Preparation and Properties of a Complex of Adenosine Triphosphate with Myosin, Actomyosin, and Subfragment 1*

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ABSTRACT: Myosin, heavy meromyosin, and subfragment 1 are labeled with radioactivity when precipitated in the presence of [^{14}C]- or [γ-^{32}P]adenosine triphosphate by the addition of $(NH_4)_2SO_4$ solution. When pH 4.5 acetate buffer is used to precipitate the myosin, no significant radioactivity is found in the protein. Actomyosin at low ionic strength is also labeled by radioactive adenosine triphosphate. The apparent K_m of the labeling is 1.3×10^{-5} M which is considerably higher than the value observed for myosin alone, but in agreement with the K_m observed for Mg^{2+}-adenosine triphosphate hydrolysis catalyzed by actomyosin. Practically no labeling of F-actin occurs under identical conditions. Pretreatment of myosin with *p*-mercuribenzoate causes a similar loss of both labeling value and Mg^{2+}-adenosine triphosphatase activity when the molar ratio of *p*-mercuribenzoate to myosin rises above five. The addition of nonradioactive adenosine triphosphate to a solution of myosin or heavy meromyosin preincubated with radioactive adenosine triphosphate results in a first-order loss of label. The rate constant for the decomposition of the complex is affected by salt and temperature in the same manner as the steady state hydrolytic activity. These findings suggest that the observed adenosine triphosphate–myosin complex is an intermediate in the hydrolysis of Mg^{2+}-adenosine triphosphate.

Gruda *et al.* (1962) reported that myosin and heavy meromyosin (EC 3.6.1.3, ATP:phosphohydrolase), precipitated from a solution of radioactive ATP by the addition of unneutralized $(NH_4)_2SO_4$ to 55% saturation, contained significant quantities of radioactivity. This finding was confirmed and shown to be due to the binding of ATP to a specific site on the myosin molecule (Schliselfeld and Bárány, 1968). Because ATP appeared to form a complex with finite stability, this procedure was referred to as the "labeling of myosin by radioactive ATP." The use of the word "label" did not necessarily imply that the ATP or its products were covalently bound.

This report describes some further properties of the labeling of myosin with [^{14}C]ATP and [γ-^{32}P]ATP. It also shows that both subfragment 1, the smallest enzymatically active fragment of myosin, and actomyosin can be labeled with ATP by this procedure. The demonstration of the turnover of the ATP–myosin complex is compatible with a role as an intermediate in the Mg^{2+}-ATPase activity of myosin. A preliminary report on a portion of this work has been given (Schliselfeld and Bárány, 1969).

Materials and Methods

Commercial ATP, [8-^{14}C]ATP, and [γ-^{32}P]ATP were obtained or prepared as described previously (Schliselfeld and Bárány, 1968). The PMB[1] was purchased from Calbiochem. Phospho(enol)pyruvate and pyruvate kinase (EC 2.7.1.40, ATP:pyruvate phosphotransferase) were obtained from Sigma Chemical Co.

Myosin purified by chromatography on a phosphocellulose column and heavy meromyosin purified from tryptic digests of myosin were prepared as described previously (Schliselfeld and Bárány, 1968). Subfragment 1 was prepared by the digestion of heavy meromyosin with trypsin (Bailin and Bárány, 1968). F-actin was prepared from saline-insoluble rabbit muscle acetone powder purchased from Pel-Freeze Biologicals, Inc., by a modified potassium iodide extraction procedure (Bárány *et al.*, 1957). Acetone powder (5 g) was extracted at 4° for 30 min with 150 ml of 0.30 M KI, 10^{-3} M ATP, 0.01 M Tris-HCl (pH 7.3), and 5×10^{-4} M thioglycerol. The mixture was centrifuged at 12,500*g* for 10 min. The residue was extracted once more with 100 ml of the KI–ATP solution at 4° for 30 min. After centrifugation at 12,500*g* for 10 min, the two extracts were combined and dialyzed for 2 hr against 2–4 l. of 10^{-3} M $MgSO_4$ at 4°. The combined extracts were then made 0.10 M with KCl and mixed frequently at room temperature for 2 hr to polymerize the actin. The viscous solution was centrifuged at 63,000*g* for 4 hr and the supernatant fluid was discarded. The F-actin in the pellet was further purified by the procedure of Carsten and Mommaerts (1963) in the presence of Ca^{2+}, as recommended by Rees and Young (1967). The pellet was homogenized in a solution of 5×10^{-4} M ATP, 5×10^{-4} M $CaCl_2$, and 5×10^{-4} M thioglycerol (pH 7), and allowed to stir for 10 min to permit maximum depolymerization. This was centrifuged at 63,000*g* for 4 hr and the residue was discarded. To the supernatant KCl and $MgSO_4$ were added to 0.10 and 10^{-3} M, respectively. After polymerization was complete (2 hr) the F-actin was centrifuged down at 63,000*g* for 4 hr. The actin pellet was stored at 0° until it was to be used. Then the pellet was homogenized in sufficient 10^{-3} M $MgSO_4$ to give a protein concentration of 7 mg/ml or higher.

