin (MHH) and subfragment 1, a fraction of the label is exchanged into water. The exchange is known to result from repeated cycles of ATP hydrolysis which precede the rate-determining release of products (9). Recent measurements have shown the exchange to be essentially complete when contaminant ATPases are removed (8, 10, 11).

When the basal ATPase activity of the myosin preparation has been subtracted, the rate of intermediate exchange catalyzed by actomyosin is slower than predicted by the Lymn and Taylor hypothesis (8). Shukla and Levy (8) suggested that either a persisting conformational change in myosin near the active site slows down the reorientation of P_i oxygens in M·ADP·P_i or the P_i formed is a mixed pool coming from several ATPase activities, each with different exchange properties. Because their method of ^{18}O analysis measured the average enrichment of the P_i formed (P_i was combusted to CO_2), they were not able to resolve these two possibilities.

By measuring the distribution of phosphate species containing none, one, two, or three ^{18}O atoms per phosphate molecule, Sleep *et al.* (11) recently found that hindered rotation of P_i oxygens in M·ADP·P_i does not explain the limited intermediate exchange. They found that two pools of P_i, with different ^{18}O enrichments, were formed by actomyosin ATPase. Because acto–subfragment 1 behaved as a single ATPase activity with the properties predicted by Lymn and Taylor's hypothesis, Sleep *et al.* interpreted the results with actomyosin as being due to the filamentous structures of myosin and actin. They concluded that the intimate association of the two types of filaments produced local concentration effects which led to limited intermediate exchange.

In the present study, the intermediate exchange properties of actomyosin and acto-HMM are compared. The distributions of phosphate species containing zero to three ^{18}O atoms per phosphate molecule are analyzed with a kinetic relationship which allows the proportions of the four species to be correlated with the relative rates of intermediate exchange and of release of product ADP + P_i. The results demonstrate that there are two interdependent ATPase activities in both actomyosin and acto-HMM.

MATERIALS AND METHODS

Materials. All commercial enzymes were purchased from Boehringer Mannheim. ^{18}O-Enriched water was purchased from Miles, and PCl_5 was from Baker. ^{18}O-Enriched P_i was prepared by hydrolysis of the PCl_5 in [^{18}O]H_2O (12). Diazald and a diazomethane-generating apparatus were from Aldrich.

Myosin, HMM, and Actin. Myosin was extracted from the white muscles of the back and hind legs of rabbit (13), and actin was prepared and stored as described by Spudich and Watt (14). HMM was prepared by limited tryptic digestion (15) and later by chymotryptic digestion in the presence of Ca^{2+} (16). Protein

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Abbreviation: HMM, heavy meromyosin.

was measured with the biuret reagent.

Isolation and Analysis of $[^{18}O_n]P_i$. After incubation under the conditions described in the text, the reaction was stopped by addition of $HClO_4$ to 0.4 M. The $[^{18}O_n]P_i$ was purified (17) and precipitated with magnesia (18). After treatment with Bio-Rad AG-50(H^+), the $[^{18}O_n]H_3PO_4$ was methylated with diazomethane (freshly prepared according to the manufacturer's instructions) and analyzed as described (19). Each sample was analyzed twice and the standard deviations were less than 1% of the area of each peak. A 1% error is assumed for the calculations in Fig. 1.

[γ-^{18}O]ATP. Labeled ATP was prepared as described by Penefsky *et al.* (20). For ^{18}O analysis, it was converted to P_i with dihydroxyacetone and glycerokinase (19).

Equations for Analysis of Intermediate Exchange. Assume the following four-step sequence in which steps 2 and 4 are irreversible and the rate constant for reversal of hydrolysis (k_3) equals the rate constant for intermediate exchange (in the equation shown, AM_1 can replace M_1, etc.):

$$M_1 + ATP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} M_1{\cdot}ATP \xrightarrow{k_2} M_2{\cdot}ATP$$
$$+ H_2O \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} M_2{\cdot}ADP{\cdot}P_i \xrightarrow{k_4} M_1 + ADP + P_i.$$

It remains to be determined directly if the last assumption is correct, but the currently accepted value for k_{-3} of 10–15 sec^{-1} [at pH 7.0, 20°C, and 50 mM KCl (21)] is close to the value of 13–16 sec^{-1} for intermediate exchange at pH 7.4, 25°C, and 50 mM KCl which can be calculated with Eq. 1 from the acto-subfragment 1 data of Shukla and Levy (8).

