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STEADY-STATE MAGNESIUM ION-ADENOSINE TRIPHOSPHATASE ACTIVITIES OF MYOSIN AND ITS SUBFRAGMENT 1 DERIVATIVE *

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Summary

The Mg^{2+}-ATPase activity of myosin and its subfragment 1 (ATP phosphohydrolase, EC 3.6.1.3) always followed normal Michaelis-Menten kinetics for ATP concentrations less than 10 μM. The average K_m values at pH 7.4 and 25°C are 0.33 ± 0.04 μM for myosin and 0.43 ± 0.11 μM for subfragment 1. At low salt concentration myosin yields a second hyperbolic increase in Mg^{2+}-ATPase activity as the ATP rises from 10.2 μM to 153 μM: V doubles with a K_m of 11 ± 5 μM. This second low-salt-dependent increase in Mg^{2+}-ATPase activity occurred between pH 6.8 and pH 8.7. It was not affected by the presence of 0.10 M EGTA to remove Ca^{2+} contamination. Solubilization of the catalytic sites by assaying myosin for ATPase activity in the presence of 0.60 M NaCl or by conversion of myosin to subfragment 1 eliminated the secondary hyperbolic increase.

Subfragment 1 has a significantly different pH-activity curve from that of myosin. Subfragment 1 has an activity peak at pH 6.0, a rising activity as the pH goes from 8.7 to 9.8, and a deep activity valley between pH 6.8 and pH 8.4. Myosin has a very shallow trough of activity at pH 6.8 to 8.4, and in 1.0 mM ATP its activity drops as the pH decreases from 6.8 to 6.0. NaCl is a noncompetitive inhibitor of the Mg^{2+}-ATPase activity of myosin and subfragment 1. Myosin has a greater affinity for NaCl (K_i = 0.101 ± 0.004 M) than does subfragment 1 (K_i = 0.194 ± 0.009 M).

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* For a preliminary report of part of these results see Ref. 8.

Abbreviations: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

Introduction

In 1970 Lymn and Taylor [1] proposed that the Mg^{2+}-ATPase activity of myosin (ATP phosphohydrolase, EC 3.6.1.3) consists of the very rapid formation of the enzyme-ATP complex, the rapid hydrolysis of the protein-bound ATP, and the rate limiting dissociation of ADP and P_i from the protein. This reaction mechanism has been extended to seven reaction steps, each of which has been studied [2]. Lymn and Taylor [1] claimed that the steady state Mg^{2+}-ATPase activity of myosin has two K_m values of 0.03 μM and 1.0 μM; the smaller K_m could be calculated from the rate constants for the proposed mechanism. However, this conflicts with reports in many laboratories that myosin has only one high affinity K_m which is significantly greater than 0.03 μM [3—7].

In order to resolve this difference a study of the steady state Mg^{2+}-ATPase activity of myosin was undertaken. As a result of these studies it was found that below 10 μM ATP the Mg^{2+}-ATPase activity of myosin and of the subfragment 1 obeys normal Michaelis-Menten kinetics with a K_m in the pH range of 7 to 9 of 0.12—0.56 μM. The only modification of this finding occurs with myosin at low salt concentration, where a second hyperbolic increase in Mg^{2+}-ATPase activity occurs as the ATP concentration rises above 10.2 μM. This second increase of the Mg^{2+}-ATPase activity has a K_m of 11 μM.

Materials

The following is a list of substances purchased for these studies and their sources: non-radioactive ATP from P-L Biochemicals, Inc.; Cellex-P from Bio-Rad; $[^{32}P]H_3PO_4$ in 0.02 N HCl from New England Nuclear and from Amersham Corp.; chymotrypsin from Worthington; all crystalline enzymes except chymotrypsin, phenylmethylsulfonyl fluoride, dithiothreitol, NAD, NADP and DEAE-Sephadex-A25 from Sigma Chemical Co.; and DE52 resin plus DE81 paper from Whatman. The purest available forms of all other chemicals were purchased from local chemical supply houses. The triethylamine was purified by distillation before being neutralized with gaseous CO_2 released from dry ice in order to prepare triethylammonium bicarbonate solutions. Myosin was prepared from the back and hind leg muscles of male rabbits by the procedure of Barany and Oppenheimer [9]. It was then chromatographed on Cellex-P at 5°C in 0.04 M Tris-HCl and 0.40 M KCl at pH 7.8 as described previously [10].

