THE INITIAL PHASE OF ACTOMYOSIN-ADENOSINETRIPHOSPHATASE*

II. FACTORS INFLUENCING ACTIVITY

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SUMMARY

1. The same initial rapid splitting of ATP by myosin B is repeatedly obtained upon adding fresh ATP, if the ATP added previously has been completely hydrolyzed.

2. The quantity of the initial rapid splitting of ITP is very small, as compared with that of ATP.

3. The initial ATP hydrolysis by non-dialysed myosin B preparations is only slightly influenced by the addition of Ca^{++} or Mg^{++}.

4. The initial burst of P-liberation is annihilated by dialysis of the myosin. It is restored by adding Mg^{++} or Mn^{++}, but not by adding Ca^{++}.

5. It is also abolished by adding small amounts of EDTA, capable of chelating only a negligible part of the Mg^{++} present. It is also decreased by the addition of *p*-chloromercuribenzoate.

6. When sufficient Mg^{++} is added to a reaction mixture containing ATP and EDTA, a burst of P-liberation is observed.

INTRODUCTION

In a previous paper[1] we described an investigation into the initial rapid liberation of P from ATP by myosin B. The phenomenon was first observed by WEBER AND HASSELBACH[2]. By means of a special apparatus constructed for the measurement of a rapid reaction, it has been revealed that the inorganic phosphate liberated in the initial rapid phase originates from the terminal phosphate group of the ATP added and that the addition of EDTA leads to complete suppression of the initial phase.

In subsequent experiments an attempt has been made to explore the reaction mechanism of the initial phase of the myosin B ATPase action more extensively, and it has been found that Mg^{++} and the SH groups of myosin B are essential for the initial burst of P-liberation.

Abbreviations: ATP, adenosine triphosphate; ATPase, adenosine triphosphatase; ITP, inosine triphosphate; P, inorganic orthophosphate; EDTA, ethylenediaminetetraacetic acid; CyDTA, 1,2-diaminocyclohexane-N,N'-tetraacetic acid; PCMB, *p*-chloromercuribenzoate.

MATERIALS AND METHODS

Myosin B was prepared from rabbit skeletal muscle according to the standard practice used in this laboratory. ATP and ITP were Sigma preparations. EDTA, PCMB and other reagents were commercial products of the best reagent grades available.

The dephosphorylation rate was measured either by using the apparatus described in detail in the previous paper[1] or by the conventional method, as occasion demanded. The intercept of the ordinate obtained by extrapolating the linear P-liberation to zero time was defined as the quantity of the initial burst. The spectrophotometric method of BOYER[3] was employed to follow the reaction of protein-SH with PCMB, assuming the molecular change of optical density at 255 mμ as 6,000.

The contents of Mg^{++} and Ca^{++} in the myosin B preparations were determined colorimetrically by a slight modification of the YANAGISAWA method[1]. The dye Plasma Colinth B was purchased from Sumitomo Chemical Co. The protein denatured in the presence of 20 % of $HClO_4$ was removed by filtration through ash-free filter paper. The filtrate was neutralized with KOH. The precipitated $KClO_4$ was also removed by filtration . The determinations were further performed exactly according to YANAGISAWA's procedure.

Other methods used were the same as described in the previous paper unless otherwise stated.

RESULTS

The relationship between the rate of the liberation of P from ATP by myosin B and the amount of the enzyme is shown in Fig. 1. It is clearly seen in this figure that the rates of P-liberation in both the steady and the initial phases are proportional to the concentration of the enzyme.

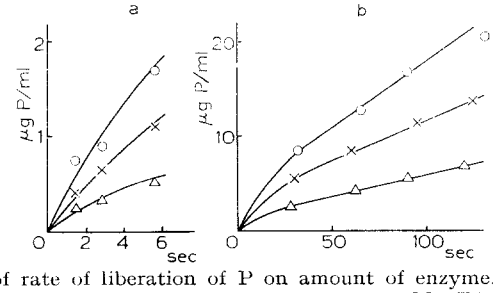
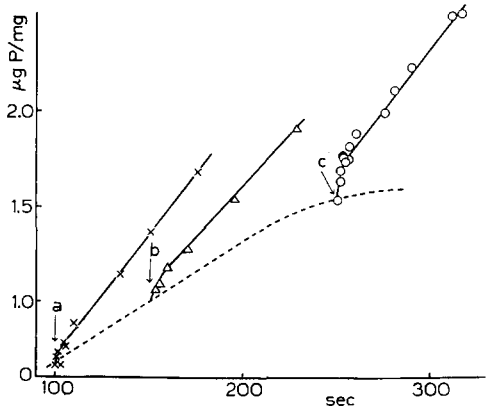