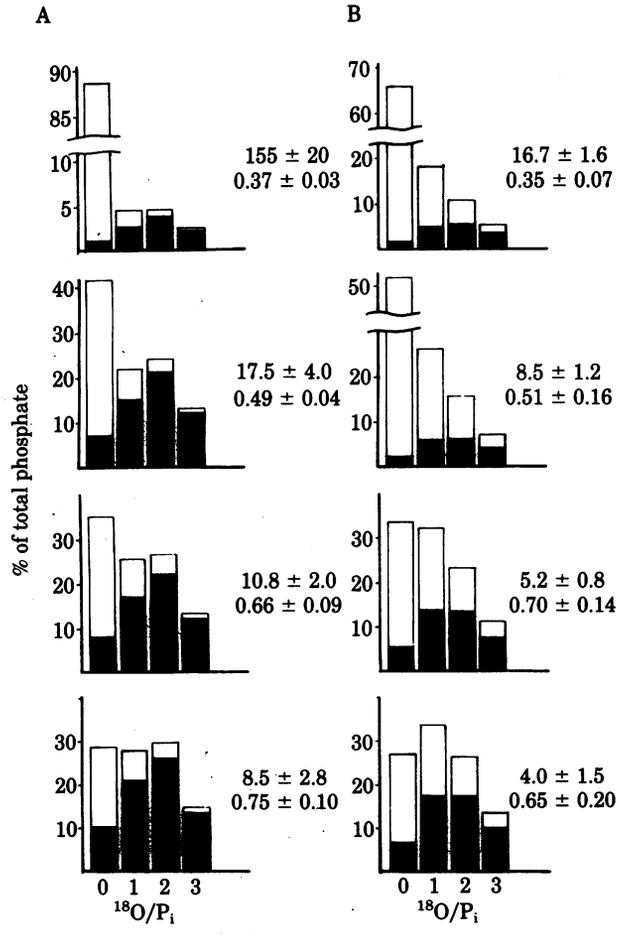


FIG. 1. Analysis of the data in Tables 1 and 3 with Eq. 2 and assuming two pathways of hydrolysis. The R values (upper for ATPase 1; lower for ATPase 2) next to each bar graph give the best fit of the data; the errors (±) are the range of R in which the error is ±1%. □, ATPase 1; ■, ATPase 2. (*A*) Actomyosin. (*B*) Acto-HMM.

Given that the γPO_3 of ATP has the labeling $^{18}O_3/(^{18}O_2, ^{16}O_1)/(^{18}O_1, ^{16}O_2)/^{16}O_3 = a_0/b_0/c_0/d_0$ in which $a_0 + b_0 + c_0 + d_0 = 100$, and the P_i released in the steady state has the labeling $a/b/c/d$ in which $a + b + c + d = 100$. $E_0 = a_0 + (2/3)b_0 + (1/3)c_0$; $E = a + (2/3)b + (1/3)c$. The fraction of label retained after intermediate exchange will be $F = (E/E_0)$. A certain fraction (X) of the P_i molecules released from $M_2{\cdot}ADP{\cdot}P_i$ will retain all the ^{18}O label of the starting ATP; this fraction is given by the ratio of rate constants $X = k_4/(k_{-3} + k_4)$. A smaller fraction, $X(1 - X)$, will be released after one cycle of exchange, and a still smaller fraction, $X(1 - X)^y$, will be released after y cycles of exchange. The oxygen labeling will be E_0, $(3/4)E_0$, and $(3/4)^y E_0$. The average labeling of P_i formed in the steady state will be

$$E = E_0X + (3/4)E_0X(1 - X) + \ldots + (3/4)^yE_0X(1 - X)^y$$

or

$$E = E_0X \sum_{y=0}^{y=\infty} (3/4)^y(1 - X)^y.$$

Because

$$\sum_{y=0}^{y=\infty} (o/q)^y = q/(q - p) \text{ when } (p/q) < 1,$$

$$F = (E/E_0) = \left(\frac{4}{R + 4}\right) \quad [1]$$

in which $R = k_{-3}/k_4$.

