# Heterogeneity in the Actin Activation of Myosin†

Yoshihide Ikeuchi‡ and Christian F. Midelfort*

*Department of Biochemistry, Albert Einstein College of Medicine of Yeshiva University, Bronx, New York 10461*

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ABSTRACT: The soluble proteolytic fragments of myosin, heavy meromyosin and subfragment 1, were prepared with varying amounts of the proteases chymotrypsin and papain, respectively. The kinetics of actin-activated ATP hydrolysis were examined with oxygen-18-labeled ATP. Each preparation of heavy meromyosin and subfragment 1 displayed two pathways of ATP hydrolysis, called respectively the high and low oxygen exchange mechanisms. The contributions of the two mechanisms were found to be sensitive to the potassium chloride concentration. With a fixed concentration of actin (300 $\mu$M), the contribution of the low-exchange mechanism decreased from a maximum of 90% of the ATP hydrolysis at 10 and 20 mM KCl to 12% at 180 mM KCl. The results suggested that the two mechanisms were competing reactions catalyzed by a single species of myosin.

The application of an approach such as oxygen isotope labeling would be expected to provide evidence for the elucidation of the actomyosin ATPase[1] mechanism. The intermediate oxygen exchange during hydrolysis of ATP by myosin has been evaluated on the basis of the reversible hydrolysis of a bound ATP at the active site of myosin (Levy & Koshland, 1959). Analysis of the oxygen exchange by the older method of $[^{18}O]P_i$ analysis, where the $P_i$ is converted to $CO_2$, gives information only about the average value for the $^{18}O$ content of the $P_i$ pool. Recently, analysis of the intermediate oxygen exchange has been improved by information gained from the analysis of the four $[^{18}O_n]P_i$ species ($n$ = 3, 2, 1, and 0) derivatized in the form of trialkyl phosphate ($R_3PO_4$).

The kinetic model of $Mg^{2+}$-dependent myosin ATPase suggests that reversible ATP hydrolysis follows immediately upon binding of ATP in the active site of myosin (Bagshaw & Trentham, 1973):

$$M + ATP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} M(ATP) \xrightarrow{k_2}$$
$$M^*(ATP) \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} M^*(ADP\cdot P_i) \xrightarrow{k_4} M + ADP + P_i \quad (1)$$

M* represents one or more conformations of myosin with increased protein fluorescence. The relatively rapid equilibrium of the reverse cleavage step (step 3) compared with the rate-limiting release of products (step 4) allows the oxygen-labeled $P_i$ to exchange with water oxygens over and over until the $P_i$ is released from $M^*(ADP\cdot P_i)$. The extent of exchange is controlled by the ratio of rates of the $P_i$ releasing and ATP re-forming steps ($R = k_{-3}/k_4$), assuming the rate constant for re-formation of ATP equals that for intermediate exchange.

Theories of the actomyosin ATPase mechanism that are based on the rapid kinetic model suggest that the actomyosin crossbridge dissociates before each cycle of ATP hydrolysis and that reversible hydrolysis is catalyzed by dissociated myosin (Lymn & Taylor, 1971) or by both dissociated myosin and the actomyosin complex, which are in a state of rapid equilibrium with each other (Stein et al., 1979). Actin accelerates ATP hydrolysis at the rate-determining step (step 4) so that the lifetime of the intermediate exchange step is too short to allow extensive incorporation of water oxygens into $P_i$. The assumption that a rapid equilibrium exists between the actomyosin complex and dissociated myosin during each cycle of ATP hydrolysis predicts that the intermediate oxygen exchange would be correlated with the observed ATPase activity. However, older oxygen exchange data where $P_i$ was combusted to $CO_2$ are not consistent with this prediction (Levy & Koshland, 1959; Shukla & Levy, 1978). Recent studies on the distributions of $[^{18}O_n]P_i$ species suggest that this inconsistency arises from the existence of two oxygen exchange pathways in the actomyosin ATPase reaction.

As described previously (Midelfort, 1981), kinetic analysis of the oxygen exchange catalyzed by myosin and heavy meromyosin shows two pools of phosphate formed when actin is added. One pool is the product of a "high-exchange pathway", which has the kinetic properties expected of the Lymn and Taylor mechanism. The other pool is the product of a "low-exchange pathway", where the ratio of rate constants for intermediate exchange and for release of products is approximately 0.5. This ratio is independent of actin concentration, which suggests that the intermediate stages of ATP hydrolysis are initiated after binding to actin.

In attempting to rationalize the low-exchange pathway, there is the complication that, as shown in the rapid kinetic model, myosin can catalyze the burst of ATP hydrolysis at a rapid rate. However, the rate can vary greatly from one preparation to the next. This has been observed with cardiac myosin (Taylor & Weeds, 1976) and with fractions of skeletal muscle subfragment 1 (Inoue & Tonomura, 1976). The intermediate oxygen exchange can also vary from one preparation to the next. Differences between preparations of subfragment 1 and heavy meromyosin (Shukla & Levy, 1977, 1978) and of heavy meromyosin (Midelfort, 1981) have been

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‡ Present address: The Graduate School of Science and Technology, Kobe University, Kobe 657, Japan.

[1] Abbreviations: A or actin, F-actin; ATP, adenosine 5′-triphosphate; ATPase, adenosinetriphosphatase; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HMM, heavy meromyosin; $k_{cat}$, the turnover number per subunit of myosin; $LC_1$ and $LC_3$, the alkaline light chains of myosin; $LC_2$, the DTNB or regulatory light chain of skeletal muscle myosin; M, myosin; $P_i$, inorganic phosphate; SDS, sodium dodecyl sulfate; subfragment 1 or S-1, heavy meromyosin subfragment 1; TCA, trichloroacetic acid.

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reported. It has been suggested that the disappearance of the low-exchange pathway might result from proteolytic damage to a component of myosin (Shukla et al., 1983; Midelfort, 1981).

In the present study, we prepared subfragment 1 and heavy meromyosin by protease treatment of myosin. The oxygen exchange properties of subfragment 1 and heavy meromyosin retaining the $LC_2$ were compared with those depleted in the $LC_2$. The result in this paper demonstrated that the two pathways of hydrolysis were present in all subfragment 1 and heavy meromyosin preparations. In addition, the contributions of the two pathways were sensitive to the KCl concentration. Conditions were found where either one pathway or the other was approximately 90% of the ATP hydrolysis.

## Materials and Methods

Myosin was prepared in the presence of protease inhibitors because, in their absence, the DTNB light chain ($LC_2$) content varied from one preparation to the next and declined during storage. The Guba–Straub buffer contained 1 mM diisopropyl fluorophosphate, 0.1 mM phenylmethanesulfonyl fluoride, and 0.01 mg/mL soybean trypsin inhibitor. Otherwise, the procedure of Mommaerts & Parish (1951) was followed. The third myosin precipitate was dissolved to a concentration of 30 mg/mL in 150 mM potassium phosphate buffer, pH 6.5, and 300 mM KCl, mixed with an equal volume of glycerol, and stored at −20 °C.

Heavy meromyosin was prepared with chymotrypsin by a modification of the procedure of Weeds & Pope (1977), and subfragment 1 was prepared with papain (Margossian et al., 1975). The soluble myosin fragment was concentrated by ammonium sulfate precipitation between 45 and 60% saturation. The precipitate was dissolved in 20 mM imidazole hydrochloride, pH 7.0, containing 0.5 mM DTT and dialyzed against several changes of this buffer. It was stored frozen at −80 °C without apparent loss of activity.

