Hvidt, A. (1964), *Compt. Rend. Trav. Lab. Carlsberg 34*, 299.
Hvidt, A., and Nielsen, S. O. (1966), *Advan. Protein Chem. 21*, 288.
Klotz, I. M., and Frank, B. H. (1965), *J. Am. Chem. Soc. 87*, 2721.
Lees, C., and von Hippel, P. H. (1968), *Biochemistry 7*, 2480.
Leichtling, B. H., and Klotz, I. M. (1966), *Biochemistry 5*, 4026.
Lewin, S., and Pepper, D. E. (1965), *Arch Biochem. Biophys. 109*, 192.
Linderstrom-Lang, K. (1955), *Chem. Soc. (London) Spec. Publ. No. 2.*
Lyons, J. W., and Kotin, L. (1965), *J. Am. Chem. Soc. 87*, 1781.
McConnell, B., and von Hippel, P. H. (1968), *Federation Proc. 27*, 802.
Olins, D. E., Olins, A. L., and von Hippel, P. H. (1967), *J. Mol. Biol. 24*, 157.
Peacocke, A. R., and Walker, I. O. (1962), *J. Mol. Biol. 5*, 550.
Printz, M. P. (1967), *7th Intern. Congr. Biochem., Tokyo.*
Printz, M. P., and Trout, J. (1968), *12th Natl. Meeting Biophys. Soc. Pittsburgh, Pa.*
Printz, M. P., and von Hippel, P. H. (1965), *Proc. Natl. Acad. Sci., U. S. 53*, 363.
Scarpa, J. S., Mueller, D. D., and Klotz, I. M. (1967), *J. Am. Chem. Soc. 89*, 6024.
Schildkraut, C., and Lifson, S. (1965), *Biopolymers 3*, 195.
von Hippel, P. H., and Printz, M. P. (1965), *Federation Proc. 24*, 1458.
Wingert, L., and von Hippel, P. H. (1968), *Biochim. Biophys. Acta 157*, 114.

The Binding of Adenosine Triphosphate to Myosin*

L. H. Schliselfeld and M. Bárány

ABSTRACT: NaCl is a noncompetitive inhibitor of the Mg^{2+}–adenosine triphosphate phosphohydrolase activities of myosin and heavy meromyosin. By employing gel filtration chromatography in the presence of [γ-^{32}P]-adenosine triphosphate and 1.50 M NaCl, both myosin and heavy meromyosin were observed to bind 1.6 and 1.7 moles of adenosine triphosphate per mole of protein, respectively. The binding of adenosine triphosphate by myosin required Mg^{2+}, was inhibited by adenosine diphosphate and inorganic pyrophosphate but was unaffected by inorganic phosphate. Both myosin and heavy meromyosin appeared to be labeled when precipitated in the presence of [^{14}C]- or [^{32}P]adenosine triphosphate by rapid addition of neutral $(NH_4)_2SO_4$.

The labeling of myosin with radioactive adenosine triphosphate required Mg^{2+} and showed a typical adenosine triphosphate saturation curve. It was also inhibited by adenosine diphosphate. When myosin labeled with [γ-^{32}P]adenosine triphosphate was dissolved in 10 M urea or washed with cold 0.020 M acetate and 1.50 M NaCl (pH 4.5), the radioactivity was released as inorganic phosphate.

The ATP-binding sites of rabbit skeletal muscle myosin and heavy meromyosin (EC 3.6.1.3, ATP phosphohydrolase) have been investigated by various methods. A number of investigations on the binding of ADP and PP_i to myosin and heavy meromyosin suggested two or three sites per protein molecule (Nauss and Gergely, 1967; Young, 1967; Luck and Lowey, 1968; Kiely and Martonosi, 1968). Nanninga and Mommaerts (1960) determined the ATP–myosin complex concentration by the decrease in free ATP resulting from the addition of myosin. They concluded that there was one ATP binding site per molecule. Burton and Lowenstein (1964) showed that when a mixture of heavy meromyosin with Mg^{2+} and ATP was passed rapidly through a Sephadex G-25 column at 2°, a significant quantity of ATP appeared in the protein peak. This last procedure showed that ATP was bound to myosin, but it did not provide quantitative data. Finally, the perturbation of the spectra of myosin and heavy meromyosin at 280 and 288 mμ caused by the binding of ATP and ADP to myosin and heavy meromyosin was employed to demonstrate that the dissociation constants of ATP for myosin and heavy meromyosin essentially were identical with their K_m values (Morita and Yagi, 1966; Sekiya and Tonomura, 1967).

Hummel and Dreyer (1962) have shown that passing a protein solution through a Sephadex column equilibrated with an ion which binds to that protein results

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in an ion concentration peak followed by a trough. The area in the peak or in the trough corresponds to the moles of the ion bound to the protein. With this method it is possible to study the binding sites of the protein. Recently the method has been described in detail for the binding of tryptophan and its derivative to BSA[1] (Fairclough and Fruton, 1966) and for the binding of nucleotides to phosphofructokinase (Kemp and Krebs, 1967). Employing 1.50 M NaCl as a noncompetitive inhibitor of the Mg^{2+}–ATPase activity, we have applied this method to myosin and to heavy meromyosin. In addition we have studied the incorporation of [^{14}C]ATP or [^{32}P]ATP into myosin or heavy meromyosin precipitated in neutral 67% saturated $(NH_4)_2SO_4$ and washed with neutral 67% saturated $(NH_4)_2SO_4$ containing carrier P_i and ATP. A preliminary account of this work has appeared (Schliselfeld and Bárány, 1968).