Subfragment 1 was prepared by the digestion of synthetic filaments of myosin (15 mg/ml) by chymotrypsin (0.1 mg/ml) in 0.12 M NaCl, 10 mM NaH_2PO_4, 10 mM Na_2HPO_4, and 1.0 mM EDTA at pH 6.8 and 25°C for 20 min [11]. The reaction was stopped by addition of an excess of phenylmethylsulfonyl fluoride, dialyzed against 30 mM Tris-HCl at pH 7 and 5°C, and then centrifuged at 50 000 rev./min for 30 min to remove insoluble protein. The supernatant was transferred to a 2.5 × 40 cm column of D52 chloride equilibrated at 5°C in 10 mM Tris-HCl and 1 mm EDTA (pH 7.8). The protein was eluted by a linear gradient of 0 to 0.50 M NaCl in 10 mM Tris-HCl and 1 mM EDTA (pH 7.8). Two poorly separated peaks of ATPase activity were obtained.

Each peak was concentrated to about 20 ml by ultrafiltration using an Amincon UM10 membrane filter.

Initially [γ-^{32}P]ATP was prepared by the procedure of Glynn and Chappell [3,12]. As reported by these authors this preparation contained 2% of its total radioactivity as $^{32}P_i$ and ^{32}P in the β-phosphoryl atom of ATP. Because 0.20 N HCl is needed to elute the [γ-^{32}P]ATP from Dowex-1 chloride, it was necessary to add NaOH to neutralize the nucleotide, which resulted in the presence of 0.2 M NaCl. In order to obtain a salt-free [γ-^{32}P]ATP with less than 1% of the radioactivity consisting of $^{32}P_i$ contamination and ^{32}P in the β-phosphoryl group of ATP in high yield, the procedure of Glynn and Chappell [12] was modified by adding NAD to activate glyceraldehyde-3-phosphate dehydrogenase which permits reduced enzyme concentrations, and changing the chromatographic purification procedure.

A 5.0 ml reaction solution containing 0.05 M Tris-HCl, 6 mM $MgCl_2$, 6 mM ATP, 0.03 mM NAD, 0.5 mM 3-phosphoglycerate, 12 mM dithiothreitol, 0.20 mM potassium phosphate, 2 mCi/ml [^{32}P]H_3PO_4, 60 μg/ml rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, and 36 μg/ml 3-phosphoglycerate phosphokinase at pH 8.0 was incubated at 30°C for 60 min. At the end of this time 92% or more of the $^{32}P_i$ had been incorporated into the ATP. The reaction solution was transferred quantitatively to a 1 × 4 cm column of DEAE-Sephadex-A25 bicarbonate. The column was then washed with the following triethylammonium bicarbonate solutions (pH 7.9): 200 ml of 0.02 M buffer; 50 ml of 0.10 M buffer to remove most of the $^{32}P_i$ and AMP; 50 ml of 0.15 M buffer to remove traces of $^{32}P_i$, NAD, and ADP; and 100 ml of 0.40 M buffer to elute [γ-^{32}P]ATP. The fractions containing ATP were combined and taken to dryness under vacuum at 30—38°C on a rotary evaporator. The residue was twice dissolved in 25 ml of 25% (v/v) methanol and taken to dryness under vacuum to remove all of the triethylammonium bicarbonate. The residue was dissolved in sterilized distilled water and stored in a freezer when not in use. Only one ultraviolet light absorbing spot, that had the same R_F as ATP and contained 99% of the radioactivity, was obtained after chromatography on DE81 paper [10]. The yields are 90—95% of the starting nucleotide and 82—90% of the starting radioactivity. This salt free neutral preparation contains less than 0.5% of its radioactivity as $^{32}P_i$ and less than 0.5% of its radioactivity in the β-phosphoryl group. This stock solution of [γ-^{32}P]ATP was standardized by measuring the reduction of NADP at pH 8 in the presence of Mg^{2+}, glucose, hexokinase, and glucose-6-phosphate dehydrogenase as described previously [3]. The initial specific activity of the γ-phosphoryl group of the ATP was $6 \cdot 10^8$ cpm/μmol.

Methods

Mg^{2+}-ATPase activity assay procedures

When the ATP concentration was 1.0 mM, the Mg^{2+}-ATPase activity was measured by the photometric procedure described previously [13].

When the ATP concentration was less than 0.20 mM, Mg^{2+}-ATPase activity was measured by the formation of $^{32}P_i$ from [γ-^{32}P]ATP [4,13]. This involves adding to a 2.0 ml reaction sample 0.40 ml of 5.0 N $HClO_4$/0.50 mM P_i and 1.60 ml of 0.050 M Na_2MoO_4 to form phosphomolybdic acid. This was mixed

with 4.00 ml of isopropylacetate on a Vortex test tube shaker for at least 30 s to extract the phosphomolybdic acid into the isopropylacetate.