The $LC_2$ contents of various myosin, heavy meromyosin, and subfragment 1 preparations were assayed by the light chain kinase–calmodulin–[$\gamma$-$^{32}$P]ATP system (Yazawa & Yagi, 1978). In addition, the presence or absence of the $LC_2$ was checked by SDS–polyacrylamide gel electrophoresis with a 15% acrylamide gel (Laemmli, 1970). The gel was stained with Coomassie Blue.

F-Actin was prepared by the method of Spudich & Watt (1971). Before use in oxygen exchange experiments, the actin was precipitated by ultracentrifugation and resuspended in 10 mM imidazole hydrochloride, pH 7.0, containing 2 mM $MgCl_2$ and 0.5 mM DTT. In some cases, the centrifugation step was repeated to further remove inorganic phosphate and ATP. For determination of inorganic phosphate content, a sample of the washed actin was precipitated with 200 mM $HClO_4$, and the supernatant was treated with charcoal (10 mg/mL final concentration) to remove ADP and ATP. The inorganic phosphate was then determined by the method of Ames (1966). Tropomyosin was prepared by the method of Greaser & Gergely (1969), and the tropomyosin/troponin complex was prepared as described by Spudich & Watt (1971).

Protein concentrations were measured by the extinction coefficient at 280 nm for a 1% solution of 5.3 for myosin, 6.47 for heavy meromyosin (Young et al., 1964), 7.8 for subfragment 1 (Johnson & Taylor, 1978), and 11.5 for actin (Eaton et al., 1975).

*[$^{18}O$]ATP. Method 1.* The procedure of Penefsky et al. (1960) was followed except that the ATP was labeled in both the $\beta$- abd $\gamma$-phosphates by starting from AMP and a catalytic amount of ADP. Adenylate kinase was present at a concentration of 0.005 mg/mL. the [$^{18}$O]ATP was isolated as described under Method 2.

*Method 2.* The reaction mixture contained, in a total volume of 100 mL, 20 mM triethanolamine hydrochloride, pH 7.2, 8 mM $MgCl_2$, 7 mM AMP, 0.02 mM ADP, 15 mM dilithium acetyl [$^{18}$O]phosphate, 0.01 mg/mL acetate kinase, and 0.005 mg/mL adenylate kinase. After 20 min at room temperature, the reaction was stopped by the addition of a 100% TCA solution to 5% final concentration. Denatured protein was removed by centrifugation in the cold, and the TCA was extracted from the supernatant with ether. The water layer was neutralized and chromatographed on a DEAE-cellulose column (Whatman DE-52, 2.6 × 60 cm) in the bicarbonate form, equilibrated with 100 mM triethylammonium bicarbonate. The column was developed with a 4-L gradient of triethylammonium bicarbonate from 100 to 350 mM. The ATP was concentrated and desalted under reduced pressure. Traces of triethylamine were removed by several additions of methanol. The ATP was stored as a solution in methanol at −20 °C. The yield was 580 $\mu$mol (83%).

The enzymes used were purchased from Boehringer Mannheim. Oxygen-18-labeled water (99.8% $^{18}$O) was from Norsk Hydro (New York), and $PCl_5$ was from Baker. Dipotassium [$^{18}$O]phosphate was prepared from $PCl_5$ and [$^{18}$O]$H_2O$ as described by Hackney et al. (1980). Acetyl [$^{18}$O]phosphate was prepared from the dipotassium [$^{18}$O]-phosphate by incubation with a slight excess of acetic anhydride in 50% aqueous pyridine, and it was crystallized as the dilithium salt (Stadtman, 1957).

*Steady-State ATPase Assays.* The reaction mixtures contained, in a total volume of 1 mL, 50 mM KCl, 20 mM imidazole hydrochloride, pH 7.0, 5 mM $MgCl_2$, 3 mM [$\gamma$-$^{32}$P]ATP [(0.5–2) × $10^5$ cpm/$\mu$mol], 0–300 $\mu$M actin, and 1–4 $\mu$M myosin, heavy meromyoxin, or subfragment 1. Incubations were for various times at 25 °C, and aliquots of 0.2 mL were removed and mixed with 0.1 mL of cold 0.2 N $HClO_4$. The unreacted ATP was absorbed to charcoal (10 mg/mL final concentration) at 0 °C, and, after low-speed centrifugation, an aliquot of the supernatant was counted.

*[$^{18}O$]ATP Hydrolysis and Analysis of [$^{18}O$]$P_i$.* Oxygen-18-labled ATP, containing a trace of [$\gamma$-$^{32}$P]ATP, was incubated at 25 °C with heavy meromyosin or subfragment 1 in unenriched water under the conditions described in the tables. Trials were performed to calculate the time required for 90% reaction. In addition, 20-$\mu$L samples were removed from the reaction mixtures to confirm the hydrolysis rate. The reactions were quenched on ice at 75–100% of ATP hydrolysis by the addition of $HClO_4$ to 0.2 N in a 4-mL final volume. Denatured protein was removed by centrifugation at 10 000 rpm. The supernatant was treated twice with washed charcoal (10 mg/mL final concentration) in the cold. The [$^{18}$O]$P_i$ in the charcoal supernatant was precipitated as the molybdate complex with triethylammonium chloride, crystallized as magnesium ammonium phosphate, and dried in vacuo (Boyer & Bryan, 1967). The sample was dissolved with a few beads of Bio-Rad AG-50 ($H^+$), passed through a 0.5-mL column of AG-50, and dried in vacuo. It was dissolved in 0.1 mL of methanol and methylated with a fresh diazomethane solution in ether.

The relative abundances of the four $^{18}O_n$-labeled trimethyl phosphate species ($n$ = 0–3) were measured by chemical ionization mass spectrometry (Finnigan Model 3300) using an SE-30 capillary GC column with helium as carrier gas and isobutane as ionizing gas. The areas of the peaks at $m/e$ 141,

Table I: $P_i$ from [$^{18}$O]ATP Hydrolysis by Acto Heavy Meromyosin[a]

| HMM | [actin] ($\mu$M) | $k_{cat}$ ($s^{-1}$)[b] | [$^{18}O_n$]$P_i$[c] | | | | low-exchange pathway | | high-exchange pathway | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | *a* | *b* | *c* | *d* | *R* | % | *R* | % |
| starting ATP[d] | | | 78.1 | 9.0 | 1.1 | 11.8 | | | | |
| 75% $LC_2$ content | 0 | 0.041 | 18.5 | 10.1 | 9.1 | 62.3 | 0.41 | 26 | 26 | 74 |
| | 1 | 0.12 | 30.4 | 15.6 | 10.3 | 43.7 | 0.45 | 46 | 16 | 54 |
| | 4 | 0.24 | 30.8 | 16.0 | 11.2 | 42.0 | 0.42 | 45 | 12 | 55 |
| | 20 | 0.68 | 35.6 | 19.5 | 13.8 | 31.1 | 0.40 | 50 | 7.0 | 50 |
| | 100 | 2.38 | 41.3 | 21.7 | 12.8 | 24.2 | 0.40 | 57 | 5.0 | 43 |
| 6% $LC_2$ content | 0 | 0.065 | 11.7 | 6.7 | 6.5 | 75.1 | 0.40 | 16 | 50 | 84 |
| | 1 | 0.17 | 22.2 | 14.2 | 9.4 | 54.2 | 0.77 | 41 | 30 | 59 |
| | 4 | 0.39 | 19.0 | 13.5 | 11.5 | 60.0 | 0.75 | 31 | 20 | 69 |
| | 20 | 1.14 | 19.6 | 17.8 | 19.0 | 43.6 | 0.52 | 17 | 7.1 | 83 |
| | 100 | 4.6 | 36.6 | 26.8 | 16.2 | 20.4 | 0.70 | 41 | 2.5 | 59 |

[a] The reaction mixtures contained, in a total volume of 2 mL, 50 mM KCl, 25 mM imidazole hydrochloride, pH 7.0, 2 mM $MgCl_2$, 1 mM [$^{18}$O]ATP, 1.2 $\mu$M heavy meromyosin, and the indicated amounts of actin. [b] The turnover number per subunit of heavy meromyosin ($M_r$ 170 000). [c] See equations under Materials and Methods. [d] The [$^{18}$O]ATP was prepared by method 1. The abundances of the four [$^{18}O_n$]$PO_3$ species in the $\gamma$-phosphoryl group of ATP were determined by using the $Ca^{2+}$-ATPase activity of myosin, which proceeds with little or no intermediate oxygen exchange (Levy & Koshland, 1959).

