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THE OPTICAL-ROTATORY DISPERSION OF MYOSIN A

III. EFFECT OF ADENOSINE TRIPHOSPHATE AND INORGANIC PYROPHOSPHATE

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SUMMARY

The effects of PP_i and ATP on the spectrophotometric titration curve and the optical-rotatory dispersion curve of myosin A were investigated. In 0.5 M KCl the number of "abnormal" tyrosine increased from 3.6–3.7 moles to 6.2–7.0 moles and 5.5 moles per 10^5 g protein on adding PP_i and ATP, respectively, while it did not change on adding EDTA. In 0.5 M NaCl "normal" and "abnormal" tyrosine could not be distinguished, and no significant change in the dissociation state of tyrosine could be observed on adding ATP. The electrostatic interaction factor, w, of the dissociation of "normal" tyrosine was measured under various conditions.

In 0.6 M KCl, PP_i decreases the helical content of myosin A, while ATP increases the helical content by several percent. However, the content does not change on adding ADP. In 0.6 M NaCl ATP increases the helical content of myosin A by several percent in the presence and absence of Mg^{2+} and even in the presence of EDTA, where ATP was not decomposed by myosin A.

INTRODUCTION

It is well known that the size and shape of reconstituted actomyosin and myosin B are changed by the addition of ATP and PP_i[1–4]. The size and shape of myosin A are, however, not changed by ATP and PP_i and binding of ATP and PP_i with myosin A has been investigated by the luciferin–luciferase method[5] and the equilibrium-dialysis method[6,7]. The conformation change of enzyme by its binding with substrate has been generally suggested only on the basis of indirect evidences[8–11]. In the case of myosin A the change in the intramolecular structure on its binding with ATP or PP_i has been inferred from their protecting effect to the heat inactivation of ATPase[12] and the temperature dependence of ATPase and ITPase[13]. Therefore, it seems to be very important to demonstrate by more direct evidences the structural change of myosin A by its binding with ATP and PP_i. In the present studies the spectrophotometric titration and the optical-rotatory dispersion curves of myosin A were measured and it was found that the amount of "abnormal" tyrosine and excess right-handed α-helical content of myosin A change on its binding with ATP and PP_i.

EXPERIMENTAL PROCEDURE

Materials

Myosin A was prepared from rabbit skeletal muscle according to the method of PERRY[14] with slight modifications[15]. Its molecular weight was taken to be 420000 (see refs. 16, 17). Crystalline disodium salt of ATP was the product of Sigma Chemical Co. Guanidine hydrochloride and urea were recrystallized by the method of KOLTHOFF *et al.*[18]. Other reagents were of reagent grade.

Methods

The solution for spectrophotometric titration contained 0.8–1 mg myosin A/ml and 50 mM piperidine buffer, of which pH was adjusted by careful addition of appropriate amount of acid or base. The ionic strength of the solution was brought to 0.5 by adding KCl. The absorbancy at 295 mμ was measured at 25 ± 1° by a Shimazu type QB-50 Spectrophotometer 1 h after changing pH from 8.0 to the desired one. Acid–base titration was made on a Radiometer pH meter (Type TTT Ic) equipped with a glass electrode (G 202 B). All measurements were carried out at 25°.

Optical-rotatory dispersion measurements of myosin A solution were made at 20° and pH 7 by the method described in ref. 19 and the results are expressed in terms of the equation of MOFFITT AND YANG[20]. The excess right-handed helical content was obtained by dividing the b_0 term of the MOFFITT–YANG equation by −580, as suggested by DOTY[21]. The molecular rotatory power of ATP is different from that of ADP[22]. However, the change in rotatory power by splitting of ATP into ADP and P_i was neglected, since under our experimental conditions the change caused by splitting of even 60% of the ATP added was found to be within experimental error (± 2°/1000)).

ATPase activity was measured by determining the amount of P_i liberated by the method of MARTIN AND DOTY[23].

RESULTS AND DISCUSSION

CRAMMER AND NEUBERGER[24] developed a spectrophotometric procedure to investigate the ionization of phenolic groups in ovalbumin and insuline. Similar investigations

have since been performed on serum albumin[25], ribonuclease[26,27], lysozyme[28,29] chymotrypsinogen[30], trypsinogen[31] and trypsin[32]. The spectrophotometric titration of myosin A has already been made by STRACHER[33]. Fig. 1 shows the ultraviolet absorption spectra of myosin A solution at different pH, which were measured by a Hitachi model EPS 2 recording spectrophotometer, and Fig. 2 the experimental results of spectrophotometric titration of myosin A under various conditions. As reported by STRACHER, one type of tyrosyl residue ionizes at pH 10, whereas another type ("abnormal" tyrosine) at pH 11.6. In the presence of 1.2 M urea and 5 M guanidine·HCl (see ref. 34), the titration curve is given by a dissociation curve of a single group whose pK is 9.85. According to KOMINZ *et al.*[35], myosin A contains 18 moles of tyrosine per 10^5 g of protein. From the value of Fig. 2 the amount of molar extinction change at 295 mμ, which is due to dissociation of tyrosyl residue, is given as $2 \cdot 10^3$, in good agreement with that of free tyrosine, $2.3 \cdot 10^3$ (ref. 24).