When the concentration of protein in the 2.00 ml sample was 10 μg/ml or less, the phosphomolybdic acid was extracted quantitatively into the isopropylacetate. Then the amount (nmol) of radioactivity in the isopropylacetate is equal to the amount (nmol) of $^{32}P_i$ in the reaction. When the 2.00 ml reaction sample had 50 μg/ml protein, 19.4% of the phosphomolybdic acid is bound to the protein and cannot be extracted into the organic phase. The total $^{32}P_i$ in the reaction sample for this condition is equal to the amount (nmol) of radioactivity in the isopropylacetate divided by 0.806.

All reaction mixtures contained 0.020 M Tris-HCl or Mes-NaOH buffer, 1.00 mM $MgSO_4$, and ATP. Mes-NaOH was used at pH 5 through 6. When the concentration of the enzyme in the reaction was 10 μg/ml or less, sufficient bovine serum albumin was added to give a total protein concentration of 50 μg/ml. The bovine serum albumin was added to prevent non-specific denaturation, which might occur at extremely low enzyme concentrations. The reaction temperature was 25°C, and the reaction pH was 7.4 unless stated differently. Zero-time samples were prepared by adding the appropriate acid solution to the buffered substrate solution before adding the enzyme solution. When no more than 25% of the substrate was hydrolyzed, the amount (nmol) of P_i formed was a linear function of time. Therefore the specific activity is expressed as nmol P_i formed per min per mg protein.

Yount et al. [7] reported that distilled water sometimes contains traces of a microorganism that can hydrolyze [γ-^{32}P]ATP. I have also found that sometimes distilled water contained such a microorganism but its hydrolysis of [γ-^{32}P]ATP was significant only at concentrations less than 0.20 μM [10]. In order to prevent this the distilled water used to dissolve the [γ-^{32}P]ATP and to prepare the low substrate concentration reaction solutions was sterilized by filtration on a Nalgene disposable bacterial filter. [γ-^{32}P]ATP in enzyme-free reaction solutions prepared with sterilized distilled water showed no hydrolysis after 1 h at 25°C.

Other analytical procedures

Protein concentration was determined by precipitating it in 5% trichloroacetic acid and assaying for biuret protein [3].

The radioactivity in the β-position of [γ-^{32}P]ATP was determined as follows. Samples of [γ-^{32}P]ATP (10 to 30 nmol) were mixed with 0.05 M Tris-HCl, 1 mM $MgCl_2$, 5 mM glucose, and yeast hexokinase at pH 8 and 30°C. After 15 min the reaction was terminated with absolute methanol to 50% (v/v) followed by sufficient ATP, ADP and AMP to make each nucleotide 7 mM. Then 0.01 ml of the solution was spotted on a 17.8 × 17.8 cm square of DE81 paper, which was developed in 0.60 M formic acid and 5 mM EDTA neutralized with NH_4OH to pH 3.1 [10]. The percent of the total radioactivity migrating with the ultraviolet light absorbing spot for ADP is the percent radioactivity in the β-phosphoryl position.

Radioactivity was measured either in a thin-window Geiger counter or in a scintillation counter as previously described [13].

The results of all K_m determinations were analyzed by a linear regression

[14] of [ATP] vs. [ATP]/v. An F-test was performed on some of the data to test for equality of the ordinate intercepts and equality of the slopes [15]. The mean of the slopes and adjusted mean of ordinate intercepts were calculated from the analysis of covariance [15].

Results

Effect of ATP concentration on the Mg^{2+}-ATPase activity of myosin

Fig. 1A shows the Mg^{2+}-ATPase specific activity of myosin in 0.03 M KCl in the ATP concentration range of 0.200 to 153 μM. These assays were performed at low salt concentration in order to minimize its non-competitive inhibition of myosin's catalytic activity. In order to present these results for such a wide concentration range the abscissa is plotted as log[ATP]. The specific activity rises from 0.20 μM until it plateaus at 2—10 μM. As the ATP concentration rises above 10 μM the specific activity rises to a new plateau at 153 μM ATP. Fig. 1B shows that the [ATP] vs. [ATP]/v plot for 0.20—4.0 μM ATP fits a straight line with a correlation coefficient of 0.999. The K_m is 0.351 ± 0.059 μM and a V of 7.0 ± 0.2 nmol P_i/min per mg. Fig. 1C shows a similar plot for 10.2—153 μM ATP, which fits a straight line with a K_m of 11 ± 5 μM and a V of 13.9 ± 0.7 nmol P_i/min per mg with a correlation coefficient of 0.996.