143, 145, and 147 were measured by selected-ion monitoring with a Finnigan 6000 data system. The percentages were corrected for natural $^{18}$O abundance (0.204%) and for a variable $P_i$ contamination introduced along with the actin.

*Analysis of the Oxygen Exchange Reaction.* The relationship between the percentages of the four [$^{18}$O]$P_i$ species and the kinetics of ATP hydrolysis by myosin ATPase is given by the following equations (Midelfort, 1981). For [$^{18}O_3$]$P_i$

$$a = a_0\left(\frac{4}{3R + 4}\right)$$

For [$^{18}O_2$]$P_i$

$$b = (a_0 + b_0 - a)\left(\frac{2}{R + 2}\right)$$

For [$^{18}O_1$]$P_i$

$$c = (a_0 + b_0 + c_0 - a - b)\left(\frac{4}{R + 4}\right)$$

For [$^{18}O_0$]$P_i$

$$d = 100 - (a + b + c)$$

The percentages in the starting ATP are $a_0$, $b_0$, $c_0$, and $d_0$, and the percentages in the $P_i$ formed are $a$, $b$, $c$, and $d$. The kinetic ratio $R = k_{-3}/k_4$ is the ratio of rate constants for reversal of ATP hydrolysis and for release of products. The smaller the value of $R$, the more rapid the release of products and the more the $^{18}$O labeling in $P_i$ "shifts to the left", resembling the starting ATP. The larger the value of $R$, the slower the release of products and the more extensive the intermediate oxygen exchange. The labeling in $P_i$ then "shifts to the right" toward unlabeled $P_i$ ([[$^{18}O_0$]$P_i$] = $d$).

The percentages of the four [$^{18}$O]$P_i$ species were fit by assuming two pathways of hydrolysis of ATP by myosin ATPase because they deviated systematically from the amounts predicted by a single pathway. With the aid of a Hewlett-Packard 97 programmable calculator, the observed ratio of $a/b$ was matched to a value of $R_1 = k_{-3}/k_4$, which produced the same ratio of $a/b$, and an entire set of $a_1/b_1/c_1/d_1$ was calculated. These were subtracted from $a/b/c/d$, and the difference, $c_2/d_2$, was used to calculate a value of $R_2$ for the second pathway of hydrolysis. An entire distribution of $a_2/b_2/c_2/d_2$ was then calculated and subtracted from $a/b/c/d$ to give a more realistic value for $a_1/b_1$. This process was continued until an exact fit to the data was obtained. There was enough information contained in the percentages of the four [$^{18}$O]$P_i$ species for $R_1$ and $R_2$ and the contribution of each pathway to the observed ATP hydrolysis to be calculated.

The data were also fit to two pathways of hydrolysis with a computer program developed at the Albert Einstein Computing Center, which employed statistical methods. The results were equivalent to those obtained with the more laborious method described in the preceding paragraph.

## Results

In previous experiments (Midelfort, 1981), a difference between the intermediate oxygen exchange catalyzed by chymotryptic and tryptic heavy meromyosins was described. The properties of actin-activated chyrotryptic heavy meromyosin more closely resembled those of actomyosin in displaying two pathways of ATP hydrolysis, while those of actin-activated tryptic heavy meromyosin showed mainly a single pathway. Since tryptic digestion is known to produce a more heterogeneous population of heavy meromyosin species, it was suggested that the disappearance of the second pathway was the result of proteolytic damage. We decided to monitor the $LC_2$ content since it has been suggested that the presence or absence of $LC_2$ may alter the actin affinity of skeletal muscle myosin (Margossian et al., 1975) and may influence the oxygen exchange properties of acto heavy meromyosin and acto subfragment 1 (Shukla et al., 1983).

*Heavy Meromyosin.* Soluble myosin was digested in high-salt solution with varying concentrations of chymotrypsin in the presence of $Ca^{2+}$ to protect the $LC_2$ (Weeds & Pope, 1977). Figure 1A shows an SDS gel electrophoretic profile of the digestion products. There was a decrease in the $LC_2$ content accompanied by a darkening of the $LC_3$ band. The increase in the latter presumably represented the comigration of the $LC_3$ with the "truncated" $LC_2$, which had lost the light chain kinase specific phosphorylation site but retained the divalent metal ion binding site (Bagshaw, 1977). When the highest chymotrypsin concentration was added (0.1 mg/mL), the digested product contained such small amounts of the $LC_2$ (6%) that we could not detect it on 15% gels. On the other hand, there was no obvious change in the heavy chains even at the highest concentration of chymotrypsin.

The experimental distributions of $^{18}$O-labeled $P_i$ formed by hydrolysis of [$^{18}$O]ATP in normal water are given in Table I. The two heavy meromyosin preparations used contained 75 and 6% of the theoretical 2 mol of $LC_2$/myosin dimer. The

Table II: $P_i$ from [$^{18}$O]ATP Hydrolysis by Acto Subfragment 1[a]