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[1] Abbreviation used that is not listed in *Biochemistry 5*, 1445 (1966), is: PMB, *p*-mercuribenzoate.

TABLE I: Effect of Precipitating Agent on the Labeling of Myosin with ATP.[a]

Precipitating Agent at 3°	Wash Solution at 3°	Mole of ^{32}P Bound/ Mole of Myosin
Satd $(NH_4)_2SO_4$ (pH 7)	2.4×10^{-3} M P_i, 6.0 $\times 10^{-4}$ M ATP, and 67% satd $(NH_4)_2SO_4$ (pH 7)	0.69 0.70
Unneutralized satd $(NH_4)_2SO_4$	2.4×10^{-3} M P_i, 6.0 $\times 10^{-4}$ M ATP, and unneutralized 67% satd $(NH_4)_2SO_4$	0.59 0.54
1.5 M NaCl and 0.02 M sodium acetate (pH 4.5)	1.5 M NaCl and 0.02 M sodium acetate (pH 4.5)	0.06 0.05

[a] Reaction mixtures containing 100 μmoles of Tris-HCl (pH 7.4), 5.0 μmoles of $MgSO_4$, 7500 μmoles of NaCl, 300 μmoles of KCl, and 5.80 mg of myosin in 4.90 ml were incubated at 25° for 5 min. Then 0.10 ml of 1.12×10^{-3} M [γ-^{32}P]-ATP was added to each mixture, mixed, and incubated at 25° for 1.00 min. The reaction was stopped by the addition of two volumes of the precipitating agent. The resulting suspension was mixed and placed at 0° for 15 min. The suspensions were centrifuged at 12,500g for 10 min and the supernatants were discarded. Each precipitate was washed twice with 15 ml of the indicated wash solution. Then each precipitate was dissolved in 5.00 ml of 0.10 N NaOH and assayed for both radioactivity and protein.

TABLE II: Effect of pH on the Labeling of Myosin with [8-^{14}C]ATP.[a]

pH	Mole of ^{14}C Bound/ Mole of Myosin
5.89	0.54
6.38	0.51
6.88	0.51
7.40	0.48
7.91	0.48
8.40	0.50
8.85	0.48

[a] The reaction mixtures contained 0.02 M Tris, 1.50 M NaCl, 1×10^{-3} M $MgSO_4$, 0.03 M KCl, 2.24×10^{-5} M [8-^{14}C]ATP, and 5.8 mg of myosin in 5.0 ml. The pH of the mixtures was adjusted with HCl to the indicated value as measured on a Beckman research pH meter. The reaction mixtures were incubated at 25° for 5 min before adding rapidly two volumes of neutral saturated $(NH_4)_2SO_4$. Each resulting suspension was placed at 0° for 15–60 min. The precipitates were centrifuged down at 12,500g for 10 min and the supernatants were discarded. Each precipitate was washed twice with 15 ml per washing of 2.7×10^{-3} M P_i, 6.7×10^{-4} M ATP, and neutral 67% saturated $(NH_4)_2SO_4$, and was washed once with 15 ml of neutral 67% saturated $(NH_4)_2SO_4$. Each precipitate was dissolved in 0.50 M NaCl and 0.01 N NaOH to determine radioactivity and protein.

The procedure for the isolation of myosin labeled by radioactive ATP was the same as described previously (Schliselfeld and Bárány, 1968; also see the footnote to Table II). A similar procedure was used for the experiments with subfragment 1 and actomyosin. In previous studies the washed radioactive ATP–myosin precipitate was dissolved in either dilute NaOH or 10 M urea. In most of the present studies the protein was dissolved in 0.50 M NaCl and 0.01 N NaOH. A 0.50-ml sample of the protein solution was counted in 15 ml of the dioxane-based scintillation fluid described by Kemp and Krebs (1967), using a Nuclear-Chicago Unilux liquid scintillation counter. No quenching of the radioactivity of ^{32}P was observed. Some quenching of the radioactivity of ^{14}C was found; this quenching was corrected by counting a known quantity of [8-^{14}C]ATP prepared in the salt solution used to solubilize the precipitates. Wherever possible counting was carried out to yield a standard error of 1% or less. However, very low counting samples were counted to yield a standard error of 5% or less.

