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THE OPTICAL-ROTATORY DISPERSION OF MYOSIN A

II. EFFECT OF DIOXANE AND *p*-CHLOROMERCURIBENZOATE

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SUMMARY

Effects of dioxane and *p*-chloromercuribenzoate (PCMB) on the optical-rotatory dispersion of myosin A were measured in 0.6 M KCl at pH 7.0 and compared with those on the ATPase activity. The α-helical content of myosin A estimated from the b_0 term of the MOFFITT–YANG plot was 57–61 %. On adding 8–10 volume percent of dioxane, increase in the helical content by several percent and pronounced activation of ATPase were firstly observed and were followed by gradual decreases in the helical content and the ATPase activity. 2 h after the addition of dioxane, the helical content decreased only by a few percent, while the ATPase activity disappeared completely. Immediately after the addition of dioxane, specific rotatory power at 5000 Å showed its maximum at about 10% of dioxane in accordance with the activation of ATPase. The helical content of the alkaline-inactivated myosin A, however, remained constant on the addition of dioxane. On adding PP_i before and at various times after the addition of dioxane, shift in the helical content caused by dioxane was depressed completely and the content remained constant during the measurements. On adding 3–4 moles PCMB per 10^5 g of myosin A, the maximum velocity and the Michaelis constant of ATPase at 20° were increased, respectively, from 0.22 to 0.44 mmoles P_i/min/g and from 1.3 to $1.5 \cdot 10^{-4}$ M, and temperature dependence of the maximum velocity was increased significantly, while on adding 8 moles PCMB the ATPase activity was completely inhibited. The helical content of myosin A increased by several percent on addition of 4 moles PCMB and decreased by several percent on 8 moles PCMB per 10^5 g. On adding PCMB in the presence of ATP or PP_i, the helical content fell in between those in the presence of either of the two. On the basis of these and other observations, it was suggested that conformation of the active site is very susceptible to influences of dioxane and PCMB and that, accordingly, a minute change in the helical content induces a pronounced change in the ATPase activity.

INTRODUCTION

Several investigations have been reported on the connection of enzymic activity with molecular structure of myosin A. LEVY *et al.*[1] inferred from temperature dependences

Abbreviation: PCMB, *p*-chloromercuribenzoate.

of the rates of hydrolysis of ATP and ITP that conformation of the active site changes on its binding with the substrate. The present authors[2] have recently observed the changes in the number of "abnormal" tyrosine and in the excess right-handed helical content of myosin A on its binding with PP_i and ATP. The activation and inhibition of ATPase by PCMB was firstly observed by KIELLEY AND BRADLEY[3]. BLUM[4] deduced from his kinetic studies on the activation of ATPase by PCMB that the binding of PCMB induces structural changes in the active site of myosin A. KOMINZ[5] has observed a remarkable change in the sedimentation coefficient of myosin A on its treatment with methylmercuric hydroxide. In the previous paper[6] the effects of dioxane on the molecular shape and the ATPase activity of myosin A were reported. Soon after the addition of 10 volume percent dioxane to 0.6 M KCl solution of myosin A, an enhancement of ATPase activity, an increase in the radius of gyration, and a decrease in the viscosity were observed concomitantly. These changes were followed by a gradual decrease in the radius of gyration, an increase in the viscosity, and inhibition of ATPase activity. Furthermore, it was found that the properties of enhancement of ATPase by dioxane are very similar to those by PCMB. On the basis of these results, changes in the ATPase activity and the optical-rotatory dispersion of myosin A solution on adding dioxane and PCMB were investigated to clarify the relation between the ATPase activity and the secondary structure of myosin A.

EXPERIMENTAL PROCEDURE

Myosin A solutions were obtained by the method of PERRY[7] after minor modifications[6]. The solutions were clarified by centrifugation at $10^5 \times g$ for 2 h before use.

Crystalline disodium salt of ATP was obtained from Sigma Chemical Company. PCMB and other chemicals were commercial products of guaranteed grade. Since MORALES AND HOTTA[8] have pointed out that dioxane contains a small amount of peroxide and the dioxane effect on ATPase is mimicked by hydrogen peroxide, guaranteed grade dioxane was purified by the following procedures[9]: 300 ml of dioxane was boiled in the presence of 3–4 g of Ag_2O for 2.5 h, and distilled after addition of fused KOH. A fraction was distilled again in the presence of Na metal. 20 g of iron powder was added to 250 ml of the distillate, and distilled after boiling for 2 h. Purified dioxane was kept as 80% aqueous solution in a refrigerator. By these procedures the content of peroxide was reduced from 1.5 to lower than 0.25 μmoles/ml.