| | S-1 | [actin] ($\mu$M) | $k_{cat}$ ($s^{-1}$) | [$^{18}$O]$P_i$ | | | | low-exchange pathway | | high-exchange pathway | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *a* | *b* | *c* | *d* | *R* | % | *R* | % |
| starting ATP[b] | | | | 78.1 | 9.0 | 1.1 | 11.8 | | | | |
| | Mg–S-1 | 0 | 0.028 | 27.5 | 15.5 | 12.8 | 44.2 | 0.40 | 37 | 12 | 63 |
| | 72% $LC_2$ | 2 | 0.12 | 32.8 | 17.4 | 12.8 | 37.0 | 0.38 | 45 | 9.5 | 55 |
| | | 6 | 0.16 | 34.1 | 19.0 | 13.9 | 33.0 | 0.40 | 46 | 7.2 | 54 |
| | | 20 | 0.36 | 37.2 | 21.7 | 14.4 | 26.7 | 0.42 | 48 | 5.0 | 52 |
| | | 100 | 0.70 | 48.2 | 23.6 | 11.2 | 17.3 | 0.35 | 59 | 2.3 | 41 |
| | EDTA–S-1 | 2 | 0.18 | 37.8 | 16.9 | 10.0 | 35.3 | 0.35 | 55 | 12.0 | 45 |
| | 21% $LC_2$ | 6 | 0.30 | 35.4 | 17.6 | 11.8 | 35.2 | 0.38 | 50 | 10.0 | 50 |
| | | 20 | 0.50 | 29.6 | 19.1 | 15.5 | 35.8 | 0.45 | 38 | 7.2 | 62 |
| | | 100 | 1.25 | 41.6 | 25.3 | 14.7 | 18.4 | 0.35 | 36 | 2.2 | 64 |
| | EDTA–S-1 | 0 | 0.043 | 23.1 | 13.6 | 12.1 | 51.2 | 0.41 | 31 | 15.5 | 69 |
| | 6% $LC_2$ | 2 | 0.19 | 29.8 | 15.9 | 11.6 | 42.7 | 0.40 | 43 | 13.0 | 57 |
| | | 6 | 0.31 | 27.2 | 17.2 | 14.1 | 41.5 | 0.47 | 37 | 9.8 | 63 |
| | | 20 | 0.51 | 31.3 | 21.4 | 17.7 | 39.6 | 0.47 | 35 | 6.8 | 65 |
| | | 100 | 1.25 | 40.8 | 25.3 | 15.3 | 18.6 | 0.40 | 37 | 2.2 | 63 |
| starting ATP[c] | | | | 97.4 | 2.0 | 0.1 | 0.5 | | | | |
| | chymotryptic S-1 | 0 | 0.10 | 10.4 | 6.3 | 11.0 | 60.7 | 0.51 | 11 | 45 | 89 |
| | | 3 | 0.42 | 19.3 | 13.6 | 14.0 | 53.1 | 0.78 | 22 | 17 | 78 |
| | | 8 | 0.98 | 25.9 | 18.4 | 17.4 | 38.3 | 0.78 | 29 | 10 | 71 |
| | | 20 | 1.51 | 25.1 | 20.4 | 20.6 | 33.9 | 0.75 | 20 | 6.9 | 80 |
| | | 100 | 5.31 | 46.3 | 29.2 | 16.8 | 7.8 | 0.58 | 20 | 1.9 | 80 |

[a] The reactions were carried out under the same conditions as in Table I except that subfragment 1 was present at a concentration of 3.8 $\mu$M. [b] See Table I. [c] The ATP was prepared by method 2. The oxygen-18 labeling was measured as described previously (Midelfort & Rose, 1976).

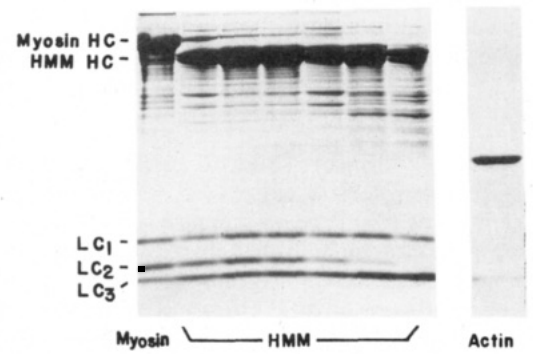



FIGURE 1: SDS gel electrophoresis of heavy meromyosin. Soluble myosin (20 mg/mL) was digested with varying amounts of chymotrypsin in a buffer containing 400 mM KCl, 20 mM potassium phosphate, pH 7.0, and 10 mM $CaCl_2$ for 15 min at 25 °C, and the reaction was stopped with phenylmethanesulfonyl fluoride (1 mM final concentration). After a 10-fold dilution with cold distilled water, the samples were centrifuged at 30000*g* for 40 min. The $LC_2$ contents were measured by the light chain kinase assay. A similar amount of protein (60 $\mu$g) from each supernatant was applied to the gel. Myosin (90 $\mu$g) and actin (15 $\mu$g) were used as markers. The amounts of chymotrypsin and the $LC_2$ contents were as follows (left to right): 0.002 mg/mL, 75%; 0.004 mg/mL, 72%; 0.008 mg/mL, 68%; 0.02 mg/mL, 42%; 0.04 mg/mL, 22%; 0.1 mg/mL, 6%.

right side of Table I shows the values of $R$ for the two mechanisms of ATP hydrolysis that fit each set of data. Also, the contribution of each pathway is given as the percentage of the total ATP hydrolysis.

The data for chymotryptic heavy meromyosin retaining 75% $LC_2$ content show that each pathway contributed approximately 50% to the observed ATP hydrolysis over a range of actin concentrations from 1 to 100 $\mu$M. These results are similar to those of Shukla et al. (1983), who found a constant stoichiometry between the two pathways, although in their experiments the low-exchange mechanism made approximately a 30% contribution. The kinetic ratio $R = k_{-3}/k_4$ for the low-exchange mechanism did not change with actin concentration. By contrast, the kinetic ratio $R$ for the high-exchange mechanism decreased from 26 in the absence of added actin to 5 at 100 $\mu$M actin. The change in $R$ was as expected if intermediate exchange was catalyzed by dissociated myosin and the release of products occurred upon the reassociation with actin.

The $LC_2$-deficient heavy meromyosin, obtained by digestion with a high concentration of chymotrypsin, had a diminished low-exchange pathway compared to the heavy meromyosin retaining the $LC_2$, although the decrease was not as large as the decrease in the $LC_2$ content. This suggests that the $LC_2$, at least that portion which contains the $LC_2$ light chain kinase specific site, was not what determined the proportions of the two mechanisms of hydrolysis. However, the $LC_2$-deficient heavy meromyosin probably retained some of the "truncated" $LC_2$, which may have influenced the oxygen exchange properties.

*Subfragment 1.* Subfragment 1 containing various $LC_2$ contents was obtained by papain treatment of filamentous myosin either in the presence of $MgCl_2$ or EDTA (Margossian et al., 1975). The SDS gel electrophoretic profile of the subfragment 1 preparations is shown in Figure 2. There was a substantial decrease in the $LC_2$ content in EDTA–subfragment 1, even in the earliest time points. On the other hand, $MgCl_2$ protected the $LC_2$, in agreement with the report of Margossian et al. The intensity of the $LC_2$ band did not agree with the $LC_2$ light chain kinase measurements, suggesting that an unidentified band of this molecular weight was formed from the heavy chains or the $LC_1$ light chain. Even though there was a darkening of the $LC_3$ band, a truncted $LC_2$ apparently did not remain in EDTA–subfragment 1 (Bagshaw & Reed, 1976).

The intermediate oxygen exchange catalyzed by several of the subfragment 1 preparations is shown in Table II. The results with Mg–subfragment 1 were similar to those obtained with heavy meromyosin in that the low-exchange mechanism made approximately a 50% contribution over a wide range of actin concentrations. The two $LC_2$-deficient EDTA–subfragment 1 preparations exhibited a decreased contribution of the low-exchange mechanism, although the decrease was not as large as the change in $LC_2$ content. We also examined the intermediate oxygen exchange catalyzed by a preparation of chymotryptic subfragment 1 (Weeds & Taylor, 1975). This

Table III: Heavy Meromyosin in the Absence of Actin[a]

| [KCl] (mM) | $k_{cat}$ ($s^{-1}$) | [$^{18}$O]$P_i$ | | | | low-exchange pathway | | high-exchange pathway | |
|---|---|---|---|---|---|---|---|---|---|
| | | *a* | *b* | *c* | *d* | *R* | % | *R* | % |
| 10 | 0.086 | 11.9 | 5.8 | 6.7 | 75.6 | 0.38 | 13 | 48 | 97 |
| 50 | 0.095 | 10.6 | 5.6 | 6.5 | 77.3 | 0.42 | 10 | 52 | 90 |
| 100 | 0.088 | 11.1 | 5.6 | 6.6 | 76.7 | 0.40 | 11 | 50 | 89 |
| 150 | 0.085 | 11.4 | 5.7 | 6.5 | 76.4 | 0.40 | 11 | 50 | 89 |

[a] The reaction mixtures contained, in 0.5 mL, 20 mM imidazole hydrochloride, pH 7.0, 5 mM $MgCl_2$, 4 mM [$^{18}$O]ATP, 30 $\mu$M heavy meromyosin (10 mg/mL, 70% $LC_2$ content), and the indicated amounts of KCl. The reactions were at 25 °C for 25 min. The ATP preparation was by Method 2 (see the bottom of Table II).