The determination of the Mg^{2+}-ATPase activity with [γ-^{32}P]ATP and the biuret protein analysis (performed on protein precipitated in 5% trichloroacetic acid) were carried out as described previously (Schliselfeld and Bárány, 1968). The maximum specific activity for Mg^{2+}-ATPase determined by this procedure for myosin is identical with that specific activity determined by the procedure of Fiske and Subbarow (1925).

The following molecular weights were employed in this report: 5.1×10^5 daltons for myosin (Chung *et al.*, 1967), 3.80×10^5 daltons for heavy meromyosin, and 1.20×10^5 daltons for subfragment 1 (Young *et al.*, 1965).

Results

Precipitating Agent. Gruda *et al.* (1962) reported that if myosin or heavy meromyosin was precipitated from a radioactive ATP solution by the addition of sodium acetate (pH 4.6) to 0.5 M, the resulting precipitate contained radioactivity equal to or greater than that obtained with unneutralized 55% saturated $(NH_4)_2SO_4$. Table I summarizes the effect of precipitating agent on the labeling of myosin by radioactive ATP. Neutralized $(NH_4)_2SO_4$ is the most effective precipitating agent. Unneutralized $(NH_4)_2SO_4$ is 81% as effective. The precipitation of myosin in 1.5 M NaCl and 0.013 M sodium acetate buffer (pH 4.5) at 0° yields only 8.0% of the maximum labeling value. This agrees with the previously reported finding that, when myosin labeled with [γ-^{32}P]ATP and precipitated by $(NH_4)_2SO_4$ is washed once with 1.5 M NaCl and 0.02 M sodium acetate buffer (pH 4.5), all of the radioactivity is released as P_i (Schliselfeld and Bárány, 1968). In the studies of Gruda and coworkers the concentration of ATP used was 20- to 40-fold greater than the ATP concentration employed for the results in Table I. Gergely and Maruyama (1960)

TABLE III: The Effect of Temperature on the Labeling of Myosin with ATP.[a]

Expt	Temp (°C)	Labeling Value (Mole of ^{32}P Bound/ Mole of Myosin)	Mg^{2+}-ATPase Activity Recovered (%)	Corrected Labeling Value[b] (Mole of ^{32}P Bound/ Mole of Myosin)
1[c]	0	0.32		
		0.30		
	11	0.43		
		0.37		
	25	0.46		
		0.48		
2[d]	0	0.21	62.0	0.34
		0.35	77.6	0.45
	25	0.42	50.2	0.84
		0.51	62.9	0.81

[a] The reaction mixtures contained 100 μmoles of Tris-HCl, 5.0 μmoles of $MgSO_4$, 7500 μmoles of NaCl, 300 μmoles of KCl, and 5.5 mg of myosin in a total volume of 4.90 ml at pH 7.4. They were equilibrated at the indicated temperatures for 5 min or more. Then 0.10 ml of a solution of [γ-^{32}P]ATP was added (final [γ-^{32}P]ATP concentrations were 2.60×10^{-5} M in expt 1 and 1.33×10^{-5} M in expt 2). After a 1.00-min incubation two volumes of neutral saturated $(NH_4)_2SO_4$ was added rapidly and each resulting suspension was placed at 0° for 15–60 min. The precipitates were centrifuged down at 12,500*g* for 10 min and the supernatants were discarded. Each precipitate was washed as described in the footnote to Table II. [b] Labeling value/% Mg^{2+}-ATPase activity recovered $\times 10^{-2}$. [c] Washed precipitates were each dissolved in 5.00 ml of 0.50 M NaCl and 0.01 N NaOH. [d] Washed precipitates were each dissolved in 5.00 ml of 0.001 M Tris-HCl (pH 7.4) and 0.50 M NaCl.