The change in the spectrophotometric titration curve of enzyme on adding substrate or substrate analog has so far been studied only in case of the binding of polyvalent amine and polyamine with ribonuclease[36]. The effect of PP_i and ATP on the titration curve of myosin A has therefore been investigated. The experiment could

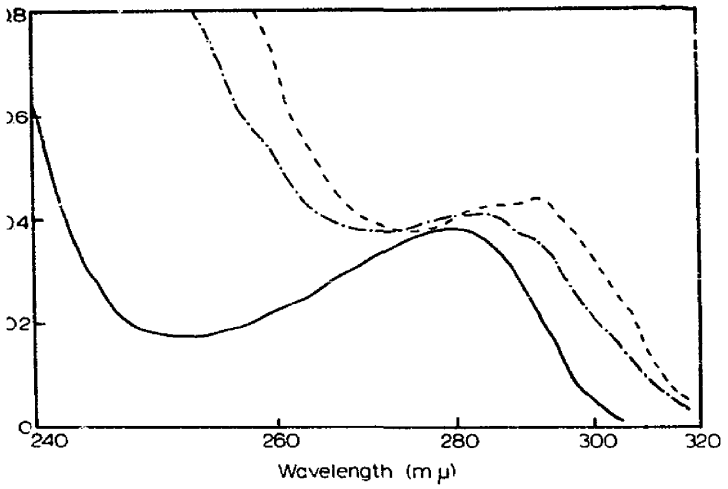

Fig. 1. Ultraviolet absorption spectrum of myosin A as a function of pH. $\mu = 0.5$, 0.538 mg myosin A/ml at 25°. ———, pH 6.90; –·—·–, pH 10.88; – – – –, pH 12.31.

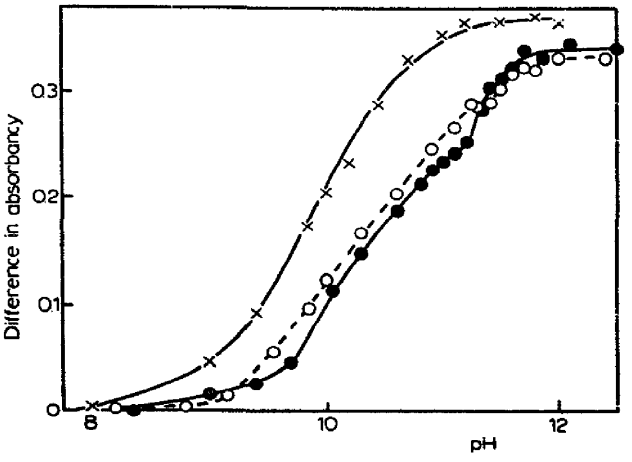

Fig. 2. Spectrophotometric titration of myosin A. 0.5 M KCl, 50 mM piperidine buffer, 1 mg myosin A/ml, 25°. O--O, control; ×—×, 1.2 M urea–5 M guanidine·HCl. The line is a theoretical one for dissociation of a single group whose pK is 9.85; ●—●, 0.5 mM Mg^{2+}-PP_i.

be carried out only under limited conditions*, since about 1 h was required to the measurement, protein concentration was high (1 mg/ml), and the solubility of phosphate compound in the presence of divalent cation was very low at high pH.

As represented in Fig. 2, the amount of "abnormal" tyrosine increased greatly on adding 0.5 mM Mg^{2+}-PP_i. The result was unchanged by decreasing concentration of PP_i to 0.1 mM. Similar results were also obtained in the presence of 0.1 mM Mg^{2+} and 1 mM ATP as shown in Fig. 3. In the presence of ATP, the transition from "normal" to "abnormal" tyrosine was less distinct than in the presence of PP_i. To measure the optical density at 295 mμ of myosin A the value due to Mg^{2+}-PP_i and Mg^{2+}-ATP was subtracted from that of the reaction mixture at respective pH. In Table I are summarized the results obtained under various conditions. As evident from this table, the number of "abnormal" tyrosine increases from 3.6–3.7 moles to 6.2–7.0 moles and 5.5 moles/10^5 g protein on adding PP_i and ATP, respectively.