In considering the individual phosphate species, $[^{18}O_n]P_i$, similar logic leads to the following equations:

$$[^{18}O_3]P_i\text{: } a = a_0\left(\frac{4}{3R + 4}\right)$$
$$[^{18}O_2]P_i\text{: } b = (a_0 + b_0 - a)\left(\frac{2}{R + 2}\right) \quad [2]$$
$$[^{18}O_1]P_i\text{: } c = (a_0 + b_0 + c_0 - a - b)\left(\frac{4}{R + 4}\right)$$
$$[^{18}O_0]P_i\text{: } d = 100 - (a + b + c).$$

in which $R = k_{-3}/k_4$.

RESULTS

The ^{18}O-labeling of P_i formed by hydrolysis of [γ-^{18}O]ATP by myosin and actomyosin is shown at the top of Table 1. Of interest is the fact that $^{18}O_3$-$P_i/^{18}O_2$-$P_i/^{18}O_1$-P_i (i.e., $a/b/c$) remained almost constant over a 30-fold variation in k_{cat} (k_{cat} is number of molecules of ATP hydrolyzed per myosin subunit per sec); the only pronounced shift was in the relative amount of $[^{18}O_0]P_i$ formed in the four samples. From Eq. 2, the proportions of the four P_i species should change in a predictable manner depending on the proportions in the starting ATP, $a_0/b_0/c_0/d_0$, and on the value of R. The ratios a/b suggest that there is one pathway of ATP hydrolysis in which R increases from 0.6 to 0.9 with increasing actin concentration; the ratios c/d suggest that there is a second pathway in which R decreased from 65 to 3. Thus, there appear to be two or perhaps more ATPase activities in actomyosin with different exchange properties.

Table 1. Myosin, actomyosin, and actin ATPases

Myosin, μM	Actin, μM	k_{cat},* sec^{-1}	$[^{18}O_n]P_i$ $n=3$	$n=2$	$n=1$	$n=0$
Starting [γ-$^{18}O_n$]ATP			29.8	40.0	21.2	9.00
3.5‡	—	0.019	2.44	4.53	4.53	88.5
1.76§	1.1	0.18	12.8	24.1	21.7	41.4
1.76	4.4	0.30	13.1	26.3	25.6	35.1
1.76	11	0.56	14.5	29.4	27.7	28.4
—	240¶	—	23.0	31.3	16.5	29.4
Predicted if R =			(a)	(b)	(c)	(d)
0.67			12.8	24.1	18.6	8.9
1.08			11.5	24.1	21.7	12.2
6.4			4.1	12.3	21.7	41.4

* The turnover number per subunit of myosin (M_r = 235,000).
† Natural ^{18}O abundance (0.204%) has been subtracted.
‡ The sample with myosin alone was in 20 ml total volume containing 2 μmol of [γ-^{18}O]ATP and 40 μmol of $MgCl_2$. Other conditions were the same as for the actomyosin experiments. Incubation was for 20 min.
§ The reaction mixtures contained, in 1.0 ml total volume, 50 μmol of KCl, 25 μmol of Tris·HCl (pH 7.4), 4 μmol of $MgCl_2$, 2 μmol of [γ-^{18}O]ATP, 4 μmol of [γ-^{32}P]ATP (5.6×10^5 cpm), and the indicated amounts of F-actin and myosin. Incubation was for 20–40 min at 25°C. The extent of reaction was measured by adsorption of nucleotides on charcoal and measurement of $^{32}P_i$ in the supernatant.
¶ The reaction mixture contained 4.0 ml of G-actin (11 mg/ml) in Buffer A (14) from which excess ATP, ADP, and P_i had been removed by dialysis. Polymerization was initiated at 25°C by the addition of 3 μmol of [γ-^{18}O]ATP, 8 μmol of $MgCl_2$, and 200 μmol of KCl. The reaction was stopped after 10 min with $HClO_4$. The G-actin contained 1.0 μmol of bound, unlabeled ATP.