Table IV: Actomyosin ATPase at Three KCl Concentrations[a]

| [KCl] (mM) | [actin] ($\mu$M) | $k_{cat}$ ($s^{-1}$) | [$^{18}$O]$P_i$ | | | | low-exchange pathway | | high-exchange pathway | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | *a* | *b* | *c* | *d* | *R* | % | *R* | % |
| 50 | 0 | 0.042 | 14.3 | 6.2 | 6.1 | 73.4 | 0.39 | 16 | 53 | 86 |
| | 6 | 1.21 | 56.3 | 20.6 | 10.3 | 12.7 | 0.42 | 69 | 6.3 | 31 |
| | 15 | 1.65 | 60.8 | 20.9 | 9.3 | 9.0 | 0.40 | 74 | 5.0 | 26 |
| 100 | 0 | 0.045 | 9.1 | 4.1 | 4.3 | 82.5 | 0.40 | 10 | 85 | 90 |
| | 6 | 0.37 | 31.0 | 15.3 | 12.7 | 41.0 | 0.51 | 37 | 15 | 63 |
| | 15 | 0.55 | 34.0 | 16.3 | 13.6 | 36.1 | 0.46 | 39 | 12 | 61 |
| 150 | 0 | 0.035 | 5.5 | 2.8 | 3.1 | 88.6 | 0.42 | 6 | 121 | 94 |
| | 6 | 0.083 | 11.4 | 6.5 | 7.6 | 74.6 | 0.45 | 12 | 42 | 88 |
| | 15 | 0.121 | 13.9 | 7.8 | 9.0 | 69.3 | 0.44 | 15 | 33 | 85 |

[a] The reaction mixtures contained, in 2 mL, 20 mM imidazole hydrochloride pH 7.0, 4 mM $MgCl_2$, 2 mM [$^{18}$O]ATP, 1 $\mu$M myosin (0.47 mg/mL), and the indicated amounts of KCl and actin. The actin stock solution (5.8 mg/mL, 130 $\mu$M) contained 8 $\mu$M $P_i$. The myosin was purified by ammonium sulfate fraction to remove actomyosin (Starr & Offer, 1971) and was used immediately after isolation from the rabbit. The [$^{18}$O]ATP was by Method 2 (see Table II).

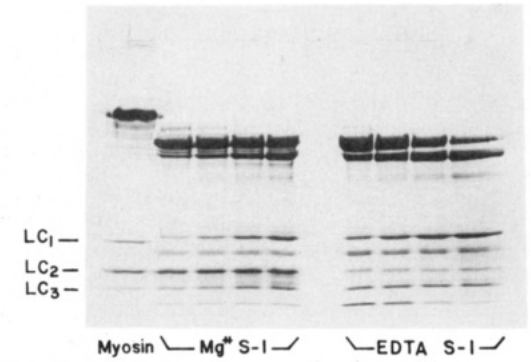



FIGURE 2: Time course of papain digestion of myosin filaments (30 mg/mL) in the presence of 2 mM $MgCl_2$ or 2 mM EDTA. Myosin filaments were formed by dialysis against 200 mM ammonium acetate, pH 7.2, and digested with papain (0.06 mg/mL) at 25 °C for various times. The reactions were terminated by the addition of iodoacetamide to 1 mM final concentration. After centrifugation at 25000*g* for 60 min, the supernatants were removed for examination by SDS gel electrophoresis and measurement of $LC_2$ content. The amounts of protein applied to the gel were as in Figure 1. The times of digestion and the $LC_2$ contents were as follows (left to right): (Mg–subfragment 1) 2 min, 74%; 5 min, 70%; 10 min, 72%; 20 min, 70%; (EDTA–subfragment 1) 2 min, 21%; 5 min, 12%; 10 min, 9%; 20 min, 6%.

preparation contained 6% $LC_2$ content by the light chain kinase assay. The low oxygen exchange mechanism was approximately 20% of the ATP hydrolysis. Thus, there was at least some correlation between the $LC_2$ content and the contribution of the low-exchange mechanism.

*The Actin-Independent Low-Exchange Pathway.* The preparations of heavy meromyosin and subfragment 1 displayed two pathways of hydrolysis in the absence of added actin. For example, for the two heavy meromyosin preparations in Table I, 26 and 16% of the hydrolysis was by a low-exchange pathway. As shown in Table III, this component was not sensitive to the concentration of KCl. The insensitivity to KCl concentration distinguished it from the actin-dependent low-exchange mechanism, which was sensitive to KCl (see below). Shukla et al. (1983) called this pathway an "induced slow exchange". The term slow exchange indicates that the observed rate of ATP hydrolysis is slow compared to the actin-activated low-exchange pathway. We will adopt this term in the following discussion.

*Sensitivity of the Actin-Dependent Low-Exchange Mechanism to KCl Concentration.* The potassium chloride concentration is an important parameter in studying actomyosin ATPase. The maximum activation of filamentous myosin by actin is observed in the range of 30–80 mM KCl. At higher KCl concentrations, the activation is greatly reduced. Table IV shows actomyosin ATPase at three KCl concentrations. The actin-activated ATPase activity decreased approximately 20-fold over this range. The oxygen-18-labeling data show that the low-exchange mechanism was also sensitive to the KCl concentration. Its contribution was highest (74%) at 50 mM KCl and lowest (15%) at 150 mM KCl. Thus, there was a significant correlation between the actin-activated ATPase activity and the contribution of the low-exchange mechanism.

The oxygen exchange in the absence of added actin was also examined. In an attempt to minimize the contamination by actin, the myosin preparation was purified by ammonium sulfate fractionation (Starr & Offer, 1971). The data in Table IV show that the contribution of the low-exchange mechanism decreased from 16 to 6% in going from 50 to 150 mM KCl. Since the induced slow exchange was not sensitive to KCl concentration, the results show that less than 6% of the ATP hydrolysis was due to the induced slow exchange form of myosin. On the other hand, the myosin preparation appeared still to contain a small actin contamination, which was responsible for most of the low oxygen exchange pathway at 50 mM KCl.