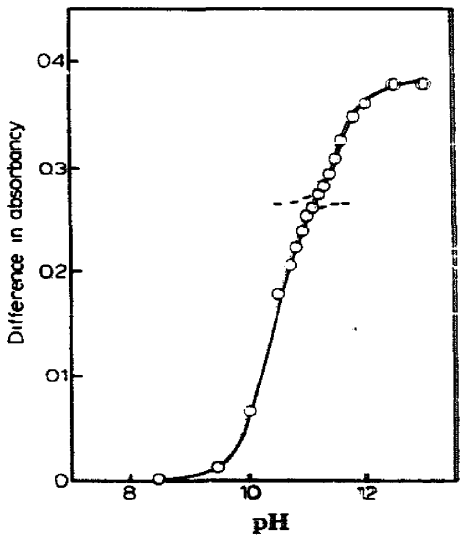

Fig. 3. Spectrophotometric titration of myosin A in the presence of ATP. 0.5 M KCl, 0.1 mM Mg^{2+}, 1 mM ATP, 50 mM piperidine buffer, 1 mg myosin A/ml, 25°.

Addition of 1 mM EDTA to 0.5 M KCl solution of myosin A did not change the titration curve. The direct binding of PP_i or ATP with tyrosine might be a cause of the increase of "abnormal" tyrosine on adding PP_i or ATP. However, the amount of binding of PP_i and ATP with myosin A is 1–2 moles/$4.2 \cdot 10^5$ g of myosin A[5–7], which is much smaller than the amount of the increase in "abnormal" tyrosine on adding PP_i and ATP. Accordingly, it may be concluded that the binding of PP_i or ATP changes the secondary and tertiary structure of the myosin A molecule as a whole and, as a result, increases the number of tyrosine which is buried in the protein structure, though it is not obvious whether "abnormal" tyrosines are hydrogen-bonded with carboxyl groups[36,39] or embedded in the myosin A molecule making hydrophobic bonds[40]. In 0.5 M NaCl the distinction between "normal" and "abnormal" tyrosine

* In the presence of Mg^{2+}, K_m of myosin A ATPase was $5 \cdot 10^{-5}$ M in 0.6 M KCl and at 13° and pH 7.0, and it decreased in alkaline region. The dissociation constant of the binding of ADP with myosin A, inferred from the conventional Michaelis kinetics on the competitive inhibition of ATPase by ADP[37,38] was about 30 times as high as K_m. The dissociation constant of the binding of PP_i to myosin A was $10^{-5.5}$ M at pH 7.5 and 5° (ref. 7). Therefore, under our experimental conditions all the binding sites of myosin A were occupied by ATP or by PP_i.

was not so remarkable as that in 0.5 M KCl, and the significant change in dissociation state of tyrosine was not observed on adding ATP.

In the control solution the absorbancy changed immediately after changing pH to 11.1–11.8 and remained constant for more than 2 h. In the presence of PP_i, the absorbancy changed instantly to constant value at pH 11 and 11.3, whereas at pH 11.63 it increased gradually with time to a constant value. Therefore, even in the presence of PP_i the amount of "normal" tyrosine could be precisely calculated by our method, since "abnormal" tyrosine begins to ionize in the region of pH 11.0. Effect of alkaline denaturation[41] is, however, involved in the titration of "abnormal" tyrosine.

TABLE I

EFFECT OF ATP AND PP_i ON DISSOCIATION OF TYROSINE OF MYOSIN A
0.5 M KCl, 50 mM piperidine buffer at 25°.

Condition	*Preparation*	*Number of tyrosine (moles/10^5 g)*	
		"Abnormal"	*"Normal"*
Control	c	3.6	14.4
Control	d	3.7	14.3
Control	f	3.7	14.3
Guanidine·HCl + urea	a	0	18
Guanidine·HCl + urea	b	0	18
Guanidine·HCl + urea	d	0	18
Guanidine·HCl + urea	f	0	18
PP_i	b	6.2	11.8
PP_i	c	6.2	11.4
PP_i	d	7.0	11.0
PP_i	f	6.5	11.5
ATP	e	5.5	12.5

Therefore, the electrostatic interaction factor, w, in the LINDERSTRØM–LANG equation[39,42,43] was calculated only on the dissociation of "normal" tyrosine.