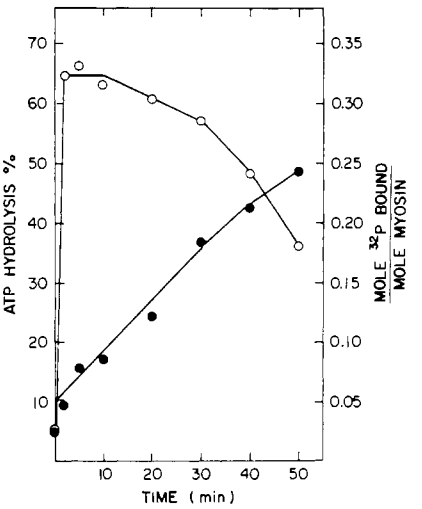

FIGURE 1: Time course of the labeling of myosin and the hydrolysis of [γ-^{32}P]ATP at 0°. A 5.00-ml solution of myosin, 1.14 mg/ml, was incubated at 0° in the presence of 1.5 M NaCl, 0.02 M Tris-HCl (pH 7.4), 1×10^{-3} M $MgSO_4$, and 1.33×10^{-5} M [γ-^{32}P]ATP, precipitated by addition of two volumes of neutral saturated $(NH_4)_2SO_4$, and washed as described in the footnote to Table II. The precipitates were dissolved in 5.00 ml of 0.50 M NaCl and 0.01 N NaOH for radioactivity and protein analyses. Zero-time samples were prepared by adding the radioactive ATP after adding the two volumes of neutral saturated $(NH_4)_2SO_4$, and then treating the precipitates as described above. The per cent hydrolysis was determined as the per cent [^{32}P] released in the initial $(NH_4)_2SO_4$ precipitation supernatant: (●—●) ATP hydrolyzed %; (○—○) moles of ^{32}P bound/mole of myosin.

have reported binding of P_i by myosin precipitated at pH 4.0 that required high ATP concentration. It is possible that a similar binding of P_i would explain the results of Gruda and coworkers.

Effect of pH, Ionic Strength, Protein Concentration, Reaction Time, and Temperature on the Labeling of Myosin with Radioactive ATP. The extent of labeling of myosin within a single experiment is seen in Table II to be constant with pH between 5.9 and 8.8. Similarly the labeling was constant at ionic strength from 0.06 through 1.5, and at myosin concentrations ranging from 0.54 through 2.16 mg per ml.

Table III summarizes the effect of temperature on the extent of labeling. Experiment 1 shows a significant decrease of labeling as the temperature drops from 25 to 0°. In expt 2 the protein precipitate was dissolved in neutral 0.5 M NaCl in order to assay the myosin for enzymatic activity. From the Mg^{2+}-ATPase activities of the labeled myosin precipitates compared with the initial untreated myosin, it was found that significant denaturation had occurred during the $(NH_4)_2SO_4$ treatment. Since denaturation causes the release of bound ATP and hydrolysis products (Schliselfeld and Bárány, 1968), the labeling values were calculated on the basis of the percent active protein. With this correction the values at 25° yield an average of 0.83 mole of ^{32}P bound/mole of myosin. At 0° the corrected value gives an average of 0.40 mole of ^{32}P bound/mole of myosin.

Figure 1 illustrates the time course of the labeling and hydrolysis of ATP by myosin at 0° in the presence of 1.5 M NaCl. The labeling value is constant for 10 min with 88% remaining at 30 min. The per cent hydrolysis is a linear function of time for at least 30 min. After this time the labeling value and the hydrolytic activity decrease.

Labeling of Subfragment 1 with Radioactive ATP. The work of Johnson *et al.* (1967) and of Slayter and Lowey (1967) have established that two molecules of subfragment 1 are derived from one molecule of myosin or heavy meromyosin. Since both myosin and heavy meromyosin were labeled with radioactive ATP, it was of interest to determine the extent of labeling with subfragment 1. It is shown in Table IV that subfragment 1 is labeled by either [^{14}C]ATP or [γ-^{32}P]ATP to a value of 0.18–0.20 mole of radioactivity/mole of subfragment 1. If this is doubled, it falls within the experimentally determined values for myosin or heavy meromyosin (Schliselfeld and Bárány, 1968).

Labeling of Actin and Actomyosin with [^{14}C]ATP. Purified F-actin and actomyosin, reconstituted from F-actin and myosin, were treated for their ability to be labeled by [^{14}C]-ATP. To minimize the loss of [^{14}C]ATP by hydrolysis, phospho(enol)pyruvate and pyruvate kinase were added as an