The reaction mixture for measurement of ATPase activity contained 0.6 M KCl, 7 mM $CaCl_2$ as an activator, 10 mM Tris–maleate buffer (pH 7.0) and 0.5 mM ATP and 0.2–0.3 mg/ml of myosin A. Measurements were usually carried out at 20°. The reaction was stopped by adding trichloroacetic acid at measured intervals of time and P_i liberated was determined by the MARTIN–DOTY method[10].

Optical-rotatory dispersion of 0.6 M KCl solution of myosin A was measured by means of a model 200S-80 photoelectric spectropolarimeter with an oscillating polarizer prism (O. C. Rudolph and Sons (U.S.A.)) in the range of wave-length from 3200 to 5500 Å. A xenon compact arc lamp was used as the source for continuous spectra. The polarimeter can be set to $\pm$ 0.0015°, if the same end plates are used at fixed positions throughout one series of experiments. The concentration of myosin A was about 5 mg/ml and the angle of rotation was from —0.15 to —1.50. Polarimeter tube was 10 cm in length. Its temperature was maintained usually at 20 $\pm$ 0.2° by

circulating water from an ultrathermostat Haak (Germany) through the jacket surrounding the tube. All the results are expressed in terms of the equation of MOFFITT AND YANG[11]:

$$[m'] = \frac{3}{n^2+2}\frac{M_0}{100}[\alpha] = \frac{a_0\lambda_0^2}{\lambda^2-\lambda_0^2} + \frac{b_0\lambda_0^4}{(\lambda^2-\lambda_0^2)^2}$$

where $[m']$ is the so-called effective residue rotation at any wave-length, λ, this being the observed specific rotation, $[\alpha]$, corrected for the effects of the refractive index, n, measured by an Abbe type refractometer, and the average molecular weight of the single residue, M_0, calculated to be 117 (see ref. 12). The adjustable parameter, λ_0, was taken as constant and equal to 2140 Å. Excess right-handed helical content was estimated by dividing the $-b_0$ term by 580 (see ref. 13), since the a_0 term varies not only with the helical content but also with changes of the medium.

Concentration of the protein was calculated by multiplying the nitrogen content, determined by the micro-Kjeldahl method or by the micro-Dumas method (Coleman's nitrogen analyzer), by a factor of 6.

RESULTS

Effect of dioxane

Dispersion data at various times after the addition of 8 volume percent of purified dioxane are presented in Fig. 1 in the forms of the plot recommended by MOFFITT AND YANG[11]. As clearly seen in the figure, slight decrease in the $-a_0$ term and slight increase in the $-b_0$ term were observed immediately after the addition of dioxane, and then a gradual increase in $-a_0$ and a decrease in $-b_0$ with time were observed. Similar results were also obtained by adding unpurified dioxane into myosin A solution. These changes in a_0 and b_0 were observed without exception for all eight preparations tested.

In Fig. 2 are shown the time rate of the changes in excess right-handed helical content of myosin A after the addition of 8–10 volume percent dioxane together with

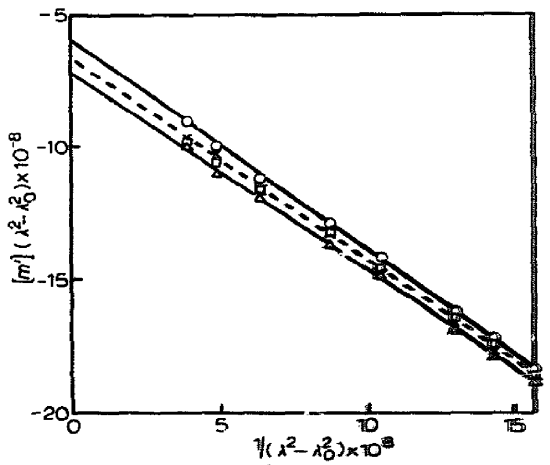

Fig. 1. MOFFITT–YANG plots of typical examples of dispersion data at various times after addition of 8 volume percent purified dioxane. 0.6 M KCl, myosin A No. 89, 3.25 mg/ml, at 20° (pH 7.0). ×—×, control; ○—○, 30 min; □—□, 120 min; △—△, 240 min after addition of dioxane.

those of the ATPase activity. The excess right-handed helical content of myosin A in 0.6 M KCl fluctuated from one preparation to another in the range from 57 to 61%. Comparing the results in Fig. 2 with those presented in the previous paper[6] (see especially Fig. 8 of ref. 6), it is clear that the helical content changes parallel to the ATPase activity and the radius of gyration but inversely to the reduced viscosity.