Materials and Methods

ADP and ATP were obtained from P & L Biochemicals, Inc. [8-^{14}C]ATP was purchased from Schwarz BioResearch, Inc. [γ-^{32}P]ATP was prepared as described previously (Glynn and Chappel, 1964; Bárány *et al.*, 1967). NADP, phosphocellulose, edestin, glucose 6-phosphate dehydrogenase, and hexokinase were purchased from Sigma Chemical Co. BSA was obtained from Pentex. Twice-crystallized salt-free trypsin and crystalline soybean trypsin inhibitor were purchased from Worthington Biochemical Corp. Sephadex G-50 Fine was obtained from Pharmacia Fine Chemicals, Inc.

Rabbit skeletal muscle myosin was prepared as described by Bárány and Oppenheimer (1967). This then was chromatographed on a phosphocellulose column to remove adenylic acid deaminase activity (Harris and Suelter, 1967). The specific activity of the ATPase was increased 20% or more in the chromatographed myosin. The adenylic acid deaminase activity contaminating the myosin before and after chromatography was determined at room temperature in 0.05 M sodium citrate, 0.50 M KCl, 0.001 M mercaptoethanol, and 5.0×10^{-5} M AMP (pH 6.5) by a spectrophotometric procedure (Kalckar, 1947; Smiley *et al.*, 1967). The specific activity of the deaminase before chromatography was 40.6×10^{-3} μmole of AMP deaminated/min per mg of myosin and after chromatography 5.32×10^{-3} μmole of AMP deaminated/min per mg of myosin.

Heavy meromyosin was prepared by a 6-min trypsin digestion at room temperature and pH 6.3 with a ratio of myosin to trypsin of 289:1 (Lowey and Cohen, 1962). The digestion was stopped by the addition of 2 mg of trypsin inhibitor/mg of trypsin. The digest then was dialyzed overnight at 3° against 200 volumes of 0.020 M Tris-HCl (pH 7.4). The resulting precipitate was removed by centrifuging at 37,000*g* for 10 min. The heavy meromyosin was precipitated by addition of an equal volume of neutral 3° saturated $(NH_4)_2SO_4$. After 15 min at 0° the precipitated heavy meromyosin was collected by centrifugation at 37,000*g* for 10 min. The precipitate was dissolved in 25 ml of 0.020 M Tris-HCl (pH 7.4) and dialyzed for 5 hr at 3° against 500 ml of 0.020 M KCl (pH 7.0). The solution was then centrifuged in the Spinco Model L ultracentrifuge at 105,000*g* for 60 min to remove insoluble material.

The binding of ATP to myosin was carried out by a modification of the procedure described by Kemp and Krebs (1967). A Sephadex G-50 column (1.5 × 20 cm) was equilibrated with 75 ml or more of a solution of 0.020 M Tris-HCl, 1.0×10^{-3} M $MgSO_4$, 1.50 M NaCl, and [γ-^{32}P]ATP of varying concentrations (pH 7.40). Immediately before use a solution of the protein to be tested in the equilibration solution was prepared, and 1.00 ml of this solution was applied to the top of the column. After it entered the resin, the column was washed with more of the equilibration solution. The flow rates were 0.8–1.0 ml/min. The studies were carried out at room temperature (23°). Fractions of 1.5 ml or more, preferably 2.5 ml, were collected by hand in graduated centrifuge tubes, from which the volume of each fraction was read. After each fraction had been mixed, samples were plated, dried, and counted. From the average of the total net counts per minute in the radioactivity peak and trough, the specific activity of the ATP, and the amount of protein on the column, the moles of ATP bound per gram of protein, r, was calculated.

The labeling of myosin and heavy meromyosin with radioactive ATP was carried out by the following procedure. A 5.00-ml solution consisting of 0.020 M Tris-HCl, 1.0×10^{-3} M $MgSO_4$, 1.50 M NaCl, myosin or heavy meromyosin (usually 1–2 mg/ml), and either [^{14}C]ATP or [^{32}P]ATP was incubated at 25° for 0.25 or 5.0 min. Then 10 ml of neutral 3° saturated $(NH_4)_2SO_4$ was added with mixing, and the mixture was placed at 0° for 15–60 min to precipitate the protein. The mixture was centrifuged at 37,000*g* for 10 min, and the supernatant solution was discarded. The precipitate was washed twice with neutral, cold 67% saturated $(NH_4)_2SO_4$ containing 6.3×10^{-4} M ATP and 6.7×10^{-3} M P_i. In one experiment, which will be indicated, the wash solution also contained 6.7×10^{-4} M ADP. The precipitate was then washed once with neutral, cold 67% saturated $(NH_4)_2SO_4$. The precipitate was dissolved in 5.00 ml of dilute NaOH or 10 M urea. The resulting solutions were assayed for protein and plated for determination of radioactivity. From the counts per minute per milliliter, the specific radioactivity of the ATP, and the protein concentration, the moles of radioactive nucleotide bound/10^6 g of protein were calculated. This procedure resulted in a recovery of 50–80% of the protein originally employed.