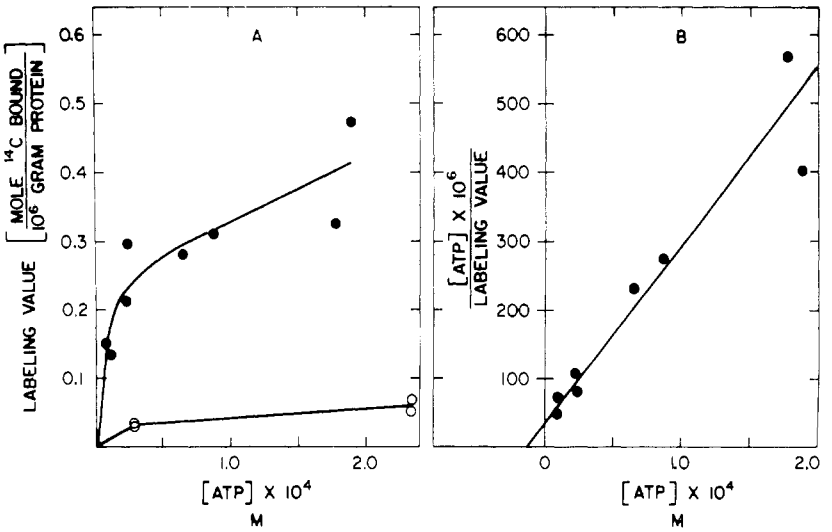

FIGURE 2: The labeling of F-actin and actomyosin as a function of [8-^{14}C]ATP concentration. Reaction mixtures of 100 μmoles of Tris-HCl, 5.0 μmoles of $MgSO_4$, 210 μmoles of KCl, 13.8 μmoles of phospho(enol)pyruvate, 9.5 μg of pyruvate kinase, and either F-actin (5.75 mg) or actomyosin (5.70 mg of myosin and 5.75 mg of F-actin) at pH 7.4 were incubated at 25° for 5 min. [8-^{14}C]ATP was added rapidly to give a total volume of 5.00 ml and the reaction mixtures were incubated at 25° for 30 sec. At 30 sec, 0.10 ml of the reaction mixture was added to 1.40 ml of 53.5% (v/v) methanol containing 10.5 μmoles of each adenine nucleotide (ATP, ADP, and AMP). Simultaneously, 10 ml of neutral saturated $(NH_4)_2SO_4$ was added to the remainder of the reaction mixture. The $(NH_4)_2SO_4$ precipitates were recovered, washed, and analyzed as described in Table II. Samples of the methanolic ATP, ADP, and AMP solutions were spotted on DEAE-cellulose paper (Whatman DE81; 17.8 × 17.8 cm) and chromatographed by the ascending method with 0.60 M formic acid and 5×10^{-3} M EDTA, neutralized with NH_4OH to pH 3.1, in mason jars (Morrison and Cleland, 1966). After 2 hr the papers were removed from the jars and air dried. Standardized [8-^{14}C]ATP was diluted in the methanol–nucleotide solution and chromatographed as described for the other solutions. The ultraviolet light absorbing spots were located, cut out, and counted in 15 ml of a toluene-based scintillation fluid (Bárány *et al.*, 1969). The analytically determined [^{14}C]ATP concentrations are employed in these figures. A shows the effect of increasing ATP concentration on the labeling values of F-actin and actomyosin: (○—○) F-actin, 1.15 mg/ml; (●—●) actomyosin, 1.14 mg/ml of myosin and 1.15 mg/ml of F-actin. B is a replot of the points for actomyosin in A for determining the maximum ATP-labeling value and the K_m for actomyosin.

ATP-regenerating system and the reaction time was reduced to 30 sec. The ATP concentration at the time of addition of the $(NH_4)_2SO_4$ solution was measured as described in the legend of Figure 2. This was done to ensure that [^{14}C]ATP was present and to permit a determination of the ATP-labeling constants. Figure 2A shows the effect of increasing ATP concentration on the labeling values for F-actin and actomyosin at low ionic strength. F-actin contains only a trace of radioactivity, but actomyosin is significantly labeled.

The data for the labeling of actomyosin in Figure 2A are replotted in Figure 2B as [ATP]/labeling value *vs.* [ATP], where the reciprocal of the slope equals the maximum labeling value and the intercept of the abscissa is equal to $-K_m$. The maximum labeling value is 0.38 mole of ^{14}C bound/10^6 g of protein and the K_m is 1.3×10^{-5} M.

TABLE IV: Labeling of Subfragment 1 by Radioactive ATP.[a]

Additions (2.24×10^{-5} M)	Mole of Radioactivity Bound/Mole of Subfragment 1
[γ-^{32}P]ATP	0.20
	0.20
[8-^{14}C]ATP	0.18
	0.19

[a] Experiments were carried out as described in the footnote to Table II.

Inhibition of Labeling of Myosin by PMB. It has been shown that the incubation of myosin with PCMB can cause a loss of Ca^{2+}-ATPase activity (Kielley and Bradley, 1956) and of Mg^{2+}-ATPase activity (Sartorelli *et al.*, 1966). Figure 3 shows the effects of a 5-min preincubation of myosin with PMB at high and low ionic strengths on the Mg^{2+}-ATPase activity and the ATP labeling of myosin. At an ionic strength of 1.5 the labeling value decreases very slightly and the per cent hydrolysis of ATP increases from 30 to 50% as the molar ratio of [PMB]:[myosin] increases from 0.0 to 4.9. When the molar ratio rises above 4.9 the labeling values and the hydrolytic activity fall to zero, though not at the same rates. Similar results are seen for myosin at an ionic strength of 0.07, except that the per cent hydrolysis increases only from 20 to 25% as the molar ratio of [PMB]:[myosin] increases from 0.0 to 4.8. At higher PMB concentration there is a rapid decrease of both the ATPase activity and the extent of labeling, that supports a close relationship between labeling and ATPase activity.