In Fig. 4, $pH + \log(1-\alpha)/\alpha$ is plotted against Z, where α is the degree of dissociation and Z is the net charge of the myosin A molecule at given pH. The inserted figure in Fig. 4 is the acid–base titration curves of myosin A in the presence of 0.5 M KCl, 0.5 M KCl + 0.5 mM Mg^{2+}-PP_i and 1.2 M urea + 5 M guanidine hydrochloride. (Blank titrations were subtracted from that of the reaction mixture.) In this figure the isoionic point of myosin A under all the conditions was assumed to be 5.75 according to the result of MIHÁLYI[44]. In the control solution the w term changed from positive to negative in the range of small Z, and pK_0 and w were 10.6 and 0.00062, respectively, in the range of Z larger than 160. In the presence of Mg^{2+}-PP_i, the w term changed also from positive to negative in the range of small Z and pK_0 and w were 10.1 and zero, respectively, in the region of Z larger than 170, though strictly quantitative conclusion cannot be drawn because of inaccuracy in measuring α.

A typical example of the effect of PP_i on the optical-rotatory dispersion of myosin A is shown in Fig. 5. A small but distinct decrease in the $-b_0$ term of the MOFFITT–YANG equation was observed on adding 1 mM Mg^{2+}-PP_i, though the rotatory power of myosin A was not changed on adding 5 mM Mg^{2+} alone. Thus, the excess right-handed helical content of myosin A (about 58%, see ref. 45) decreased by several percent on adding Mg^{2+}-PP_i.

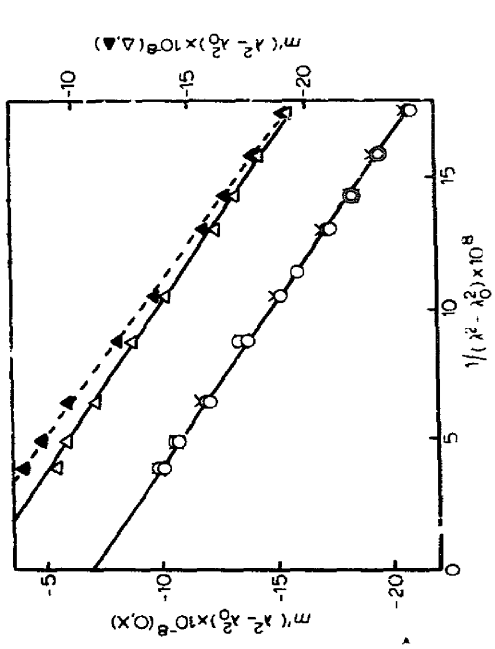

Fig. 4. Plot of data of Fig. 2 according to LINDERSTRØM-LANG's equation. Ordinate: pH + log $(1-\alpha)/\alpha$. Abscissa: net charge per $4.2 \cdot 10^5$ g myosin calculated from acid-base titration curve. O—O, control; ●—●, 0.5 mM Mg^{2+}-PP_i. The inserted figure are acid-base titration curves of myosin A in 0.5 M KCl and at 25°. – – –, control; ———, 0.5 mM Mg^{2+}-PP_i; –·—·–, 1.2 M urea–5 M guanidine·HCl.

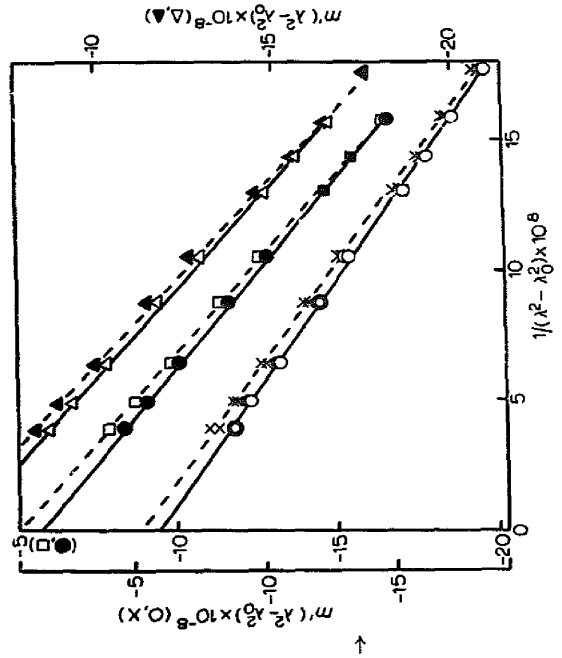

Fig. 6. MOFFITT-YANG plot of dispersion data in presence of Mg^{2+}-ADP and Mg^{2+}-ATP. 0.6 M KCl, 20 mM Mg^{2+}, 3.81 mg myosin A/ml, pH 7.0, 13°. O—O, control; ×—×, 3 mM ADP; △—△, control; ▲- - -▲, 3 mM ATP.