The Mg^{2+}–ATPase activity was determined by the release of $^{32}P_i$ from [γ-^{32}P]ATP by a modification of the procedure of Wahler and Wollenberger (1958). The 2.00-ml reaction mixture at 25° contained 0.020 M Tris-HCl, 1.0×10^{-3} M $MgSO_4$, 0.005% BSA, 0.025 or 1.52 M NaCl, [γ-^{32}P]ATP of varying concentrations, and 0.5–5 μg/ml of myosin or heavy meromyosin (pH 7.4). The reactions were stopped by addition of 0.40 ml of 5 N $HClO_4$ containing 5.0×10^{-4} M P_i. Zero-time reactions were prepared by adding the solution of perchloric

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: BSA, bovine serum albumin; ATPase, adenosine triphosphate phosphohydrolase.

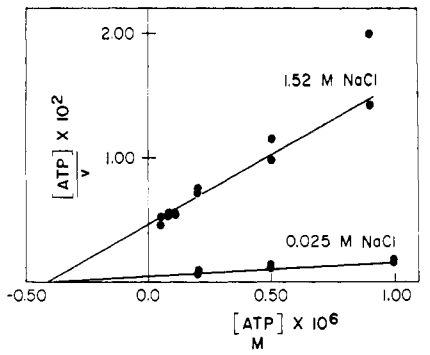

FIGURE 1: Inhibition of Mg^{2+}–ATPase activity of myosin by NaCl.

acid before the myosin or heavy meromyosin. Then 1.60 ml of 0.050 M Na_2MoO_4 and 4.00 ml of isopropyl acetate were added followed by mixing on a Vortex test-tube shaker for at least 30 sec. An aliquot of the upper phase was plated, dried, and counted. At the indicated concentrations of protein and P_i, it was found that only 80.6% of the $^{32}P_i$ could be extracted, whereas in the absence of protein nearly complete extraction occurred. The specific activities were calculated and corrected for this incomplete extraction. When the hydrolysis was less than 25%, zero-order kinetics resulted.

Nonradioactive ATP and ADP were determined by their absorbancy at 259 mμ (Bock *et al.*, 1956). [γ-^{32}P]-ATP and [^{14}C]ATP were analyzed by the reduction of NADP catalyzed in the presence of glucose by hexokinase and glucose 6-phosphate dehydrogenase (Regen *et al.*, 1964). Radioactive P_i was determined by combining a 2.00-ml aliquot with 0.4 ml of 5.0 N $HClO_4$, 1.60 ml of 0.050 M Na_2MoO_4, and 4.00 ml of isopropyl acetate, mixing at least 30 sec on a Vortex test-tube shaker, and plating a sample of the upper phase for counting. The radioactivity counting was done in a Nu-

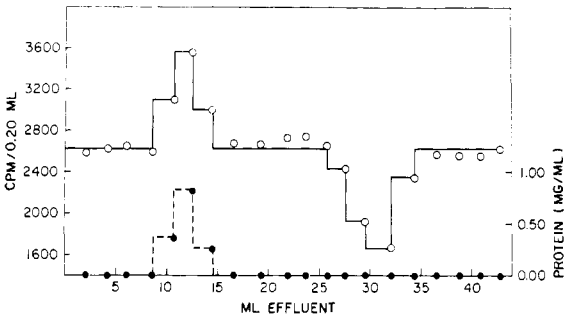

FIGURE 2: An elution profile for the binding of ATP to myosin. A Sephadex G-50 column (1.5 × 20 cm) was equilibrated with 0.020 M Tris-HCl, 1.0×10^{-3} M $MgSO_4$, 1.50 M NaCl, and 5.00×10^{-6} M [γ-^{32}P]ATP (pH 7.40). A solution of this equilibration buffer was prepared that contained 2.89 mg of myosin/ml. Immediately 1.00 ml of this myosin solution was placed on the column. The protein and radioactivity peaks appeared together in effluent volumes of 8.6–14.5 ml. The total protein recovered in the effluent was 2.81 mg. The peak r is 2.34×10^{-6} mole of ATP/g of protein. The radioactivity trough occurred in effluent volumes of 25.8–34.4 ml. The r for the trough was 3.28×10^{-6} mole of ATP/g of protein. (O——O) Radioactivity and (●—●) protein.

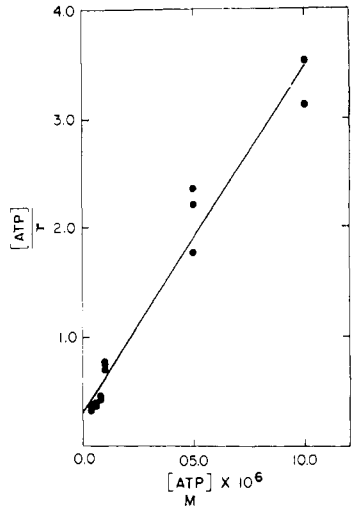

FIGURE 3: Effect of ATP concentration on the binding of ATP to myosin.

clear-Chicago thin window counter. When ^{14}C-labeled protein samples were counted, three different sizes of each solution were evenly plated, dried at room temperature overnight, and counted. A linear extrapolation of the apparent counts per minute per milliliter *vs.* milliliter plated to zero thickness gave the radioactivity in the absence of salt and protein. No such correction was needed for counting radioactive phosphate. Protein was determined by the biuret procedure of Gornall *et al.* (1949) except that the readings were made at 320 mμ in a spectrophotometer. The biuret extinction coefficient for myosin, which was standardized by analyzing for total nitrogen by a micro-Kjeldahl procedure and by assuming 16.8% nitrogen by weight, was 1.21 ml mg^{-1} cm^{-1} (Bárány and Bárány, 1959). For some of the experiments, such as the one in which concentrated urea was used, protein was determined by the procedure of Lowry *et al.* (1951) with myosin as the protein standard.