The simplest assumption is that there are two pools of ^{18}O-P_i formed. The data can be fitted by assuming two pathways of hydrolysis (Fig. 1A). For the activity that catalyzes extensive intermediate exchange (ATPase 1), R decreases steadily from 155 ± 20 with no actin to 8.5 ± 2.8 at 11 μM actin. R is almost inversely proportional to k_{cat}, as predicted for a pathway in which intermediate exchange is catalyzed by dissociated myosin and release of products occurs upon the second-order recombination of M_2·ADP·P_i with actin. The contribution of this pathway is 90% with no actin added and 30% at 11 μM actin. For the activity that catalyzes limited intermediate exchange (ATPase 2), the value of R increases steadily from 0.37 ± 0.03 at no actin to 0.75 ± 0.09 at 11 μM actin, and the contribution to the total ATP hydrolyzed increases from 9–10% to 70%. With the reasonable assumption that the rate of intermediate exchange (k_{-3}) is independent of actin concentration, the increase in R appears to be due to a 2-fold decrease in the rate at which products are released. The interpretation of this puzzling effect will be discussed below.

The analysis in Fig. 1A for the sample with myosin alone shows that the incomplete intermediate exchange observed is due to contamination by actin. Exchange experiments with two other myosin preparations which contained larger contaminations confirmed this conclusion. Sleep *et al.* first concluded that the incomplete exchange was due to a second ATPase that did not catalyze intermediate exchange, but more recently they left open the possibility that the contaminant was actin (11). The high K_m for ATP (2–3 mM) which they observed is not unexpected for actin decorated with tropomyosin (22).

Before turning to acto-HMM, a comment should be made about the contribution of actin ATPase to the observed ATP hydrolysis. The polymerization of G-actin to F-actin is accompanied by a stoichiometric hydrolysis of one molecule of ATP per actin monomer and, as shown in the bottom of Table 1, this occurs without intermediate exchange. However, in the presence of myosin, most of the [^{14}C]ATP incorporation into the actin filament as ADP occurs by exchange after hydrolysis to free ADP by myosin ATPase (23); because the amount exchanged is small, the contribution of actin ATPase will be ignored.

Acto-HMM. Anomalous intermediate exchange properties have been described not only for actomyosin, which is a heterogeneous system, but also for acto-HMM (8) and acto-subfragment 1 (24) which are homogeneous and do not precipitate from solution. More recently, acto-subfragment 1 has been shown (to a first approximation) to catalyze intermediate exchange by a single pathway in which exchange is catalyzed by dissociated M_2·ADP·P_i (8, 11). However, the observation of anomalous intermediate oxygen exchange catalyzed by acto-HMM remains (8). Table 2 shows the results of a preliminary experiment in which HMM prepared by tryptic digestion (15) was kept constant and actin was increased as in Table 1. Comparison with the predicted patterns of *a/b/c/d* given in the bottom of the table shows that the data for the lowest actin concentration cannot be fit by the assumption of a single pathway of hydrolysis. This conclusion also applies to the other three actin concentrations. Although the data can be fitted by assuming two pathways of hydrolysis, quantitative interpretation is made less significant by the knowledge that tryptic HMM is a heterogeneous product. There are probably a number of HMM species with varying affinities for actin. On the other hand, chymotryptic digestion in the presence of Ca^{2+} is known to produce a nearly homogeneous product with the DTNB light chain and the heavy chain largely intact (16).