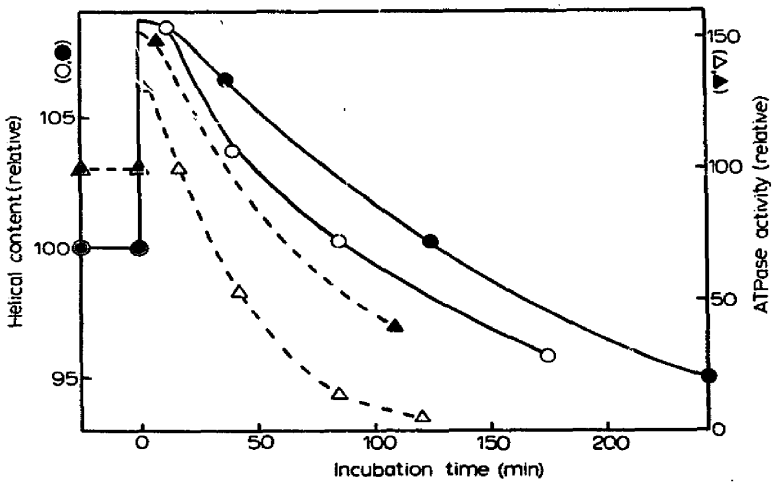

Fig. 2. Time-courses of changes in helical content (○—○, ●—●) and ATPase activity (△—△ ▲—▲) after addition of dioxane. 0.6 M KCl, at 20° (pH 7.0). Helical content was estimated from b_0 term and ATPase activity measured in 7 mM Ca^{2+}, 0.5 mM ATP and 10 mM Tris–maleate buffer. Helical content: ○—○, 10 volume percent dioxane, myosin A No. 72, 4.65 mg/ml; ●—●, 8 volume percent purified dioxane, myosin A No. 90, 3.62 mg/ml. ATPase activity: △—△, 10 volume percent dioxane, myosin A No. 72, 0.2 mg/ml; ▲—▲, 8 volume percent purified dioxane, myosin A No. 89, 0.2 mg/ml.

The magnitude of changes in the helical content was, however, remarkably smaller than in the ATPase activity, the radius of gyration, and the viscosity: Immediately after the addition of 8–10% dioxane the helical content increased only by 5–10% of the control value but the ATPase activity increased to 130–170% of the original, and 100–200 min after the addition of dioxane the helical content decreased only by 4–5%, though the ATPase activity disappeared almost completely.

As shown in this figure, the effect of dioxane on the helical content and on the ATPase activity of myosin A did not change substantially before and after purification of dioxane. The effect of purified dioxane on ATPase was unaffected by the addition of 1 mM KCN, which completely inhibits the effect of peroxide[14]. Therefore, the effect of dioxane must substantially be attributed to dioxane itself but not to peroxide contained in dioxane. However, the time rate of the decrease in ATPase activity by unpurified dioxane was somewhat higher than that by purified dioxane and was retarded by the addition of 1 mM KCN.

When myosin A was incubated for 10 h at pH 10.3, the helical content measured at pH 7.0 decreased from 60 to 50.4% and the enzymic activity disappeared. The addition of 8 volume percent dioxane to the alkaline-inactivated myosin A caused a slight decrease in the $-a_0$ term but no change in the $-b_0$ term (Fig. 3). The addition of 1 mM Mg^{2+}-PP_I decreased the helical content by several percent[2]. When 8% dioxane was added to myosin A after addition of 1 mM Mg^{2+}-PP_I, the $-a_0$ term

decreased slightly at first and then returned gradually to the original value, whereas the $-b_0$ term remained constant as shown in Fig. 3. Fig. 4 shows the results obtained by the addition of 1 mM Mg^{2+}-PP_i at various times after the addition of 8% dioxane. When PP_i was added into myosin A solution 5 min after the addition of dioxane, several percent decrease in the helical content was observed but the helical content remained constant over the period of measurements. The helical content was not affected by the addition of Mg^{2+}-PP_i 150 and 350 min after the addition of dioxane and it remained constant thereafter.