Kinetics of the Breakdown of the Radioactive ATP–Myosin Complex. These studies suggested that myosin reacts with ATP to form a complex in which the nucleotide is not in rapid equilibrium with the nucleotide of the medium. If this is true then the addition of large excess of nonradioactive ATP to the radioactive ATP–myosin complex should result in a first-order loss of the labeling value. Figure 4 confirms this

TABLE V: Summary of Rate Constants for the Breakdown of the Radioactive ATP–Myosin and ATP–Heavy Meromyosin Complexes.[a]

Protein	Temp (°C)	Salt Concentration (NaCl and KCl) (M)	k[b] (min^{-1})	Half-Life (min)	Theoretical First-Order Rate Constant[c] (min^{-1})
Myosin	25	1.5	0.33 ± 0.04	2.1	0.43[d]
	25	0.12	2.89 ± 0.10	0.2	2.50[d]
	0	1.5	0.06 ± 0.02	10.7	0.05
Heavy meromyosin	25	1.5	0.39 ± 0.02	1.8	1.4[d]

[a] Data obtained from first-order plots performed as described in the legend of Figure 4. [b] Mean rate constant ± standard error. [c] Theoretical first-order rate constant = Mg^{2+}-ATPase activity/μmole of protein; where the Mg^{2+}-ATPase activity is in μmole of ATP hydrolyzed/min. [d] Calculated from the data of Schliselfeld and Bárány (1968).

for both myosin and heavy meromyosin. The rate constants determined in the presence of 1.5 M NaCl are identical for both [^{14}C]ATP and [γ-^{32}P]ATP, suggesting that both P_i and ADP moieties are lost at the same rate. Table V summarizes the rate constants and half-lives. The rate constant for myosin in the presence of 1.5 M salt is 11.5% of the constant obtained in the presence of 0.12 M salt. Decreasing the reaction temperature from 25 to 0° causes an 80% decrease in the rate constant determined in the presence of 1.5 M salt. The rate constant for heavy meromyosin is in good agreement with that of myosin under identical conditions.

A theoretical first-order rate constant can also be calculated from the ratio of the specific Mg^{2+}-ATPase activity divided by the molar protein concentration. Table V compares the experimentally determined and the theoretical rate constants. The agreement for myosin is very good, but for heavy meromyosin the calculated rate constant is less than the theoretical value.

Discussion

Myosin, heavy meromyosin, and subfragment 1 can be prepared as complexes with both [^{14}C]ATP and [γ-^{32}P]ATP. There appears to be a close relationship between the formation and decomposition of the complex and the Mg^{2+}-ATPase activity. Both require Mg^{2+} and are inhibited by ADP (Schliselfeld and Bárány, 1968). The formation of the complex and the Mg^{2+}-ATPase activity are both inhibited by PMB at similar levels. The rate of decomposition of the complex and the Mg^{2+}-ATPase activity are inhibited by increasing salt concentration and by decreasing temperature. The K_m for the ATP labeling of myosin, which is less than 2×10^{-6} M, is near the K_m for the Mg^{2+}-ATPase activity, 4.1×10^{-7} M (Schliselfeld and Bárány, 1968). Furthermore, the K_m for the ATP labeling of myosin in the presence of F-actin, 1.3×10^{-5} M, is close to the K_m reported for the actomyosin ATPase activity, 2.0×10^{-5} M (Levy and Fleisher, 1965).

When myosin is labeled with saturating concentrations of radioactive ATP, there is 0.3–0.8 mole of radioactivity bound per mole of protein. However, myosin has been shown to have two binding sites for ATP (Schliselfeld and Bárány, 1968; Morita, 1969; Eisenberg *et al.*, 1969), for inorganic pyrophosphate (Kiely and Martonosi, 1968; Nauss *et al.*, 1969), and for ADP (Kiely and Martonosi, 1969; Lowey and Luck, 1969). In part the difference between the number of binding sites and the number of labeling sites may be due to denaturation that occurred during the $(NH_4)_2SO_4$ treatment. When a correction is made for the experimentally determined denaturation, the stoichiometry still only approaches a value