Cha [16] has shown that the application of the Michaelis-Menten equation requires that the enzyme concentration be equal to or less than 0.1 K_m. It was shown previously that at low salt concentrations only 59% of the catalytic sites of myosin can bind β,γ-imido ATP [10]. At low salt concentration and 0.20—4.0 μM ATP range the myosin protein concentration was 10 μg/ml. This is equal to an effective catalytic site concentration of 0.024 μM (assuming a mol. wt. of 480 000, 2 catalytic sites per mol, and only 59% of the catalytic sites are free to bind nucleotide). Therefore this effective catalytic site concentration of myosin in the 0.20—4.0 μM ATP range satisfies the above requirement noted by Cha [16]. However, above 10 μM ATP the reaction time required to obtain sufficient $^{32}P_i$ above the zero-time sample becomes very long. In order to reduce the reaction time at the high ATP concentrations, the myosin concentration was raised to 116 μg/ml. The specific activity obtained with 116 μg/ml myosin in 10.2 μM ATP, 7.2 nmol P_i/min per mg, was within experimental error equal to the V obtained with 10 μg/ml myosin. This suggests that the appearance of two separate hyperbolic phases at low salt concentration is not due to the difference in protein concentration.

Fig. 2A shows the effect of varying pH on the specific activity of myosin in 0.01 mM and 1.0 mM ATP at low salt concentration. Between pH 6.5 and pH 8.7 the specific activity in 1.0 mM ATP is almost 100% greater than the specific activity in 0.01 mM ATP. When the pH dropped from pH 6.8 to pH 6.1 the specific activity in 1 mM ATP dropped from 21 nmol P_i/min per mg to 10 nmol P_i/min per mg, respectively, to become equal to the specific activity in 0.01 mM ATP. At pH 5.5 and pH 6.1 no difference is seen in the two specific activities. A small difference is detected at pH 5 but in later assays at pH 5 no such difference occurred.

The difference in low salt specific activities measured in 0.01 mM and 1.0 mM ATP was eliminated by the presence of 0.60 M NaCl in the reaction mix-

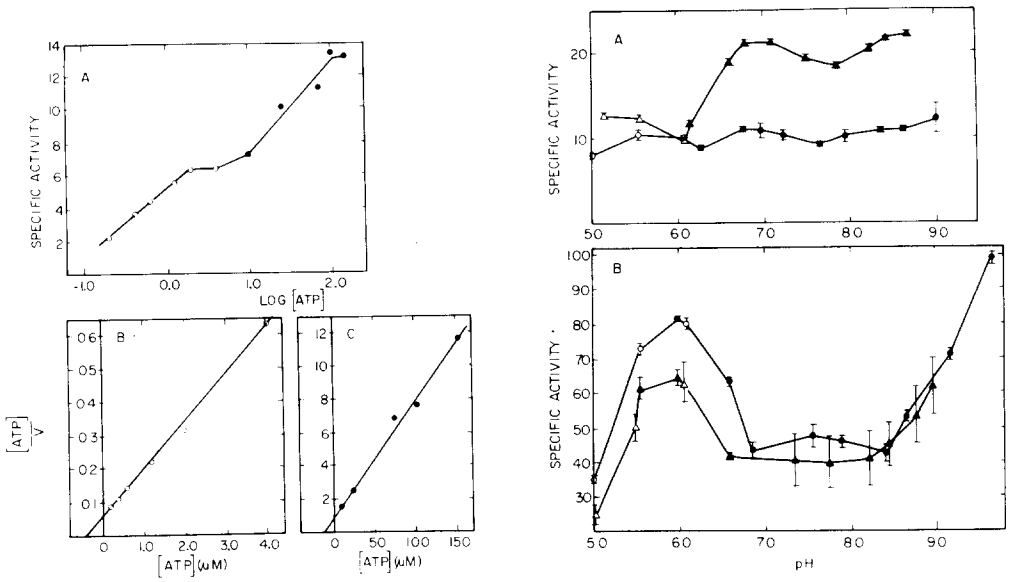


Fig. 1. Effect of varying ATP concentration on the low salt Mg^{2+}-ATPase activity of myosin. All reactions contained 0.020 M Tris-HCl, 1.0 mM Mg SO_4 and 0.030 M KCl at pH 7.4 and 25°C. Assays carried out in 0.20—4.0 μM [γ-^{32}P]ATP had 10 μg/ml myosin and 40 μg/ml bovine serum albumin, and its results are given by the open circles (○———○). Assays in the 10.2—153 μM [γ-^{32}P]ATP range had 116 μg/ml myosin, and its results are given by the close circles (●———●).