*Intermediate Oxygen Exchange at High Actin Concentrations.* The results with heavy meromyosin and subfragment 1 retaining the $LC_2$ showed that the low-exchange mechanism was approximately 50% of the ATP hydrolysis over a range of actin concentrations from 1 to 100 $\mu$M. These results might

Table V: Actin Activation of Subfragment 1[a]

| | [S-1] (μM) | [actin] (μM) | $k_{cat}$ ($s^{-1}$) | [$^{18}$O]$P_i$ a | b | c | d | low-exchange pathway R | % | high-exchange pathway R | % |
|---|---|---|---|---|---|---|---|---|---|---|---|
| starting ATP[c] | | | | 97.4 | 2.0 | 0.1 | 0.5 | | | | |
| | 0.017[d] | 10 | 2.0 | 43.6 | 21.6 | 16.2 | 18.6 | 0.41 | 43 | 5.1 | 57 |
| | 0.050[e] | 30 | 3.9 | 51.5 | 24.7 | 14.2 | 9.8 | 0.42 | 48 | 3.1 | 52 |
| | 0.083[f] | 50 | 2.8 | 55.8 | 25.0 | 12.5 | 6.6 | 0.41 | 51 | 2.4 | 49 |
| | 0.167 | 100 | 4.1 | 60.3 | 24.6 | 10.4 | 4.7 | 0.41 | 59 | 2.0 | 41 |
| | 0.33 | 200 | 4.6 | 67.4 | 22.3 | 6.9 | 3.3 | 0.44 | 86 | 2.9 | 14 |
| | 0.50 | 300 | 7.9 | 70.6 | 21.2 | 5.6 | 2.6 | 0.42 | 92 | 3.3 | 8 |
| | 1 | 10 | 0.60 | 37.1 | 21.0 | 16.7 | 25.2 | 0.52 | 39 | 6.4 | 61 |
| | 3 | 30 | 1.6 | 44.7 | 23.5 | 16.2 | 15.6 | 0.49 | 46 | 4.5 | 54 |
| | 5 | 50 | 2.4 | 51.7 | 24.3 | 14.2 | 9.8 | 0.42 | 49 | 3.3 | 51 |
| | 10 | 100 | 3.4 | 58.9 | 23.9 | 10.8 | 6.4 | 0.44 | 65 | 2.6 | 35 |
| | 20 | 200 | 6.9 | 65.0 | 23.0 | 8.1 | 3.8 | 0.43 | 78 | 2.4 | 22 |
| | 30 | 300 | 7.4 | 67.3 | 22.5 | 7.0 | 3.2 | 0.44 | 85 | 2.7 | 15 |

[a] The reaction mixtures contained, in a total volume of 0.7 mL, 20 mM imidazole hydrochloride, pH 7.0, 30 mM KCl, 5 mM $MgCl_2$, 3 mM [$^{18}$O]ATP, and the indicated amounts of subfragment 1 and actin. [b] The stock solution of actin (27 mg/mL, 600 μM) contained 0.018 mM $P_i$. [c] See Table II. [d] The reaction contained, in 5 mL, 0.4 mM [$^{18}$O]ATP and 2.4 mM $MgCl_2$. Other conditions as in footnote *a*. [e] The reaction contained, in 2 mL, 1 mM [$^{18}$O]ATP and 3 mM $MgCl_2$. Other conditions as in footnote *a*. [f] The reaction contained, in 1 mL, 2 mM [$^{18}$O]ATP and 4 mM $MgCl_2$. Other conditions as in footnote *a*.

Table VI: Heavy Meromyosin and Subfragment 1 at 300 μM Actin[a]

| [KCl] (mM) | $k_{cat}$ ($s^{-1}$) | [$^{18}$O]$P_i$ a | b | c | d | low-exchange pathway R | % | high-exchange pathway R | % |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Heavy Meromyosin | | | | | |
| 10 | 12.3 | 69.2 | 21.6 | 6.0 | 3.1 | 0.44 | 91 | 3.7 | 9 |
| 20 | 10.2 | 65.3 | 23.6 | 7.8 | 3.2 | 0.49 | 83 | 2.6 | 17 |
| 30 | 8.3 | 62.9 | 24.9 | 8.6 | 3.6 | 0.56 | 84 | 2.9 | 16 |
| 50 | 5.6 | 51.9 | 26.7 | 14.0 | 7.4 | 0.52 | 46 | 2.3 | 54 |
| 80 | 2.7 | 34.3 | 25.4 | 21.1 | 19.2 | 0.54 | 20 | 3.7 | 80 |
| 100 | 2.0 | 29.1 | 22.8 | 21.9 | 26.2 | 0.44 | 15 | 4.8 | 85 |
| 150 | 0.83 | 18.6 | 15.1 | 18.7 | 47.5 | 0.41 | 12 | 10.3 | 88 |
| 180 | 0.55 | 17.4 | 12.8 | 16.3 | 53.5 | 0.39 | 13 | 13.4 | 87 |
| | | | | Subfragment 1 | | | | | |
| 20 | 15.1 | 73.2 | 20.5 | 4.4 | 1.9 | 0.40 | 97 | 5.8 | 3 |
| 20 | 16.4 | 72.9 | 20.6 | 4.6 | 1.9 | 0.40 | 96 | 3.7 | 4 |

[a] The reactions contained, in 0.5 mL, 20 mM imidazole hydrochloride, pH 7.0, 4 mM [$^{18}$O]ATP, 5 mM $MgCl_2$, 300 μM actin, 1 μM heavy meromyosin or subfragment 1, and the indicated amounts of KCl. The actin shock solution (38.7 mg/mL, 860 μM) contained 0.103 mM $P_i$. The [$^{18}$O]ATP preparation was the same as in the bottom of Table II.

suggest that the proportion of the two pathways was a constant for a particular preparation of heavy meromyosin or subfragment 1. However, the results with actomyosin showed that the contribution of the low-exchange mechanism was sensitive to KCl concentration. KCl might also be an important parameter with heavy meromyosin and subfragment 1. Table V shows an experiment with Mg–subfragment 1, where we decreased the KCl concentration to 30 mM. The actin concentration was varied between 10 and 300 μM. The low-exchange mechanism increased to 85–92% of the ATP hydrolysis at 300 μM actin. As in the previous experiments, the kinetic properties of the low-exchange mechanism were independent of actin concentration.

In the experiments in Table V, we increased the concentrations of actin and subfragment 1 in parallel. The objective was to test whether the low-exchange mechanism was related to cooperative interactions between subfragment 1 monomers while one or both of them were bound to actin. The results show that the low-exchange mechanism made a similar contribution at a 1/300 molar ratio as at a 1/10 molar ratio of subfragment 1 to actin. Thus, there appeared to be no cooperative effects.

At 300 μM actin, there was a pronounced effect of KCl concentration on the contribution of the low-exchange mechanism catalyzed by heavy meromyosin. Table VI shows an experiment where the KCl concentration was varied continuously from 10 to 180 mM. The low-exchange mechanism varied from greater than 90% to 12% of the ATP hydrolysis. From the kinetic experiments of Eisenberg & Moos (1970), we expected heavy meromyosin to be saturated with actin at the lower KCl concentrations. Indeed, the effect was that almost all the ATP hydrolysis was by the low-exchange mechanism.

Similar results to those shown in the top of Table IV were also obtained with subfragment 1. Only the data at 20 mM KCl are shown because they establish a lower limit for the rate of intermediate oxygen exchange for the low-exchange mechanism. The observed turnover rate ($k_{cat}$) was 15–16 $s^{-1}$, which was close to the extrapolated $V_{max}$ (20 $s^{-1}$) at infinite actin concentration. Assuming that the dissociation of products ADP and $P_i$ from AM*(ADP·$P_i$) was rate-limiting, $k_4$ was 15–16 $s^{-1}$. One can then calculate that $k_{-3} = Rk_4 = 6$ $s^{-1}$. This is 40% of the rate of intermediate exchange (15 $s^{-1}$) calculated for subfragment 1 in the absence of actin (Webb & Trentham, 1981). To the extent that the release of products was not completely rate-limiting, $k_{-3}$ would be greater than 6 $s^{-1}$. Thus, the low-exchange mechanism catalyzed intermediate oxygen exchange at almost the same rate as dissociated myosin.