Results

The K_m and NaCl Inhibition of Mg^{2+}–ATPase Activity. Figure 1 shows a plot of [ATP]/v *vs.* [ATP] for the Mg^{2+}–ATPase activity. This method of plotting was recommended by Lineweaver and Burk (1934). The slope of the straight line is $1/V_m$ and the intercept on the abscissa is $-K_m$. The K_m for ATP and myosin is 4.1×10^{-7} M. NaCl is a noncompetitive inhibitor of the ATPase activity. The maximum specific activity is 8.4×10^{-3} μmole/min per mg of protein in 0.025 M NaCl and 8.4×10^{-4} μmole/min per mg of protein in 1.52 M NaCl. A similar noncompetitive inhibition of NaCl (not shown here) is found for the Mg^{2+}–ATPase activity of heavy meromyosin. The K_m for ATP and heavy meromyosin is 6.5×10^{-7} M; the maximum specific activity is 19.8×10^{-3} μmole/min per mg of protein in 0.025 M NaCl and 3.71×10^{-3} μmole/min per mg of protein in 1.52 M NaCl.

Binding of [γ-^{32}P]ATP to Myosin and Heavy Meromyosin. Figure 2 shows a determination of ATP binding with myosin by use of the gel filtration chromatography

TABLE I: Effect of P_i, PP_i, ADP, and $MgSO_4$ on the Binding of ATP to Myosin.[a]

Addition or Omission (M)	$r \times 10^6$ (moles/g)	Binding Inhibn (%)
None	1.22	
1.0×10^{-3} P_i	1.32	
2.0×10^{-5} ADP	0.50	59
1.0×10^{-4} PP_i	$\leqslant 0.12$	$\geqslant 90$
Omit $MgSO_4$	$\leqslant 0.20$	$\geqslant 84$

[a] This was carried out by the gel filtration chromatography at 1.0×10^{-6} M [γ-^{32}P]ATP.

procedure. A radioactivity peak appeared simultaneously with the protein peak, and this was followed by a radioactivity trough. The value of r is 2.81×10^{-6} mole of ATP/g of protein with a 17% deviation between the peak and the trough. This high error probably is due to the ATPase activity that remains in 1.5 M NaCl. This is minimized by employing low concentrations of myosin or heavy meromyosin and by carrying out the chromatography as rapidly as feasible. At each ATP concentration, the value of r is determined with varying protein concentrations. At too low a concentration of protein the peak and trough are too small to measure accurately; at very high concentrations so much ATP is hydrolyzed that low values for r result. Between these two extremes are those protein concentrations that give the maximum binding value and that we employ in this work. The optimum concentrations of myosin and heavy meromyosin placed on the column are about 0.3 mg/ml at 5×10^{-7} M and 5.0 mg/ml at 1.0×10^{-5} M ATP.

TABLE II: Labeling of Myosin with [γ-^{32}P]ATP.[a]

Protein (mg/ml)	Radioactivity Bound: Moles/10^6 g of Protein	Radioactivity Bound: Moles/Mole of Protein[b]
Myosin (1.22)	0.94	0.48
	0.94	0.48
Boiled myosin (1.22)	0.0043	0.0022
	0.0043	0.0022
Myosin (1.17)	1.16	0.59
	1.08	0.56
Edestin (1.31)	0.017	0.0051
	0.016	0.0048

[a] Myosin, boiled myosin, and edestin were incubated in 3.64×10^{-5} M [γ-^{32}P]ATP for 5 min, precipitated, and washed as described under Methods. [b] A molecular weight of 5.1×10^5 g for myosin was used (Chung *et al.*, 1967).

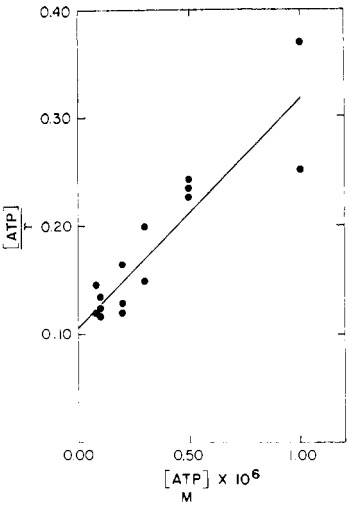

FIGURE 4: Effect of ATP concentration on the binding of ATP to heavy meromyosin.

Figures 3 and 4, respectively, show plots for myosin and heavy meromyosin of [ATP]/r *vs.* [ATP]. The slopes of the resulting straight lines are the minimum ATP binding weights; the intercept on the abscissa is $-K_D$. The best straight lines are drawn by employing the least-mean-square procedure. The values for minimum ATP binding weight and K_D are given as the average value plus or minus the standard error. For myosin the minimum ATP binding weight is 317,000 ± 16,000 g per mole of ATP, and the dissociation constant is $(8.4 \pm 2.5) \times 10^{-7}$ M. The minimum ATP binding weight for heavy meromyosin is 224,000 ± 24,000 g per mole of ATP and the dissociation constant is $(3.7 \pm 0.8) \times 10^{-7}$ M. The probability is 95% that the minimum ATP binding weight lies between 282,000 and 352,000 for myosin and between 173,000 and 274,000 for heavy meromyosin.