Fig. 2. Effect of varying pH on the low salt Mg^{2+}-ATPase of activity myosin and subfragment 1. All specific activities were measured at 25°C in 1.0 mM ATP (△ and ▲) or 0.010 mM [γ-^{32}P]ATP (○ and ●). The open triangles and circles (○ and △) represent results in Mes buffer; closed triangles and circles (● and ▲) represent results for Tris buffer. (A) presents the results for myosin. Each reaction contained 0.020 M Tris-HCl (pH 6.1 to 9.0) or 0.020 M Mes/NaOH (pH 5.0—6.1), 1.0 mM $MgSO_4$, and a constant concentration of KCl + NaCl + either HCl (for Tris buffer) or NaOH (for Mes buffer) of 0.0352 M. In 1.0 mM ATP the myosin concentration was 74.8 μg/ml; with 0.010 mM ATP, the myosin concentration was 10.0 μg/ml. (B) presents the results for subfragment 1. Each reaction contained 0.020 M Tris-HCl (pH 5.5 to 9.8) or 0.020 M Mes/NaOH (pH 5.0 to 6.1), 1.0 mM $MgSO_4$, and a constant NaCl + either HCl (for Tris buffer) or NaOH (for Mes buffer) of 0.0202 M. In 1.0 mM ATP assays the subfragment 1 concentration was 32 μg/ml; in 0.010 mM ATP the subfragment 1 concentration was also 32 μg/ml. Extra reaction mixtures for each pH, containing the enzyme, substrate, and buffer of each particular assay, were incubated at 25°C along with the mixtures employed for ATPase analyses. After ATPase analyses were completed the pH of each extra reaction mixture was measured with a Beckman digital pH-meter model 3550. These pH measurements are given on the abscissa of the above figures.

TABLE I

EFFECT OF NaCl AND EGTA ON THE Mg^{2+}-ATPase ACTIVITY OF MYOSIN

Addition	[ATP] (mM)	Specific activity (nmol P_i/min per mg)
0.60 M NaCl	0.010	5.4
	0.100	5.9
0.10 M EGTA	0.0051	5.0
	0.102	10.8

ture to solubilize the myosin (Table I). Also, low salt soluble subfragment 1 gave no significant difference in the two specific activities between pH 6.9 and pH 8.9 (Fig. 2B). Finally the addition of 0.10 mM EGTA to a low salt myosin reaction mixture to chelate all of the Ca^{2+} did not eliminate the difference in specific activities (Table I).

These results show that the specific activity difference for myosin is due to its aggregation at low salt and low ATP concentrations. Harrington and Himmelfarb [17] have shown that a high concentration of ATP can diminish the aggregation of myosin at low salt concentration. Solubilization of the catalytic sites by high salt concentration or conversion to subfragment 1 would enhance the ATP binding to the catalytic sites.

Effect of varying pH on the Mg^{2+}-ATPase activity of myosin and subfragment 1

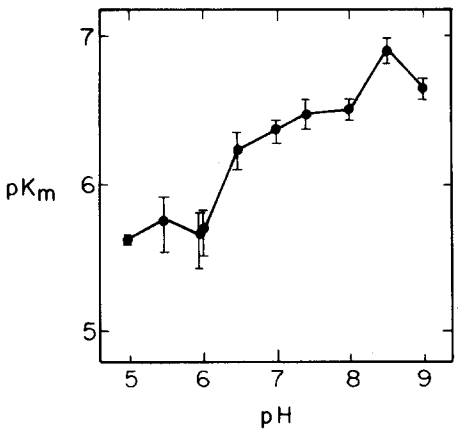
There are significant differences in the pH-activity curves for myosin (Fig. 2A) and subfragment 1 (Fig. 2B). Myosin has a very shallow trough of activity between pH 7.6 and 8.2. Also as the pH drops below 6.8 the specific activity in 1.0 mM ATP declines to a minimum between pH 5 and 6. In 0.01 mM ATP the specific activity is constant between pH 5 and pH 9 though there is a suggestion of a trough at 7.8. In sharp contrast subfragment 1 has a deep activity trough between pH 6.8 and pH 8.4, a large peak of activity at pH 6.0 and a large rise of activity as the pH rises from 8.7 to 9.8. There is no difference in the shape of the curves for subfragment 1 in 0.01 mM and 1.0 mM ATP. However, at pH 5.0—6.5 the specific activity in 0.01 mM ATP is slightly greater than the specific activity in 1 mM ATP. It appears that the tail of myosin has a significant effect on its Mg^{2+}-ATPase activity at non-physiological pH values.