*Effect of Actin-Linked Regulatory Proteins.* Since the low-exchange component was due to ATP hydrolysis initiated

Table VII: Activation by Pure and Regulated Actin[a]

| [HMM] ($\mu$M) | [actin] ($\mu$M) | $k_{cat}$ ($s^{-1}$) | [$^{18}$O]ATP | | | | low-exchange pathway | | high-exchange pathway | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | *a* | *b* | *c* | *d* | *R* | % | *R* | % |
| | | | Pure Actin | | | | | | | |
| 0.1 | 7 | 1.56 | 36.3 | 20.2 | 17.9 | 25.6 | 0.37 | 32 | 6.0 | 68 |
| 0.3 | 7 | 1.60 | 37.3 | 20.2 | 17.5 | 25.0 | 0.37 | 33 | 5.9 | 67 |
| 1.5 | 7 | 0.87 | 31.0 | 16.9 | 16.0 | 36.1 | 0.43 | 31 | 9.6 | 69 |
| 10 | 2 | 0.26 | 37.1 | 15.5 | 11.0 | 36.3 | 0.45 | 45 | 16 | 55 |
| 20 | 2 | 0.20 | 36.7 | 15.0 | 10.6 | 37.7 | 0.44 | 45 | 17 | 55 |
| 30 | 2 | 0.15 | 36.3 | 14.5 | 10.6 | 38.6 | 0.41 | 43 | 17 | 57 |
| | | | Regulated Actin | | | | | | | |
| 0.1 | 7 | 2.6 | 46.1 | 22.3 | 17.0 | 14.7 | 0.21 | 33 | 3.5 | 67 |
| 0.3 | 7 | 2.5 | 46.3 | 22.1 | 17.0 | 14.6 | 0.20 | 33 | 3.5 | 67 |
| 1.5 | 7 | 2.1 | 42.0 | 21.0 | 17.8 | 19.1 | 0.20 | 31 | 4.3 | 69 |
| 10 | 2 | 1.0 | 45.8 | 12.3 | 10.9 | 31.0 | 0.16 | 47 | 12 | 53 |
| 20 | 2 | 0.56 | 46.9 | 11.6 | 8.5 | 33.0 | 0.19 | 51 | 16 | 49 |
| 30 | 2 | 0.42 | 48.1 | 10.9 | 7.3 | 33.7 | 0.19 | 53 | 20 | 47 |

[a] The reaction mixtures contained, in 1.1 mL, 20 mM imidazole hydrochloride, pH 7.0, 30 mM KCl, 2.5 mM $MgCl_2$, 2 mM [$^{18}$O]ATP, 0.1 mM $CaCl_2$, and the indicated amounts of actin and heavy meromyosin. Where indicated, the tropomyosin/troponin complex (Spudich & Watt, 1971) was present at a concentration equal to the actin subunit concentration. The [$^{18}$O]ATP preparation was the same as that in the bottom of Table II.

by the actomyosin complex, it was of interest to determine if the properties of this component were altered by the presence of the tropomyosin/troponin complex on the actin filament. These proteins are known to activate acto heavy meromyosin ATPase under certain conditions (Lehrer & Morris, 1982). As shown in Table VII, tropomyosin/troponin activated acto heavy meromyosin in the presence of $Ca^{2+}$. In addition, they produced a significant lowering of the extent of intermediate oxygen exchange by the low-exchange mechanism. The value of *R* decreased from 0.4 to 0.2. In a separate experiment, tropomyosin alone had a similar effect in the absence of $Ca^{2+}$ (data not shown). Thus, the actin-linked regulatory proteins modified the kinetic properties of the low-exchange component. Specifically, at the intermediate stages of ATP hydrolysis, there appeared to be either an increase in the rate of release of products or a decrease in the rate of intermediate oxygen exchange.

## Discussion

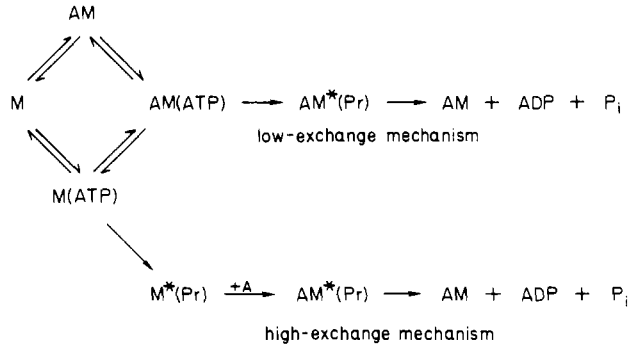
In our studies and in those of Shukla et al. (1982, 1983), a low oxygen exchange pathway was observed in the absence of added actin. This pathway was attributed to the presence of a small amount of actin (Midelfort, 1981) because actin was known to be a persistent contaminant in myosin prepared by conventional procedures and because myosin ATPase has a high affinity for actin. However, this conclusion has been shown to be incorrect by Shukla et al. (1983), who removed actin by ammonium sulfate fractionation. They described an actin-independent "induced slow exchange", which appeared after storage in low-salt buffers or during prolonged exposure to low concentrations of trypsin. This pathway could also be induced by chemical modification of the $-SH_1$ residue with *N*-ethylmaleimide or the reactive lysine residue with trinitrobenzenesulfonate (Shukla et al., 1982). The heavy meromyosin and subfragment 1 preparations used in the present study contained some of the species catalyzing induced slow exchange.

The property of induced slow exchange raises the question of whether the actin-activated low-exchange mechanism is due to this form of myosin. Shukla et al. (1983) concluded that the induced slow exchange and the actin-activated low-exchange mechanism are independent activities and that the latter is a true activity of actomyosin. Our present results have some bearing on this question. For example, a fresh preparation of native myosin was examined at several KCl concentrations. The induced slow exchange represented a small fraction of the ATP hydrolysis in the absence of actin, while the low-exchange mechanism was most of the ATP flux at a KCl concentration (50 mM) where myosin was the most highly activated by actin. To attribute the low-exchange mechanism to the induced slow-exchange form of myosin would be to say that this form was activated almost 40 times more by actin at 50 mM KCl than was the form catalyzing the Lymn and Taylor mechanism. With heavy meromyosin and subfragment 1, we found that at low KCl and high actin concentrations the low-exchange mechanism accounted for nearly all the ATP hydrolysis. The increased actin activation observed at low KCl concentration would have to be due to the form of myosin that catalyzes induced slow exchange. While it remains possible that the two pathways are catalyzed by different forms of myosin, we suggest that the most likely interpretation is that the low-exchange and high-exchange mechanisms are competing reactions catalyzed by a single species of myosin.

It has frequently been reported that subfragment 1 catalyzes ATP hydrolysis by a single pathway, the high oxygen exchange mechanism, which has the properties predicted by Lymn and Taylor mechanism (Shukla & Levy, 1978; Sleep et al., 1980; Webb & Trentham, 1981). These studies used chymotryptic subfragment 1, which has lost the $LC_2$ regulatory light chain. We found that under standard assay conditions (50 mM KCl) chymotryptic subfragment 1 has oxygen exchange properties similar to those previously described for tryptic heavy meromyosin (Midelfort, 1981): There are two pathways of hydrolysis, but the Lymn and Taylor mechanism accounts for most of the ATP flux. However, subfragment 1 prepared by limited proteolysis with papain consistently displayed two pathways of hydrolysis, and the properties of the low-exchange mechanism were similar to those observed with heavy meromyosin. The results with papain subfragment 1 are not without precedent. Shukla & Levy (1977) reported results with papain subfragment 1 that, taking into account the difference in oxygen-18-labeling methods, are very similar to ours.