Fig. 1. Dependence of rate of liberation of P on amount of enzyme. ○, 1.8 mg; ×, 1.2 mg; △, 0.6 mg dry-weight myosin B/ml. Reaction mixture: 1 mM ATP, 0.6 M KCl, 7 mM Ca^{++}, pH 6.7, 23°. (a) initial phase; (b) steady phase.

A repeated rapid splitting of ATP was found upon adding fresh ATP to the reaction mixture whenever the previously added ATP was hydrolyzed completely. Similar observations have already been made by WEBER AND HASSELBACH[2]. However, the phenomenon remained to be studied quantitatively. Therefore fresh ATP was also added before the ATP added previously had completely disappeared. At various intervals after adding 50 μM ATP to a series of identical reaction mixtures 1 mM fresh ATP was added. The result is illustrated in Fig. 2. No initial burst was observed when the remaining concentration of ATP was 25 μM (arrow a), and the

burst was about 25 % of the control value when the concentration of remaining ATP was 18 μM (arrow b), while the burst had a normal value again as soon as the ATP was exhausted (arrow c). From these results it is evident that during hydrolysis of ATP, the enzyme is quickly restored to its original state.

When ITP was the substrate in the presence of 10 mM Mg^{++} the initial burst was very small as compared with ATP hydrolysis in the presence of 1 mM Mg^{++} (see Fig. 3).



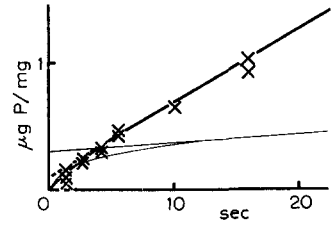


Fig. 3. Initial phase of hydrolysis of ITP. Reaction mixture: 1 mM ITP, 0.6 M KCl, 10 mM Mg^{++}, pH 6.7, 22°. Thin line, 1 mM ATP, 0.6 M KCl, 1 mM Mg^{++}, pH 6.7, 22°.

Fig. 2. Initial phase at various residues ATP concentrations. Dotted line, hydrolysis of 50 μM ATP. Arrows, additions of 1 mM ATP when concentrations of residual ATP are (a) 25 μM, (b) 18 μM and (c) 0 μM. Reaction mixture: 0.6 M KCl, 1 mM Mg^{++}, pH 6.7, 22°.

Effect of divalent cations

A typical example of the effect of the addition of Ca^{++} or Mg^{++} on ATP-hydrolysis is illustrated in Fig. 4. The activation by Ca^{++} and inhibition by Mg^{++}, which together form one of the characteristic properties of the steady phase ATPase action[5], are absent in the initial phase. The variation of the KCl concentration produced a comparable degree of effect on the initial and the steady ATPase: in the presence of

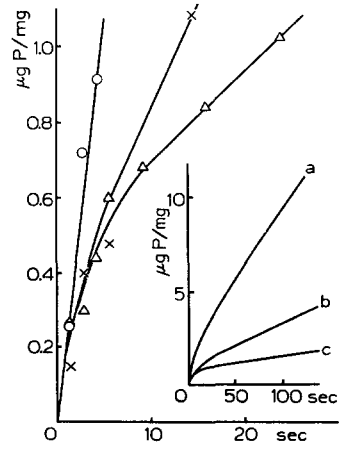


Fig. 4. Effects of Mg^{++} and Ca^{++} on initial phase. ○, 7 mM Ca^{++}; △, 4.5 mM Mg^{++}; ×, no divalent cation. 1 mM ATP, 0.6 M KCl, pH 6.7, 22°. Inserted figure, (a) 7 mM Ca^{++}; (b) no divalent cation; (c) 4.5 mM Mg^{++}.

1 mM Mg^{++} and 0.6 M KCl the velocity in the initial phase was 3 times as high as in the presence of 1 mM Mg^{++} and 0.055 M KCl, and in the steady phase 4 times.