The results of an experiment in which actin was kept constant at 2 μM and chymotryptic (Ca^{2+}) HMM was varied over a wide range (0.15–37 μM) are shown in Table 3. The k_{cat} values, expressed as number of molecules of ATP hydrolyzed per HMM subunit per sec are higher than in Table 2 because the ionic strength was lower (0.045 versus 0.080). The ionic strength was low so that more than 90% of the observed ATP hydrolysis was actin dependent. The best fit of the data (assuming two pathways of hydrolysis) along with the expected errors (assuming a 1% error in each of the $^{18}O_n$-P_i determinations, is shown in Fig. 1B. The value of R for ATPase 1 shifts from 16.7 ± 1.6 at 37 μM HMM to 4.0 ± 1.5 at 0.15 μM HMM; R for ATPase 2 appears to shift from 0.35 ± 0.07 to 0.65 ± 0.20. The contribution of ATPase 2 increases from 14% to 51% between the highest and the lowest HMM concentration.

Table 2. Acto-HMM (tryptic)

HMM, μM	Actin, μM	k_{cat},* sec^{-1}	$[^{18}O_n]P_i$ $n=3$	$n=2$	$n=1$	$n=0$
Starting [γ-$^{18}O_n$]ATP			29.8	40.0	21.2	9.00
3.7†	—	0.11	4.80	10.8	15.3	69.1
	1.1	0.20	5.14	11.4	16.0	67.5
	4.4	0.26	5.59	13.7	18.9	61.7
	11	0.40	6.23	15.6	21.0	57.3
Predicted if R =			(a)	(b)	(c)	(d)
1.4			4.80	10.8	10.6	6.43
16			2.14	7.01	15.3	69.1

* The turnover number per subunit of HMM (M_r = 150,000).
† The reaction mixtures contained, in 1.0 ml total volume, 50 μmol of KCl, 25 μmol of Tris·HCl (pH 7.4), 4 μmol of $MgCl_2$, 2 μmol of [γ-$^{18}O_n$]ATP, 0.015 μmol of [γ-^{32}P]ATP (2.6×10^6 cpm), 1.11 mg of tryptic HMM, and the indicated amounts of F-actin added as an 11 mg/ml suspension in Buffer A (14). Incubation was at 25°C for 20–50 min.

Table 3. Acto-HMM (chymotryptic)

HMM, μM	Actin, μM	k_{cat},* sec^{-1}	$[^{18}O_n]P_i$ $n = 3$	$n = 2$	$n = 1$	$n = 0$
Starting $[\gamma\text{-}^{18}O]_n$-ATP			29.2	35.2	28.6	6.99
37†	2.0	0.45	5.20	10.8	18.2	65.8
15†		1.1	6.92	15.5	26.0	51.6
1.5‡		1.8	11.2	23.1	32.1	33.6
0.15‡		2.1	13.3	26.2	33.6	26.9
Predicted if R =			(a)	(b)	(c)	(d)
1.4			5.20	10.8	13.3	7.24
13			2.50	7.58	18.2	65.8

* The turnover number per subunit of HMM (M_r = 165,000).

† The reactions contained, in 2.0 ml total volume, 70 μmol of KCl, 20 μmol of imidazole·HCl (pH 7.0), 4 μmol of $MgCl_2$, 2 μmol of $[\gamma\text{-}^{18}O_n]$ATP, 0.18 mg of F-actin, and the indicated amounts of chymotryptic HMM (M_r = 330,000). Reaction was for 15 sec at 25°C. The extent of reaction was measured with the Fiske–SubbaRow assay.

‡ The reactions contained, in 10 ml total volume, 350 μmol of KCl, 100 μmol of Imidazole·HCl (pH 7.0), 12 μmol of $MgCl_2$, 2 μmol of $[\gamma\text{-}^{18}O_n]$ATP, 0.9 mg of F-actin, and the indicated amounts of chymotryptic HMM. Reaction was for 40 sec and for 7 min at 25°C for the two samples.