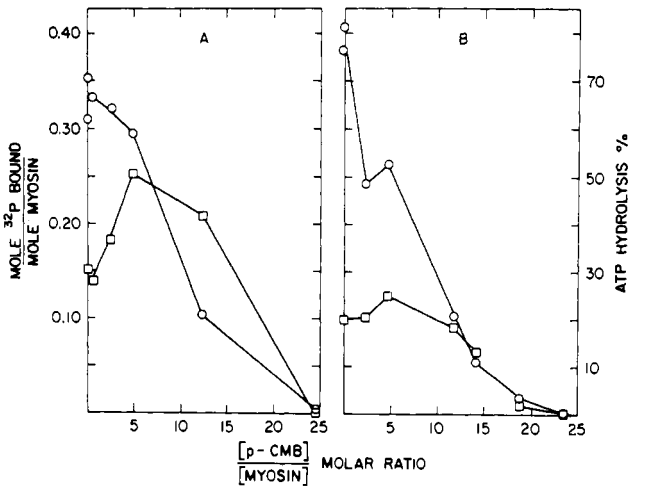

FIGURE 3: Inhibition by PMB of the labeling and hydrolytic activities of myosin. Myosin was incubated at high and low ionic strengths in 0.02 M Tris-HCl (pH 7.4) and 1×10^{-3} M $MgSO_4$ with increasing concentrations of PMB for 5 min at 25°. Then 0.113 μmole of [γ-^{32}P]ATP was added to each reaction mixture for a total volume of 5.00 ml. Each reaction was stopped by the addition of two volumes of neutral saturated $(NH_4)_2SO_4$. The precipitates were recovered, washed, and analyzed as described in the footnote to Table II. The per cent ATP hydrolysis was obtained by analyzing the first $(NH_4)_2SO_4$ precipitation supernatants for the per cent [^{32}P]P_i released. A shows the results for myosin (1.04 mg/ml) in the presence of 1.5 M NaCl, after a 5.00-min incubation with [γ-^{32}P]ATP. B shows the results for myosin (1.08 mg/ml) in the presence of 0.048 M KCl, after a 15-sec incubation with [γ-^{32}P]ATP: (○—○) labeling values; (□—□) ATP hydrolyzed %.

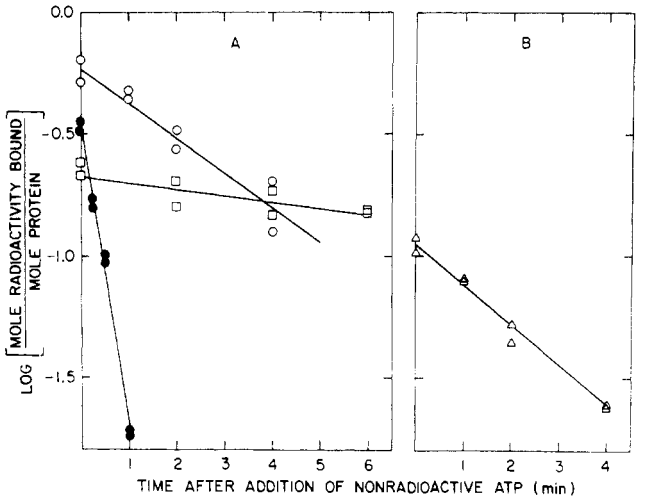

FIGURE 4: Decomposition of the radioactive ATP complex of myosin and heavy meromyosin. Myosin and heavy meromyosin were incubated with 0.02 M Tris-HCl (pH 7.4), 1×10^{-3} M $MgSO_4$, and radioactive ATP, as described below, to fully label the protein. Then an excess of nonradioactive ATP was added and the incubation was continued. The reaction was stopped by the addition of two volumes of neutral saturated $(NH_4)_2SO_4$. Zero-time samples were prepared by adding the $(NH_4)_2SO_4$ solution before adding the nonradioactive ATP. The precipitates were collected, washed, and analyzed as described in the footnote to Table II: A: (○—○) myosin, 1.04 mg/ml; NaCl, 1.5 M; [^{14}C]ATP, 1.46×10^{-5} M in 5.00 ml incubated for 1.00 min; nonradioactive ATP added, 3.7×10^{-3} M; final volume, 5.40 ml; temperature, 25°; (□—□) myosin, 1.08 mg/ml; NaCl, 1.5 M; [γ-^{32}P]ATP, 1.00×10^{-5} M in 10.0 ml incubated for 1.00 min; nonradioactive ATP added, 1.92×10^{-3} M; final volume, 10.4 ml; temperature, 0°; (●—●) myosin, 2.28 mg/ml; KCl, 0.12 M; [γ-^{32}P]ATP, 2.66×10^{-5} M in 5.00 ml incubated for 15 sec; nonradioactive ATP added, 4.55×10^{-3} M; final volume, 5.50 ml; temperature, 25°; B: heavy meromyosin, 0.83 mg/ml; NaCl, 1.5 M; [γ-^{32}P]ATP, 1.33×10^{-5} M in 5.00 ml incubated for 1.00 min; nonradioactive ATP added, 3.7×10^{-3} M; final volume, 5.40 ml; temperature, 25°.

of 1 mole of ATP bound per mole of protein. Another explanation is that there is a very slow release of radioactivity from the protein even in the presence of 67% saturated $(NH_4)_2SO_4$ (Table V, Schliselfeld and Bárány, 1968). It is not certain that even a slow release of radioactivity from the protein during isolation and washing procedures would account for the difference between the number of binding sites and the observed labeling by ATP of myosin.