Table I shows that the binding of ATP to myosin required Mg^{2+} and was inhibited by ADP and PP_i. ATP binding was not affected significantly by 1.0×10^{-3} M P_i.

The Labeling of Myosin and Heavy Meromyosin with Radioactive ATP. Table II summarizes the results from incubating myosin with [γ-^{32}P]ATP for 5 min, precipitating the protein in neutral 67% saturated $(NH_4)_2SO_4$, and washing the precipitate with neutral 67% saturated $(NH_4)_2SO_4$ containing nonlabeled ATP and P_i. The precipitated and washed myosin contained 0.94–1.16 moles of $^{32}P/10^6$ g of protein. Denaturing the myosin by heating to 100° destroyed its ability to be labeled by [^{32}P]ATP. Edestin, a globular protein with the molecular weight of 3×10^5, which did not bind ATP in the gel filtration chromatography procedure, was not labeled by [^{32}P]-ATP. This suggests that the labeling may involve an ATP binding site on the protein.

Table III shows that both myosin and heavy meromyosin were labeled with either [^{14}C]ATP or [^{32}P]ATP. The presence of 4.0×10^{-3} M P_i in the incubation solution had no effect on the labeling of myosin. In the case of heavy meromyosin, it appears that [^{14}C]ATP labels the protein at a higher level than does [^{32}P]ATP. It is

TABLE III: Labeling of Myosin and Heavy Meromyosin with Both [^{14}C]ATP and [^{32}P]ATP.[a]

Protein (mg/ml)	Nucleotide (M)	Addition (M)	Radioactivity Bound: Moles/10^6 g of Protein	Radioactivity Bound: Moles/Mole of Protein[b]
Myosin (1.36)	[^{14}C]ATP (3.64×10^{-5})		0.61	0.31
			0.57	0.29
	[^{32}P]ATP (3.64×10^{-5})		0.77	0.39
			0.72	0.37
	[^{32}P]ATP (3.64×10^{-5})	P_i	0.71	0.36
		4.0×10^{-3}	0.69	0.35
Heavy meromyosin (1.18)	[^{14}C]ATP (2.0×10^{-5})		2.00	0.76
			2.22	0.84
	[^{32}P]ATP (2.0×10^{-5})		1.18	0.45
			1.28	0.49

[a] Incubation and determination of radioactivity bound were carried out as described under Methods. [b] The molecular weights used for myosin and heavy meromyosin were 5.1×10^5 g (Chung *et al.*, 1967) and 3.8×10^5 g (Young *et al.*, 1965), respectively.

doubtful that this is significant because of the variation in experimental results; however, it is possible that the [^{14}C]ADP formed on hydrolysis of the ATP remained more tightly bound than the radioactive P_i.

Characterization of the Labeling of Myosin with Radioactive ATP. Figure 5 shows that myosin requires Mg^{2+} for labeling by radioactive ATP. This labeling is inhibited by 1×10^{-3} M EDTA. Figure 6 shows that the labeling of myosin gives a typical ATP saturation curve with 50% of the maximum labeling occurring at less than 2×10^{-6} M ATP. Figure 7 shows that it is inhibited by the presence of ADP in the incubation mixture; nonetheless, when labeled protein is washed with nonlabeled ADP in the $(NH_4)_2SO_4$ wash solution, no loss of labeling occurs.

Effect on Myosin Labeled with [γ-^{32}P]ATP of Denaturation by Urea and by a pH 4.5 Buffer. Myosin labeled with [γ-^{32}P]ATP was dissolved in 10 M urea at room temperature to denature the protein. In this manner it was hoped to stabilize a covalently bound radioactive phosphate. The urea-denatured myosin was then passed through a Sephadex G-50 column equilibrated with 5 M urea. An essentially complete separation of the radioactivity from the myosin occurred (Table IV). The radioactivity peak was found to consist almost entirely of radioactive P_i.

Myosin is insoluble and denatured at pH 4.5. Table V summarizes the effect of washing myosin labeled with [γ-^{32}P]ATP with neutral 67% saturated $(NH_4)_2SO_4$ or with cold 0.020 M acetate and 1.50 M NaCl (pH 4.5). The $(NH_4)_2SO_4$ caused only a slight loss of radioactivity and left a well-labeled protein. The pH 4.5 buffer resulted in the complete release of the radioactivity as P_i.

Discussion

The binding of ATP to myosin and heavy meromyosin has been investigated by use of gel filtration chromatog-

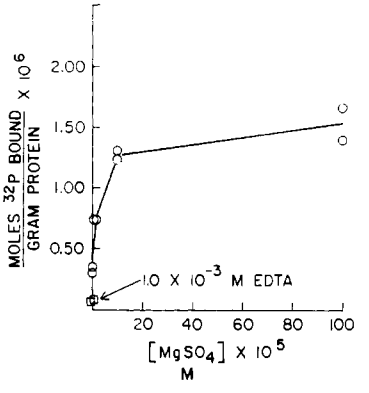

FIGURE 5: The divalent metal requirement for labeling myosin with ATP. Myosin was incubated with 2.00×10^{-5} M [γ-^{32}P]ATP in the presence of varying concentrations of $MgSO_4$ or 1.0×10^{-3} M EDTA for 5.0 min. The protein concentration was 1.32 mg/ml.