In attempting to explain the apparent absence of the low-exchange mechanism in chymotryptic subfragment 1, a critical role of the $LC_2$ light chain has been proposed (Shukla et al., 1983). We examined the effect of the $LC_2$ by comparing the oxygen exchange properties of papain subfragment 1, which retained (Mg–subfragment 1) or lacked (EDTA–subfragment

Scheme I[a]




[a] M*(Pr) is the form of myosin that catalyzes intermediate exchange and is a mixture of M*(ATP) and M*(ADP·$P_i$).

1) the $LC_2$ with those of chymotryptic subfragment 1. A change in the contribution of the low-exchange mechanism was detected, which was at least partially correlated with the $LC_2$ content. However, even chymotryptic subfragment 1 displayed two pathways of hydrolysis. Thus, it is not possible to conclude from our results that the low-exchange mechanism is due to the presence of the $LC_2$. Another component of myosin, such as a portion of the heavy chain or the presence of C protein, may also be a contributing factor.

Theories on the actomyosin ATPase mechanism have been constructed on the premise that the intermediate oxygen exchange catalyzed by actomyosin would show the property of equivalence expected for a single pathway. According to the Lymn & Taylor (1971) model, actomyosin hydrolyzes ATP through a dissociating mechanism, and the step in releasing products due to recombination with actin is rate-limiting. This model is based on the near stoichiometric burst of ATP hydrolysis observed when ATP and myosin are mixed in an isolated system. On the other hand, the abundances of the four $[^{18}O_n]P_i$ species reveal that a second pathway of hydrolysis is induced by actin, which is not included in the Lymn and Taylor model or in the modified model of Stein et al. (1979). The two pathways are observed with both dimeric heavy meromyosin and monomeric subfragment 1.

The two pathways of hydrolysis by actomyosin ATPase are linked to a heterogeneity in the kinetic properties of myosin. For a significant fraction of myosin molecules, the step, M-(ATP) → M*(Pr), which initiates intermediate oxygen exchange, is slow enough to allow binding to actin to preceed the intermediate exchange step (Scheme I). Heterogeneity in the kinetics of the rapid phosphate burst has been described for skeletal muscle myosin (Inoue & Tonomura, 1976) and for cardiac myosin (Taylor & Weeds, 1976). The hypothesis of Tonomura's group was that the two subunits of myosin have different catalytic mechanisms: one is the "burst" head, and the other is the "nonburst" head. A model where the two subunits have different functions is also favored by Shukla et al. (1983) to explain the constant stoichiometry that they observed between the two intermediate oxygen exchange pathways. We found that an important factor in determining the flux of ATP hydrolysis was the KCl concentration. For native myosin, the low-exchange mechanism was most of the ATP hydrolysis at 50 mM KCl and a small fraction at 150 mM KCl. For the proteolytic fragments of myosin, the low-exchange mechanism varied between 97 and 12% when the KCl concentration was increased from 10 to 180 mM. Thus, we suggest that the two subunits of myosin have similar kinetic properties and that a still uncharacterized inhibitor controls the rate of the irreversible step, M(ATP) → M*(Pr). In studies with purified myosin, heavy meromyosin, or subfragment 1, the low-exchange pathway makes a variable contribution because the inhibitor has been partially or completely removed.

The intermediate oxygen exchange catalyzed by glycerinated muscle fibers has been recently examined by Hubbard et al. (1985). Two pathways of ATP hydrolysis are found when contraction is activated with $CaCl_2$. The low oxygen exchange mechanism appears to be 70% of the ATP flux, and its kinetic properties are similar to those reported here.

## Acknowledgments

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**Registry No.** ATPase, 9000-83-3; ATP, 56-65-5; KCl, 7447-40-7; $O_2$, 7782-44-7.

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# Activation of the Dynein Adenosinetriphosphatase by Microtubules†

Charlotte K. Omoto‡ and Kenneth A. Johnson*

## Appendix: Kinetics of ATP-Induced Dissociation and Re-formation of the Microtubule–Dynein Complex

Kenneth A. Johnson

*Department of Molecular and Cell Biology, The Pennsylvania State University, University Park, Pennsylvania 16802*
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ABSTRACT: Previous work has indicated that following the rapid adenosine 5′-triphosphate (ATP) induced dissociation of the microtubule–dynein complex, the rate-limiting step in the ATPase cycle is product release [Johnson, K. A. (1983) *J. Biol. Chem. 258*, 13825–13832], which occurs at a rate of approximately 2–6 $s^{-1}$. In this report we complete the analysis of the ATPase cycle by examining the effect of microtubules on the rate of product release. For these studies we used repolymerized *Tetrahymena* axonemal microtubules and microtubule-associated protein (MAP) free bovine brain microtubules which were shown to be free of any measurable ATPase activity. *Tetrahymena* 22S dynein bound to these microtubules predominantly by the ATP-sensitive site and at a rate giving an apparent second-order rate constant of (0.2–1) × $10^6$ $M^{-1}$ $s^{-1}$, which is 50-fold greater than the rate observed with brain microtubules containing MAPs. ATP induced the rapid dissociation of the microtubule–dynein complex with an apparent second-order rate constant vs. ATP concentration equal to 1.6 × $10^6$ $M^{-1}$ $s^{-1}$; this value is only slightly lower than that observed in the presence of MAPs. After the ATP-induced dissociation, the dynein reassociated with the microtubules following a lag period due to the time required to hydrolyze the ATP. The duration of the lag time for reassociation decreased with increasing microtubule concentration, suggesting that microtubules increased the rate of ATP turnover. Direct measurements at steady state showed that the specific activity of the dynein increased with increasing microtubule concentration. These data provide clear evidence for activation of the dynein ATPase by microtubules in solution by a mechanism in which the rebinding of the dynein–product intermediate to the microtubules [at a rate of (1.2–6) × $10^4$ $M^{-1}$ $s^{-1}$] enhances the rate of product release.

Previous work has established the first two steps of the microtubule–dynein ATPase according to Scheme I, where M represents a microtubule and D represents dynein. The binding of adenosine 5′-triphosphate (ATP)[1] (step 1) induces a rapid dissociation of dynein from the microtubule (step 2) which is followed by a slower hydrolysis reaction (step 3) (Porter & Johnson, 1983; Johnson, 1983). The rate-limiting step in the steady state in the absence of microtubules is the release of products (step 6), which occurs at a rate of 2–6 $s^{-1}$. This reaction is too slow to be part of the in vivo mechanochemical cycle because it cannot account for one ATP per active site per beat with flagellar beat frequencies of 30–60 Hz (Brokaw & Benedict, 1968; Gibbons & Gibbons, 1972). Accordingly, one might expect that microtubules bind to the dynein–product intermediate (D·ADP·$P_i$ or D·ADP) and enhance the rate of product release.

Gibbons & Fronk (1979) showed that dynein was activated approximately 6-fold when it rebound to the axonemal lattice. Although these data strongly suggest that the ATPase rate

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*Correspondence should be addressed to this author.
‡Present address: Program in Genetics and Cell Biology, Washington State University, Pullman, WA 99164-4350.

[1] Abbreviations: ATP, adenosine 5′-triphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; GTP, guanosine 5′-triphosphate; MAP, microtubule-associated protein; $Me_2SO$, dimethyl sulfoxide; PIPES, piperazine-*N,N′*-bis(2-ethanesulfonic acid); SDS, sodium dodecyl sulfate.

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