K_m for the Mg^{2+}-ATPase activity of myosin and subfragment 1

Mg^{2+}-ATPase specific activity was determined with two separate myosin preparations in order to check the accuracy of the previously published value 0.41 μM [4]. All determinations, like the one shown in Fig. 1, yielded straight line plots for [ATP] vs. [ATP]/v. The two myosin preparations gave K_m values of 0.350 ± 0.058 μM and 0.287 ± 0.025 μM. The correlation coefficients were 0.996 or greater. The adjusted mean K_m from these two determinations is 0.330 ± 0.042 μM with a 95% confidence interval of 0.240 μM to 0.420 μM. This value is within statistically acceptable limits equal to the K_m recently reported by Taylor [18] of 0.25 ± 0.05 μM.

Fig. 3 presents the pK_m for myosin between pH 5.0 and 9.0. All of the plots to obtain these values followed normal Michaelis-Menten kinetics. At pH 5.0—6.0 the pK_m is constant (K_m varies from 1.7 μM to 2.3 μM). As the pH rises above 6.0 the pK_m rises to a new plateau in the pH range of 7 to 9. The smallest K_m, obtained at pH 8.5, is 0.123 ± 0.036 μM. According to the analysis of pK_m vs. pH plots [19] the convex inflexion point at pH 6.0 corresponds to an ionizable group on the enzyme-substrate complex with pK_a of 6.0.

Fig. 4 shows [ATP]/v vs. [ATP] plots for subfragment 1 at low salt concentration and in the presence of 1.50 M NaCl. At low salt concentration the points yield a straight line with a K_m of 0.406 ± 0.075 μM and a V of 43.0 ± 2.5 nmol P_i/min per mg. In the presence of 1.50 M NaCl the K_m is 0.451 ± 0.089 μM and the V is 6.0 ± 0.4 nmol P_i/min per mg. An analysis of covariance



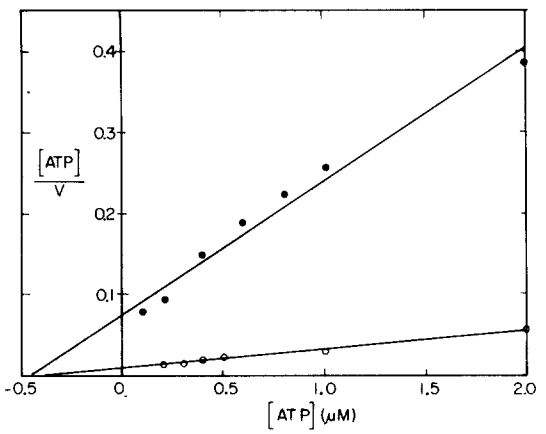


Fig. 3. Effect of varying pH on the pK_m of myosin's Mg^{2+}-ATPase activity.

Fig. 4. Effect of varying ATP concentration and NaCl on the Mg^{2+}-ATPase activity of subfragment 1. The low salt assays were carried out with 0.4 μg/ml subfragment 1 at pH 7.4 (o———o). The high salt assays were carried out in 1.50 M NaCl and 4.0 μg/ml subfragment 1 at pH 7.4 (•———•).

shows that these two K_m values are equal with an adjusted mean K_m of 0.429 ± 0.118 μM. This value is within limits of error equal to the K_m for myosin.

Table II lists some K_m values that have been determined by this investigator and in other laboratories for subfragment 1 and heavy meromyosin. This investigator has obtained K_m values for subfragment 1 of 0.43 μM to 0.56 μM between pH 7.4 and 8.3; there is no significant difference between these three K_m values. Nehei and Filipenko [6] have reported a K_m for subfragment 1 of 1.0 μM.

The K_m values for heavy meromyosin determined in this laboratory and in other laboratories are listed in Table II [7,11,18,19]. Three of these values range from 0.164 μM to 0.3 μM, which are within limits of error equal to the K_m reported here for myosin and subfragment 1. One of these K_m values, 2.4 μM, is significantly greater than the smaller three values.

TABLE II

SUMMARY OF K_m VALUES FOR SUBFRAGMENT 1 AND HEAVY MEROMYOSIN

Protein	Reaction pH	Reaction temperature (°C)	K_m (μM)	Reference
Subfragment 1	7.4	25	0.429 ± 0.118	This report
	8.0	25	0.562 ± 0.171	This report
	8.3	25	0.521 ± 0.052	This report
Subfragment 1	7.4	20	1.0	Nehei and Filipenko [6]
Heavy meromyosin	7.4	25	0.11	Yount et al. [7]
Heavy meromyosin	7.4	25	0.164 ± 0.033	Schliselfeld [10]
Heavy meromyosin	7.4	20	0.30	Nehei and Filipenko [6]
Heavy meromyosin	7.5	25	2.4	Sekiya and Tonomura [5]