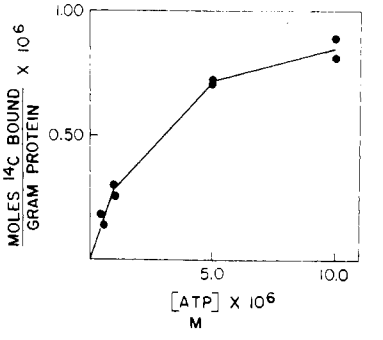

FIGURE 6: Effect of ATP concentration on the labeling of myosin. The incubation of 1.12 mg of myosin/ml in [^{14}C]-ATP lasted 15 sec. The abscissa is given as initial ATP concentration and does not take into account the probability that some of the ATP was hydrolyzed.

TABLE IV: Effect of 10 M Urea on [γ-^{32}P]ATP-Labeled Myosin.[a]

Expt No.	Sample Counted	Radioactivity Bound (moles/10^6 g of protein)
1	Myosin in 10 M urea–Sephadex chromatography	1.08
	1. Protein peak	0.022
	2. Protein-free radioactivity peak[b]	1.04
2	Myosin in 10 M urea–Sephadex chromatography	1.16
	1. Protein peak	0.016
	2. Protein-free radioactivity peak[b]	1.71
	3. Radioactive P_i in the radioactivity peak[b]	1.38

[a] [^{32}P]ATP-labeled myosin was prepared in duplicate samples as described under Methods. Each of the washed precipitates was dissolved in 5.0 ml of 10 M urea, and 4.00 ml of each solution was passed through a Sephadex G-50 column equilibrated in 5.0 M urea. [b] Normalized by dividing the total ^{32}P in the radioactivity peak by the total protein in the protein peak.

raphy. One problem with studies on ATP binding to myosin or heavy meromyosin is the hydrolysis of the substrate. In the above studies a significant but very low ATPase activity occurred even in the presence of 1.50 M NaCl. The use of gel filtration chromatography has proved valuable because the effects of substrate hydrolysis could be minimized by the rapidity of the procedure and by the use of low concentrations of protein.

The K_D and K_m were, respectively, for myosin 8.4 and 4.1 $\times$ 10^{-7} M and for heavy meromyosin 3.7 and 6.5 $\times$ 10^{-7} M. This is significantly lower than the K_m values reported by other investigators for the Mg^{2+}–ATPase activity of 2–4 $\times$ 10^{-6} M (Tokiwa and Tonomura, 1965; Sekiya *et al.*, 1967; Sekiya and Tonomura, 1967). However, Yount[2] has found a K_m value for heavy meromyosin with Mg^{2+} and ATP of 1.1 $\times$ 10^{-7} M, which is very close to the value given in the present work.

On the assumption of a molecular weight for myosin of 5.1 $\times$ 10^5 (Chung *et al.*, 1967) and for heavy meromyosin of 3.8 $\times$ 10^5 (Young *et al.*, 1965), the 95% probability range of the number of ATP binding sites per protein molecule was 1.44–1.81 for myosin and 1.39–2.20 for heavy meromyosin. This suggests that these proteins have at least two ATP binding sites per molecule. It was reported that both myosin and heavy meromyosin have two PP_i binding sites per molecule (Nauss and Gergely, 1967; Kiely and Martonosi, 1968). Young (1967) reported that myosin bound 2.77 moles of ADP/mole of protein. More recently Luck and Lowey (1968) observed two ADP binding sites per myosin molecule.

[2] Personal communication from Dr. R. G. Yount, Washington State University, Pullman, Wash.

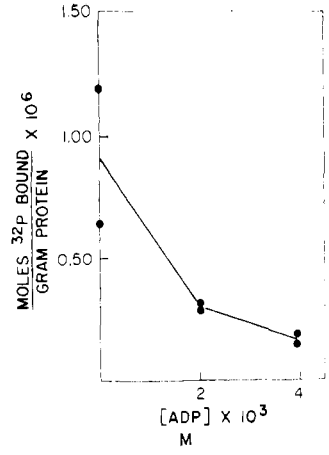

FIGURE 7: Inhibition of labeling with [γ-^{32}P]ATP by nonlabeled ADP. The myosin was incubated for 5 min with 1.0 $\times$ 10^{-5} M [γ-^{32}P]ATP in the presence of varying concentrations of nonlabeled ADP. The $(NH_4)_2SO_4$ solution contained 6.7 $\times$ 10^{-4} M nonlabeled ADP. The protein concentration was 1.51 mg/ml.

TABLE V: The Effect of pH 4.5 Buffer on [γ-^{32}P]ATP-Labeled Myosin.[a]

Final Solution	Sample Counted	Radioactivity Bound (mole/10^6 g of protein)
Neutral 67% saturated $(NH_4)_2SO_4$	Myosin[b]	0.62
		0.57
	Wash solution[c]	0.13
		0.12
0.020 M acetate and 1.50 M NaCl (pH 4.5)	Myosin[b]	0.046
		0.053
	Wash solution[c]	0.45
		0.46
	P_i in wash solution[c]	0.48
		0.48

[a] Myosin labeled with [γ-^{32}P]ATP was prepared as described. One portion was washed once with 15 ml of cold neutral 67% saturated $(NH_4)_2SO_4$; the other portion was washed once with 15 ml of cold 0.020 M acetate and 1.50 M NaCl (pH 4.5). Each of the resulting precipitates was dissolved in 5.0 ml of 0.1 N NaOH. [b] These solutions were assayed for protein and counted for radioactivity. [c] Total radioactivity in wash solution divided by total protein in dilute NaOH solution.