It is well known that the time course of ATP hydrolysis, catalyzed by myosin or actomyosin, exhibits a positive ordinate intercept (Weber and Hasselbach, 1954; Tonomura and Kitagawa, 1957; Bowen *et al.*, 1963) that corresponds to 1.0 mole of ATP bound/mole of myosin (Kanazawa and Tonomura, 1965; Sartorelli *et al.*, 1966). This so-called initial burst hydrolysis appears to be similar in many properties to the complex formed by myosin or its proteolytic fragments with ATP, *e.g.*, stoichiometry, requirement for Mg^{2+}, temperature dependency, and inhibition by PMB (Kanazawa and Tonomura, 1965). An initial burst of ADP formation was observed when the reaction was stopped by a denaturing agent (Bowen *et al.*, 1963); however, no rapid release of ADP occurred when the protein was maintained in the native state (Imamura *et al.*, 1966). This suggests that both the P_i and ADP moieties of ATP are involved in the initial burst phenomenon as well as in the ATP–myosin complex.

It has been shown that the addition of ATP or ADP to a solution of Mg^{2+} and heavy meromyosin causes a perturbation of the ultraviolet spectrum with absorbancy peaks at 280 and 288 mμ (Morita and Yagi, 1966; Sekiya and Tonomura, 1967). This change in the ultraviolet absorbancy induced by ATP and ADP has been attributed to a conformational change in the protein which causes tyrosine residues to move from a hydrophilic environment into a hydrophobic environment. The absorbancy change at 288 mμ was shown to involve 2.0 ATP binding sites/protein molecule (Morita, 1969) and 1.0 ADP binding site/protein molecule (Morita, 1967). Morita has reported that the rate of increase of absorbancy at 288 mμ upon adding ATP to heavy meromyosin is a second-order reaction with a rate constant very close to that reported by Finlayson and Taylor (1969) for the initial burst hydrolysis of ATP in the presence of Mg^{2+}. Furthermore, it has been shown that once the ATP hydrolysis is complete there is a first-order loss of absorbancy at 288 mμ (Morita, 1967; Morita, 1969). The first-order rate constant for this absorbancy decay at 288 mμ was equal to the theoretical rate constant calculated from the Mg^{2+}-ATPase activity. This strongly suggests that the ultraviolet spectrum perturbation caused by adding ATP to heavy meromyosin is closely related to the initial burst hydrolysis, and to the steady state Mg^{2+}-ATPase activity.

The present report has shown that when myosin or heavy meromyosin is incubated with radioactive ATP, and then mixed with a large excess of unlabeled ATP to prevent any further reaction with the radioactive ATP, there is a first-order loss of labeling value. The rate constant for the loss of labeling from myosin is close to the rate constant calculated from Mg^{2+}-ATPase activity (Table V). The first-order loss of labeling value can be explained by two different hypotheses. One hypothesis is that the ATP reacts with myosin to form a covalently bound intermediate which slowly hydrolyzes to release P_i and ADP. Finlayson and Taylor (1969) have shown that the inital burst of hydrolysis is complete in 0.4 sec, while the shortest half-life so far measured for the loss of labeling value is 14.4 sec. Therefore it is difficult to postulate an intermediate with a sufficient stability to account for the loss of labeling value. If the ATP and its hydrolysis products are bound to the protein only by ionic and van der Waal forces, then the addition of unlabeled ATP should cause an instantaneous loss of labeling value. The second hypothesis is that the binding of ATP to the protein causes a conformational change so that the protein blocks the release of ATP or its hydrolysis products. After hydrolysis the rate-determining step would be the return of the protein to its original conformation with the release of the hydrolysis products. This agrees with the work of Morita and Yagi (1966) and of Sekiya and Tonomura (1967) that ATP causes a conformational change (detected by a change in the absorbancy at 280 and 288 mμ); and the rate of formation and decay of this conformational change is related to the initial burst hydrolysis and to the steady-state Mg^{2+}-ATPase activity (Morita, 1967; Morita, 1969). The actomyosin-catalyzed hydrolysis of ATP may occur by a similar mechanism, where the actin increases the velocity of release of ADP and P_i from the hydrolyzed ATP–myosin complex.

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