The contents of Ca^{++} and Mg^{++} in the myosin B preparations used were 1.2–1.8 moles and 1.4–1.6 moles per 10^5 g of protein, respectively. The differences of these values from HASSELBACH's[6] (0.85 mole Ca^{++} and 0.52 mole $Mg^{++}/10^5$ g protein) will be due to the difference in the procedure of myosin B preparation. After 48 h of exhaustive dialysis of myosin B solutions against 0.6 M KCl at 0°, the amount of Ca^{++} and Mg^{++} decreased to 0.28–0.34 mole and 0.27–0.32 mole per 10^5 g, respectively. As shown in Fig. 5, no appreciable change of the velocity in the steady phase was observed, but the initial burst was abolished almost completely by dialysis. The restoring effect of several divalent cations on the initial burst, whose concentrations had been reduced by dialysis, is illustrated by Figs. 5 and 6. 5 μM Mg^{++} had no effect, while 50 μM Mg^{++} restored the burst to almost the level observed before dialysis. When 1 mM Mg^{++} was added to the dialysed preparation, the initial burst ranged from 150 to 200 % of the control value in the presence of non-dialysed myosin B. 1 mM of Mn^{++} or Sr^{++} produced the same restoring effect as 50 μM Mg^{++}, while concentrations of from 50 μM to 1 mM Ca^{++} were ineffective. Obivously it is the presence of Mg^{++} that is responsible for the initial phase. The burst was almost unaffected by three to eight repetitions of purification by the dilution–precipitation procedure. This result seems to indicate that Mg^{++} necessary to the initial phase is not removed by this procedure.

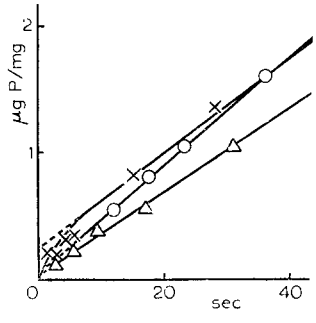


Fig. 5. Effect of dialysis on initial phase. ×, before dialysis; △, after dialysis; ○, 50 μM Ca^{++} added after dialysis. 1 mM ATP, 0.6 M KCl, pH 6.7, 21°.

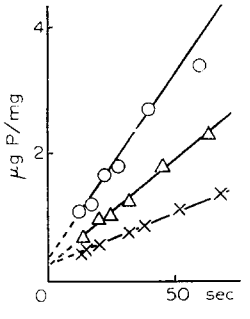


Fig. 6. Restoration of initial burst by adding divalent cations to reaction mixture containing dialysed myosin B, ×, 50 μM Mg^{++}; △, 1 mM Sr^{++}; ○, 1 mM Mn^{++}. 1 mM ATP, 0.6 M KCl, pH 6.7, 21°.

Effects of EDTA and CyDTA

The effect of EDTA already observed in our previous paper[1] was studied more thoroughly. As can be seen from the example represented in Fig. 7, the burst was reduced even at 1 μM EDTA and was almost annihilated at 10 μM EDTA, while the steady phase was almost unaffected. The concentration of the protein in these experiments was 1.4 mg/ml, and consequently the concentrations of the total and the dialysable Mg^{++} were about 20 μM and 16 μM, respectively. The inhibitory action of CyDTA was inferior to that of EDTA. 100 μM CyDTA concentration was required for the disappearance of the initial phase. It is evident from Fig. 8 that the initial phase occurs as soon as the Mg^{++} concentration is sufficiently raised in a reaction mixture containing EDTA. Furthermore, on addition of 1 mM Mg^{++} + 1 mM ATP

to a reaction mixture containing 50 μM EDTA the initial burst, was identical to that observed when 1 mM ATP was added to the reaction mixture containing 1 mM Mg^{++} + 50 μM EDTA. This means that the Mg^{++}–myosin B equilibrium is reached instantaneously.

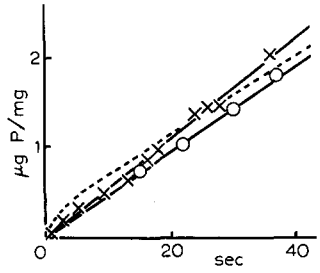


Fig. 7. Effect of chelating reagents on initial burst of P-liberation. ×, 10 μM EDTA; ○, 100 μM CyDTA; dashed line, control. 1 mM ATP, 0.6 M KCl, pH 6.7, 22°.