The results reported here clearly support the presence of two sites per molecule of myosin or heavy meromyosin. The calculations of Young were based on a molecular weight for myosin of 5.95×10^6 and on an extinction coefficient at 280 mμ of 0.540 ml mg^{-1} cm^{-1}. Evidence in the current literature strongly indicates that the molecular weight is lower (Perry, 1967). The most recently determined value with myosin purified by DEAE-Sephadex chromatography, 5.1×10^5, has been employed in this work (Chung *et al.*, 1967). In addition the extinction coefficient of myosin at 280 mμ has been found in this laboratory and in the laboratory of Dr. R. Yount[2] to be 0.670 ml mg^{-1} cm^{-1}. When the data of Young are calculated with the lower molecular weight and the higher extinction coefficient for myosin, a value of 1.91 ADP binding sites per protein molecule is obtained.

Levy and Ryan (1966) concluded from the effect of varying concentrations of ATP on the initial velocity of superprecipitation by actomyosin that there were at least two ATP binding sites per molecule. Recently Slayter and Lowey (1967) showed by electron microscopy that myosin consists of a long tail with at least two globular heads. They found that the conversion of myosin to heavy meromyosin removed most of the tail, but that the globular heads remained together. These observations are also in good agreement with the present work.

The binding of ATP to myosin requires Mg^{2+}. It is inhibited by ADP and PP_i, inhibitors of the ATPase activity (Tonomura *et al.*, 1953; Green and Mommaerts, 1956; Sekiya and Tonomura, 1967). This suggests that ATP, ADP, and PP_i may bind to the same sites. P_i has no significant effect on the binding of ATP. It has been shown that myosin catalyzed an ^{18}O exchange between H_2O and P_i that was stimulated by ATP and ADP (Dempsey *et al.*, 1963; Swanson and Yount, 1966). The lack of a significant effect of P_i on the binding of ATP suggests that either P_i and ATP do not bind to the same sites or that the dissociation constant for P_i is large.

Gruda and coworkers (1962) reported that when myosin or heavy meromyosin was incubated with [^{32}P]ATP and precipitated in either 0.5 M acetate buffer (pH 4.6) or in 55% $(NH_4)_2SO_4$, it was labeled with radioactivity. According to their results the myosin precipitated in 0.5 M acetate (pH 4.6) had severalfold more radioactive label than myosin precipitated in 55% saturated $(NH_4)_2SO_4$. On the other hand, Sartorelli and coworkers (1966) found that myosin incubated with [^{32}P]ATP and precipitated with liquid phenol (pH 7) was not significantly labeled.

In an attempt to isolate the myosin–ATP complex, myosin has been precipitated in the presence of labeled ATP and Mg^{2+} by rapid addition of neutral $(NH_4)_2SO_4$. The precipitated and washed protein appears to have between 0.3 and 0.8 mole of ATP per mole of protein. That this may be specific for myosin is suggested by the absence of labeling by ATP in denatured myosin or edestin. The requirement for Mg^{2+} in the labeling of myosin by ATP and the inhibition of labeling by ADP indicate that the labeling involves an active site on the myosin molecule.

Attempts to stabilize the radioactive myosin complex by denaturation of the myosin in 10 M urea or with acetate buffer at pH 4.5 were unsuccessful. In both cases the radioactivity was released as P_i. The finding of instability of the radioactivity bound to the protein on exposure to pH 4.5 is at variance with the results of Gruda and coworkers (1962) who have found labeled myosin after precipitation at pH 4.6.

Tokiwa and Tonomura (1965) have postulated that myosin can react with ATP to form a very labile phosphoryl myosin. This hypothesis would be in accord with the results reported here were it not for the observation that [^{14}C]ATP is bound to the precipitated myosin as effectively as is [γ-^{32}P]ATP. In the case of the formation of a phosphoryl myosin during ATP hydrolysis, it would be expected that the ADP moiety would be dissociated quickly from the myosin or diluted with ADP in the wash medium. The binding of the [^{14}C]ADP moiety suggests that ATP and its hydrolysis products may be trapped at the binding site by a folding of the protein or by aggregation on the addition of $(NH_4)_2SO_4$. The hydrolysis products would be released on denaturation of the protein with urea or pH 4.5 buffer. This hypothesis, however, certainly does not eliminate the possibility of a very labile phosphoryl myosin as an intermediate in the hydrolysis of ATP.

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References

Bárány, M., and Bárány, K. (1959), *Biochim. Biophys. Acta 35*, 293.

Bárány, M., Conover, T. E., Schliselfeld, L. H., Gaetjens, E., and Goffart, M. (1967), *European J. Biochem. 2*, 156.

Bárány, K., and Oppenheimer, H. (1967), *Nature 213*, 626.

Bock, R. M., Ling, N. S., Morell, S. A., and Lipton, L. H. (1956), *Arch. Biochem. Biophys. 62*, 253.

Burton, P., and Lowenstein, J. M. (1964), *Biochem J. 90*, 1963.

Chung, C. S., Richards, E. G., and Olcott, H. S. (1967), *Biochemistry 6*, 3154.

Dempsey, M. E., Boyer, P. D., and Benson, E. S. (1963), *J. Biol. Chem. 238*, 2708.

Fairclough, Jr., G. F., and Fruton, J. S. (1966), *Biochemistry 5*, 673.

Glynn, I. M., and Chappell, J. B. (1964), *Biochem. J. 90*, 147.

Gornall, A. G., Bardawill, C. J., and David, M. M. (1949), *J. Biol. Chem. 177*, 751.