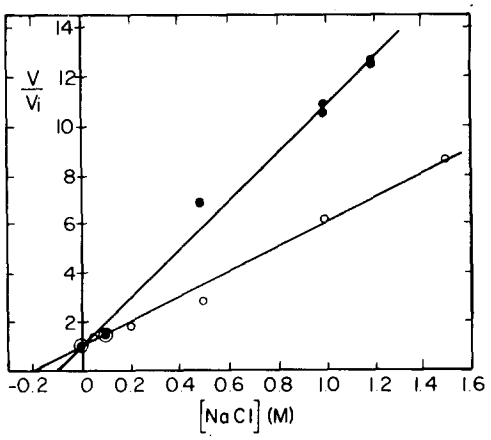


Fig. 5. Effect of varying NaCl concentration on the maximum specific activities of myosin and subfragment 1. All Mg^{2+}-ATPases activities were measured in 1.0 mM ATP at pH 7.4 and 25°C without and with increasing levels of NaCl. The ordinate provides the ratio of activity without added NaCl (V) divided by activity in the presence of NaCl (V_i). The data for each enzyme was analyzed according to the equation of Dixon [20]: $V/V_i = 1 + [NaCl]/K_i$ where K_i is the dissociation constant for NaCl. When myosin was employed (●———●) its concentration was 352 μg/ml and its specific activity without salt was 39.5 nmol P_i/min per mg. When subfragment 1 was employed (○———○) its concentration was 260 μg/ml and its specific activity without NaCl was 34.6 nmol P_i/min per mg.

Effect of salt on the activity of myosin and subfragment 1

Fig. 4 shows that NaCl is a noncompetitive inhibitor of the Mg^{2+}-ATPase activity of subfragment 1; it causes a decrease of the V but there is no significant change of the K_m. Fig. 5 shows Dixon inhibition plots [20] for myosin and subfragment 1 in 1.0 mM ATP. The slopes of the lines for subfragment 1 and myosin are not equal ($p < 0.005$) indicating that they have significantly different dissociation constants. These dissociation constants are 0.101 ± 0.004 M for myosin and 0.194 ± 0.009 M for subfragment 1. The dissociation constant calculated from the V of subfragment 1 in low salt and 1.5 M NaCl (Fig. 4) is 0.24 M which is essentially equal to the value obtained from the Dixon inhibition plot.

Discussion

Nehei and Filipenko [6] reported that at low salt and high salt concentrations the Mg^{2+}-ATPase activity of myosin has two hyperbolic phases with increasing ATP; a plateau separating these two phases occurred at 4—10 μM. The data shown in Fig. 1 and 2 confirms that at low salt the Mg^{2+}-ATPase specific activity of myosin shows two distinct activity phases as a function of ATP. The addition of 0.60 M NaCl to solubilize the myosin in this study eliminated the low affinity phase, which is contrary to the results of Nehei and Filipenko [6]. The above authors also reported that the low affinity phase did not occur with subfragment 1, but it occurred with heavy meromyosin above 1 mM ATP.

The low affinity phase is not due to Ca^{2+} present in the ATP since EGTA did not eliminate it. The low affinity phase was eliminated by solubilization in 0.60 M NaCl or conversion of the myosin to subfragment 1. In a previous publication [10] it was shown that at low salt concentration only 59% of the high

affinity nucleotide binding sites of myosin are capable of binding an analog of ATP. These results show that at low salt concentration the aggregation of myosin causes a steric inhibition of nucleotide binding to a portion of the catalytic sites. Harrington and Himmelfarb [17] found that high ATP concentration results in a decrease in the aggregation of myosin. Wagner and Yount [21] have shown that myosin has low-affinity nucleotide binding sites that may have a regulatory function. The binding of ATP at these sites may be responsible for the disaggregation at low salt, which would remove this steric inhibition of ATP binding to the catalytic sites on myosin. Since subfragment 1 does not aggregate at low salt, it will always exhibit maximum ability to bind and hydrolyze ATP.

Lymn and Taylor [1] recognized that there must be a relationship between the K_m and the rate constants for the forward and reverse steps involved in the hydrolysis of ATP. They reported K_m values of 0.03 μM and 1.0 μM for myosin's Mg^{2+}-ATPase activity, which could be related to a simple equation involving rate constants. The recent acknowledgement by Taylor [18] that the K_m is 0.25 ± 0.05 μM led him to conclude that no simple relationship could be established.