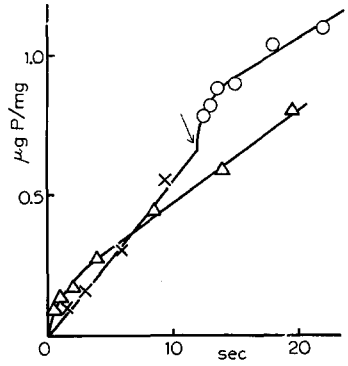


Fig. 8. Initial burst of P-liberation by adding Mg^{++} to a reaction mixture containing EDTA and ATP. ×, 50 μM EDTA and 1 mM ATP at zero time; arrow, 1 mM Mg^{++} added. △, 50 μM EDTA and 1 mM ATP, 1 mM Mg^{++} added at zero time. 0.6 M KCl, pH 6.7, 1°.

Fig. 9 shows the relationship between the quantity of PCMB bound to the SH groups of myosin B, the quantity of the burst, and the rate in the steady phase. In accordance with the results of KIELLEY AND BRADLEY[7] on myosin A, about 8 moles of PCMB were bound to 10^5 g of myosin B.

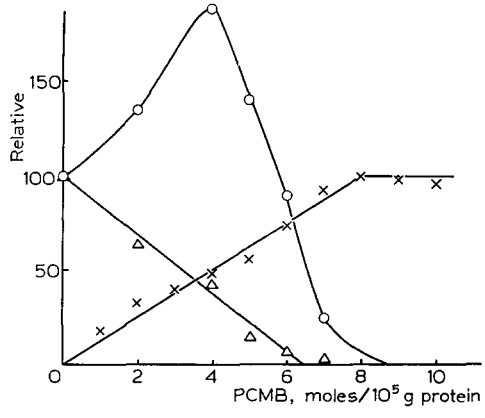


Fig. 9. Decrease of quantity of initial burst with binding of PCMB to myosin B. ×, increase of extinction at 255 mμ; ○, rate in steady phase; △, quantity of initial burst. 1 mM ATP, 0.6 M KCl, 5 mM Ca^{++}, pH 6.7, 20°.

The effect of PCMB on the steady ATPase was also quite compatible with Kielley's observations on myosin A. The most interesting point in this experiment was that a linear decrease of the burst was paraleled by a linear increase of the binding of PCMB. The burst had completely disappeared at about 80 % occupation of the SH groups by PCMB.

DISCUSSION

In the present observations the most striking feature is the effect of divalent cations on the initial phase of ATP hydrolysis: (1) using non-dialysed myosin B the rate is hardly affected by adding Mg^{++} or Ca^{++}, (2) the decrease of the burst produced by exhaustive dialysis is restored to the original level by the addition of a rather minute amount of Mg^{++}, which cannot be replaced by Ca^{++}.

It is evident that the inhibitory effect of EDTA on the initial phase is not merely due to the removal of free Mg^{++} by its chelation. Under our experimental conditions, the amount of dialysable Mg^{++} did not change appreciably* on the addition of 10 μM EDTA, a concentration that completely abolishes the initial burst. Moreover, the inhibitory effect of CyDTA on the initial burst is much smaller than that of EDTA in spite of the fact that the binding constant of CyDTA[8] to Mg^{++} is larger than that of EDTA. The minimum concentration of EDTA (1–10 μM) needed for suppressing the initial burst is of the same order of magnitude as the concentration of ATP-(or PP-)binding-sites of myosin B in the reaction mixture reported above, *viz.* $1.4/5 \cdot 10^5 M = 2.8\ \mu M$ (*cf.* Ref. 9). Subsequently it seems reasonable to assume that the initial ATPase is suppressed by some complex formation between the protein and EDTA, probably mediated by Mg^{++} firmly bound to the protein.

The disappearance of the initial phase, without appreciable decrease of the rate in the steady phase, brought about by dialysis and by addition of EDTA or CyDTA is not in accordance with the presumption that the initial burst is caused by formation of an enzyme-ADP complex as an intermediate of a conventional Michaelis–Menten type, because the above supposition would unequivocally demand that the kinetic constants of the steady state must be changed whenever the initial rate is altered considerably. As reported previously[1], this conclusion is also supported by the consideration on the quantity of the initial burst.

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* In the presence of 1 mM ATP and 16 μM Ca^{++}, 16 μM Mg^{++} is reduced by only a few per cent by the addition of 10 μM EDTA.