Green, I., and Mommaerts, W. F. H. M. (1956), *J. Biol. Chem. 210*, 695.

Gruda, J., Kakol, I., and Neimierko, W. (1962), *Acta Biochim. Polon. 9*, 215.

Harris, M., and Suelter, C. H. (1967), *Biochim. Biophys. Acta 133*, 393.

Hummel, J. P., and Dreyer, W. J. (1962), *Biochim. Biophys. Acta 63*, 530.

Kalckar, H. M. (1947), *J. Biol. Chem. 167*, 461.
Kemp, R. G., and Krebs, E. G. (1967), *Biochemistry 6*, 423.
Kiely, B., and Martonosi, A. (1968), *J. Biol. Chem. 243*, 2273.
Levy, H. M., and Ryan, E. M. (1966), *Biochem. Z. 345*, 132.
Lineweaver, H., and Burk, D. (1934), *J. Am. Chem. Soc. 56*, 658.
Lowey, S., and Cohen, C. (1962), *J. Mol. Biol. 4*, 293.
Lowry, O. H., Rosbrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Luck, S. M., and Lowey, S. (1968), *Federation Proc. 27*, 519.
Morita, F., and Yagi, K. (1966), *Biochem. Biophys. Res. Commun. 22*, 297.
Nanninga, L. B., and Mommaerts, W. F. H. M. (1960), *Proc. Natl. Acad. Sci. U. S. 46*, 1155.
Nauss, K. M., and Gergely, J. (1967), *Federation Proc. 26*, 727.
Perry, S. V. (1967), *Progr. Biophys. Mol. Biol. 17*, 327.
Regen, D. W., Davis, W. W., Morgan, H. E., and Park, C. R. (1964), *J. Biol. Chem. 239*, 43.
Sartorelli, L., Fromm, H. J., Benson, R. W., and Boyer, P. D. (1966), *Biochemistry 5*, 2877.
Schliselfeld, L., and Bárány, M. (1968), *Federation Proc. 27*, 519.
Sekiya, K., Takeuchi, K., and Tonomura, Y. (1967), *J. Biochem. (Tokyo) 61*, 567.
Sekiya, K., and Tonomura, Y. (1967), *J. Biochem. (Tokyo) 61*, 787.
Slayter, H. S., and Lowey, S. (1967), *Proc. Natl. Acad. Sci. U. S. 58*, 1611.
Smiley, Jr., K. L., Berry, A. J., and Suelter, C. H. (1967), *J. Biol. Chem. 242*, 2502.
Swanson, J. R., and Yount, R. G. (1966), *Biochem. Z. 345*, 395.
Tokiwa, T., and Tonomura, Y. (1965), *J. Biochem. (Tokyo) 57*, 616.
Tonomura, Y., Watanabe, S., and Yagi, K. (1953), *J. Biochem. (Tokyo) 40*, 27.
Wahler, B. E., and Wollenberger, A. (1958), *Biochem. Z. 329*, 508.
Young, M. (1967), *J. Biol. Chem. 242*, 2790.
Young, D. M., Himmelfarb, S., and Harrington, W. F. (1965), *J. Biol. Chem. 240*, 2428.

Biodehalogenation. Epoxidation of Halohydrins, Epoxide Opening, and Transhalogenation by a *Flavobacterium* sp.*

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ABSTRACT: Cell-free extracts of a *Flavobacterium* sp. efficiently convert 2,3-dibromopropanol into glycerol *via* the sequence: dibromopropanol → epibromohydrin → dihydroxybromopropane → glycidol → glycerin. The reactivity of a partially purified halohydrin epoxidase toward a variety of substrates has been assessed. In the presence of chloride ion, epibromohydrin is rapidly converted into epichlorohydrin by the enzyme.

Carbon–halogen bonds are present in a wide array of biocides and anesthetics. Moreover, α-halo acids are widely employed as enzymatic inhibitors (Webb, 1966; Leasure, 1964). Indeed, because of the toxicity of organic halides and their inhibitory capacities it is understandable that the full scope of enzymatic dehalogenation remains to be portrayed. Nevertheless some remarkable transformations have been recorded. Thus, carbon tetrachloride is reduced to chloroform by dogs (Butler, 1961) and converted into carbon dioxide by monkeys (McCollister *et al.*, 1951) and rat liver homogenates (Rubinstein and Kanics, 1964). The insecticide DDT undergoes dehydrohalogenation (Lipke and Kearns, 1960) and reductive dehalogenation (Bunyan *et al.*, 1966; Kallmann and Andrews, 1963) in a variety of organisms. More typically, alkyl halides are metabolized in animals to the corresponding alkylmercapturic acid (Barnsley, 1966). In addition to *N*-acetyl-3-(2-propyl)cysteine, the propyl halides and 1-chloro-2-hydroxypropane are converted into *N*-acetyl-3-(2-hydroxypropyl)cysteine (Barnsley, 1966). This latter conversion of a halohydrin represents the extent of knowledge of the fate of such compounds in biological systems. Chloroethanol undergoes a prior oxidation to the aldehyde in rats before the carbon chlorine bond is attacked (Johnson, 1967). Bacterial hydroxylation of both α-halo (Leasure, 1964; Hirsch and Stellmach-Helvorg, 1961; Davies and Evans, 1962; Goldman, 1965) and β-halo acids (Castro and Bartnicki, 1965) with whole cells has been noted, as has the cell-free hydroxylation of the substi-

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