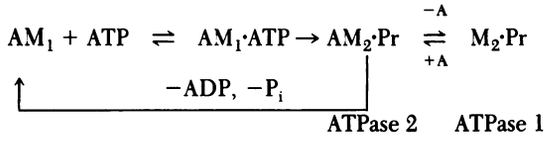
Four conclusions can be drawn: (*i*) the observation of two pathways of hydrolysis cannot be due to local concentration effects because acto-HMM is a homogeneous system (*ii*) the contributions of the two pathways of hydrolysis vary with the HMM/actin molar ratio, suggesting that the two pathways are mutually interdependent (one occurs at the expense of the other); (*iii*) R for ATPase 1 varies in inverse proportion to the observed k_{cat}, as expected if intermediate exchange is catalyzed by dissociated M_2·ADP·P_i and if the recombination with actin limits the observed rate of ATP hydrolysis; and (*iv*) R for ATPase 2 decreases with increasing HMM/actin molar ratio, as was observed for actomyosin (Fig. 1*A*), suggesting that the rate of release of products (k_4) increases when a neighboring rigor complex is present on the same actin filament.

DISCUSSION

The results presented here suggest a different interpretation for the "anomalous" intermediate oxygen exchange properties of actomyosin than made previously. Shukla and Levy (8) suggested that hindered rotation of phosphate oxygens slowed the rate of exchange in myosin when actin was present, but they left open the possibility of several pools of P_i being formed with different ^{18}O enrichments. Sleep *et al.* (11) demonstrated that two pools of P_i with different ^{18}O enrichments were formed by actomyosin ATPase, but they suggested that the limited-exchange pathway was probably due to a local concentration effect—M_2·ADP·P_i held in close proximity to actin by multiple crossbridge interactions between thick and thin filaments could rapidly recombine with actin, resulting in limited intermediate exchange ($R = k_{-3}/k_4 < 1$). However, the results with acto-HMM (Tables 2 and 3; Fig. 1*B*) rule out an interpretation of two pathways of hydrolysis based on local concentration effects.

Comparison of the data in Fig. 1 *A* and *B* shows the similar quantitative behavior of actomyosin and acto-HMM. The kinetic properties of ATPase 1 are inversely proportional to the observed k_{cat}, as expected if $k_4 = k_{cat}$; those of ATPase 2 are influenced by the myosin/actin or HMM/actin molar ratio. ATPase 1 is interpreted as ATP hydrolysis through dissociated M_2·ADP·P_i. ATPase 2 is interpreted as ATP hydrolysis by the undissociated actomyosin crossbridge for two reasons: (*i*) the kinetic properties are similar for associated myosin and actin filaments and for soluble HMM and actin filaments; and (*ii*) $R = k_{-3}/k_4$ increases with increasing actin concentration, suggesting that release of products slows down. If a dissociation/reassociation step were involved, a trend in the reverse direction would be expected, as is observed for ATPase 1.

A simplified mechanism that incorporates the results presented here is:



AM_2·Pr or M_2·Pr is the exchanging intermediate which is a mixture of M_2·ATP and M_2·ADP·P_i in the ratio of approximately 1:10. This scheme shows AM_2·Pr to be the force-generating complex which may (ATPase 2) or may not (ATPase 1) have a long enough lifetime to release products without dissociation of actin. From the data with actomyosin in Table 1 and with acto-HMM (chymotryptic) in Table 3, the ratio ATPase 1/ATPase 2 decreases as the density of rigor complexes decreases. Thus, whether AM_2·Pr releases products or dissociates to form A + M_2·Pr depends on other events on the same actin filament. If a crossbridge is formed or is already present nearby, AM_2·Pr will dissociate and the lifetime will be short. Because $R = k_{-3}/k_4$ also shifts significantly, a neighboring crossbridge also appears to accelerate the release of products.

Both types of cooperativity are advantageous in fast muscle. Mechanisms for shortening the lifetime of the crossbridge complex are necessary because a stable complex would impede fast contraction. Conversely, a stable contractile complex would help to conserve energy in muscle contracting isometrically against a heavy load (6).

Cooperativity between rigor complexes explains the two phases observed after the addition of MgATP to an actomyosin gel (5). The initial clearing phase would be observed because the density of rigor complexes is high before the addition of ATP. The subsequent increase in light-scattering would be due to ATP hydrolysis via ATPase 2.

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