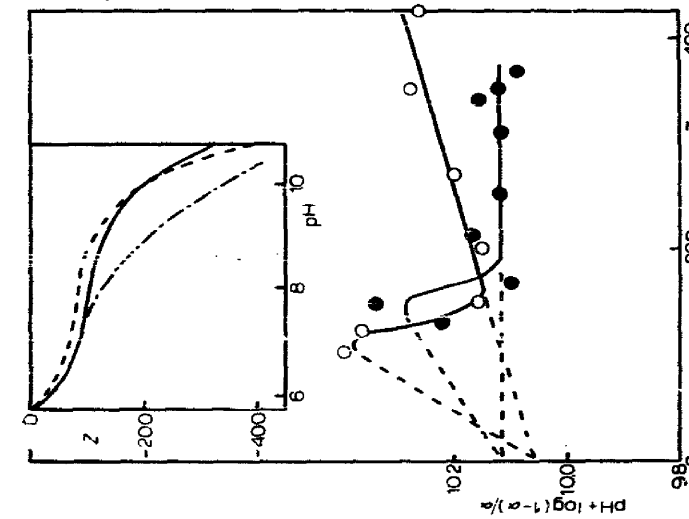

Fig. 5. MOFFITT-YANG plot of dispersion data in presence of Mg^{2+}-PP_i. 0.6 M KCl, 3.88 mg myosin A/ml, pH 7.0, 13°. O—O, control; ×—×, 1 mM PP_i + 2 mM Mg^{2+}.

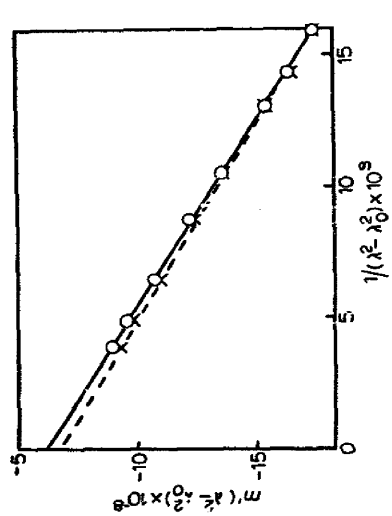

Fig. 7. MOFFITT-YANG plots of dispersion data in 0.6 M NaCl in presence of ATP under various conditions. 0.6 M NaCl (pH 7.0), at 14°. O—O, control; × - - - ×, 1 mM ATP; △—△, 5 mM Mg^{2+}; ▲- - -▲, 5 mM Mg^{2+} + 1 mM ATP; ●—●, 0.5 mM EDTA; □ - - - □, 0.5 mM EDTA + 1 mM ATP.

As represented in Fig. 6, no significant change was detected in the optical-rotatory dispersion curve of myosin A on the addition of 20 mM Mg^{2+} and 3 mM ADP (as mentioned above, the dissociation constant of the binding of ADP to myosin A was 1.5 mM).

The effect of ATP on the optical-rotatory power of myosin A could be measured under limited conditions, as in the case of the spectrophotometric titration. Contrary to the addition of Mg^{2+}-PP_i, the $-b_0$ term was increased by several percent on the addition of 3 mM Mg^{2+}-ATP in 0.6 M KCl (Fig. 6) (during the measurements 40% of ATP was decomposed but the change in rotatory power due to the decomposition could be neglected). In 0.6 M NaCl the ATPase activity was very low and the optical-rotatory dispersion could be measured under various conditions. As shown in Fig. 7, in 0.6 M NaCl solution ATP increased the $-b_0$ term of myosin A by several percent in the presence and absence of Mg^{2+} and even in the presence of EDTA where ATP was not decomposed by myosin A[46]. Accordingly, it may be concluded that the difference between the effect of ATP and PP_i is due to binding of ATP with myosin A not only at the triphosphate group but also at the adenine group[47-54] rather than hydrolysis of ATP.

As described above, in 0.5–0.6 M KCl PP_i decreased the helical content of myosin A, while ATP increased the content by several percent. However, both PP_i and ATP increased remarkably the number of "abnormal" tyrosine. It is very difficult at present to establish the mechanism of molecular change of myosin A by PP_i and ATP from these two results, since optical rotation can show only a net increase or decrease of helical content and does not reveal other conformation changes. However, these results clearly show that the secondary and the tertiary structure of the myosin A molecule change on its binding with PP_i or ATP. These results may be important for elucidating the molecular mechanism of not only ATPase activity but also the physiological function of myosin A, since myosin A has ATPase activity. PP_i is a competitive inhibitor of ATPase and the interaction of myosin A and ATP seems to be an essential step in muscle contraction (*cf.* ref. 55).

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