As a result of the work of Lymn and Taylor [1] and Trenthan et al. [2] the following mechanism has been proposed for the Mg^{2+}-ATPase activity, where M, M*, and M** represent three different conformations of the catalytic sites:

$$\mathrm{M + ATP} \overset{1}{\rightleftharpoons} \mathrm{M\ ATP} \overset{2}{\rightleftharpoons} \mathrm{M^*\ ATP} \overset{3}{\rightleftharpoons} \mathrm{M^{**}\ ADP\ P_i} \overset{4}{\rightleftharpoons} \mathrm{M^*\ ADP\ P_i} \overset{5}{\rightleftharpoons} \mathrm{M^*\ ADP}$$

$$+ \mathrm{P_i} \overset{6}{\rightleftharpoons} \mathrm{M\ ADP} \overset{7}{\rightleftharpoons} \mathrm{M + ADP}$$

Using the method of King and Altman [22] the steady state equation for this mechanism can be derived. The equation for the K_m, where k_i and k_{-i} are the forward and reverse rate constants of the ith step, is the following:

$$K_m = \frac{k_3k_4k_5(k_{-1} + k_2 + A)}{k_1(k_2k_5(k_{-3} + k_3) + B)}$$

where

$$A = k_{-2}k_{-1}(k_4k_5 + k_3k_4 + k_{-3}k_{-4})$$

and

$$B = k_{-2}(k_{-3}k_{-4} + k_{-3}k_5 + k_4k_5) + k_4\left(k_3k_5 + k_2k_5 + k_2k_3 + \frac{k_2k_3k_5}{k_6} + \frac{k_2k_3k_5}{k_7}\right.$$

$$\left. + \frac{k_2k_3k_5k_{-6}}{k_6k_7}\right) + k_2k_{-4}(k_3 + k_{-3})$$

The rate constants for subfragment 1 in 0.10 M KCl at pH 8 are summarized by Trentham et al. [2]. Because k_4, k_{-2}, and k_{-4} are very small (0.06 s^{-1}, $2 \cdot 10^{-6}$ s^{-1}, and 0.004 s^{-1}, respectively), the A and B terms can be eliminated to give the following equation:

$$K_m = \frac{k_3k_4}{(k_3 + k_{-3})}\left(\frac{k_{-1}}{k_1k_2} + \frac{1}{k_1}\right)$$

Because nobody has been able to determine the value of k_1, it is impossible to calculate the K_m from the rate constants. However, the above equation can be rearranged to solve for k_1 in terms of $k_3/(k_3 + k_{-3})$, K_1 which is equal to k_1/k_{-1}, k_2, and k_4:

$$k_1 = \frac{K_1 k_2 (k_3/(k_3 + k_{-3}))\, k_4}{K_1 k_2 K_m - k_4 (k_3/(k_3 + k_{-3}))}$$

Using the K_m for subfragment 1 at pH 8 (0.562 μM, Table II) and the data of Trentham et al. [20] ($k_3/(k_3 + k_{-3}) = 0.9$, $k_4 = 0.06\ s^{-1}$, $K_1 = 4.5 \cdot 10^3\ M^{-1}$, and $k_2 = 400\ s^{-1}$) gives $k_1 = 1 \cdot 10^5\ M^{-1} \cdot s^{-1}$ and $k_{-1} = 22\ s^{-1}$.

Taylor and Weeds [23] have reported the rate constants for homogeneous subfragment 1 isozymes A1 and A2. Using their rate constants and the K_m value in Table III gives for A1 $k_1 = 1 \cdot 10^5\ M^{-1} \cdot s^{-1}$ and $k_{-1} = 11\ s^{-1}$ and for A2 $k_1 = 0.8 \cdot 10^5\ M^{-1} \cdot s^{-1}$ and $k_{-1} = 8\ s^{-1}$. The k_1 values are essentially identical for the isozyme mixture and for the homogeneous isozymes. However, the homogeneous isozymes appear to have a smaller value for k_{-1} than does the subfragment 1 used by Trentham et al. [2].

At the present time nobody has been able to determine k_1 from pre-steady state kinetics alone. If in the future someone should develop a method capable of determining k_1 without the use of the Michaelis constant its value can be compared to the value calculated above. If a directly determined value for k_1 at 0.10 M KCl, 25°C, and pH 8 should differ from $1 \cdot 10^5\ M^{-1} \cdot s^{-1}$ by a factor of 10 or more, then the proposed reaction mechanism or the values for its rate constants should be examined for the cause of this difference.

The accuracy of the k_1 calculated above depends on the accuracy of each of the terms involved in the calculation. These rate constants can vary with salt concentration, pH, temperature, and experimental error. It is impossible at present to assess the errors that might be produced by these factors. For this reason the estimates of k_1 and k_{-1} may be improved in the future.

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