Fig. 5 shows the dependence of the specific rotatory power at 5000 Å, $[\alpha]_{5000}$,

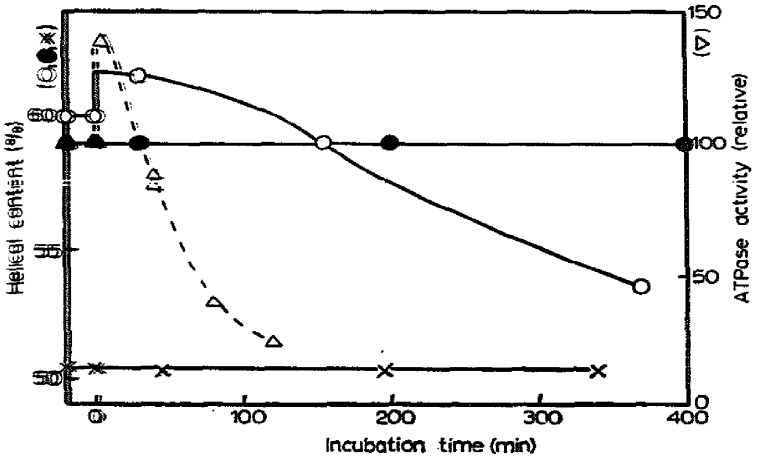

Fig. 3. Effect of dioxane on helical content of alkaline-denatured myosin A and myosin A–PP_i complex. 0.6 M KCl, myosin A No. 103, 4.26 mg/ml, at 20° (pH 7.0). 8 volume percent purified dioxane was added at time 0. ○—○, control; ×—×, myosin A denatured beforehand by incubation for 10 h at pH 10.3; ●—●, myosin A in presence of 1 mM Mg^{2+}-PP_i. △—△, ATPase activity in 7 mM Ca^{2+}.

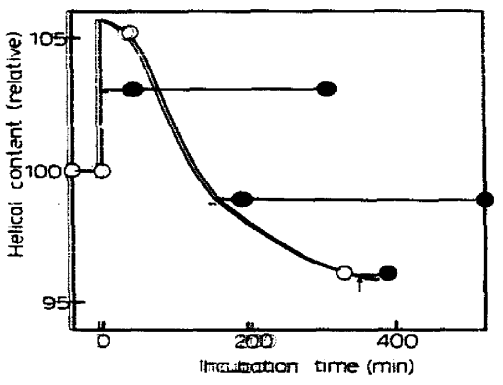

Fig. 4. Effect of PP_i on time-course of change in helical content in presence of dioxane. 0.6 M KCl, myosin A No. 105, 6.14 mg/ml, at 20° (pH 7.0). 8 volume percent purified dioxane was added at time 0. ○—○, in absence of PP_i; ●—●, in presence of 1 mM Mg^{2+}-PP_i. Arrows indicate addition of Mg^{2+}-PP_i.

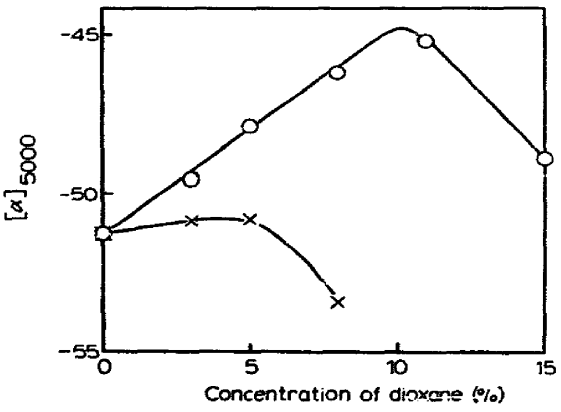

Fig. 5. Dependence on dioxane concentration (volume percent) of specific rotatory power of myosin A at 5000 Å. 0.6 M KCl, myosin A No. 81, 4.72 mg/ml, at 20° (pH 7.0). ○—○, 5–10 min; ×—×, 5 h after adding dioxane.

on the concentration of dioxane. Immediately after the addition of dioxane, $[\alpha]_{5000}$ increased with increase in the concentration of dioxane before it reached the maximum at 10%, and then it decreased gradually with increase in the concentration. A similar dependence on the concentration of dioxane has already been reported on the ATPase activity[6]. 5 h after the addition of dioxane, no increase in $[\alpha]_{5000}$ was observed, and it decreased with increase in the concentration especially above 10%.

Effect of PCMB

Since the effect of PCMB on myosin A ATPase is known to depend on temperature, the helical content was measured as a function of temperature (Fig. 6). The helical content shown in Fig. 6 was measured after 20 min incubation to equilibrate the solution at required temperature. The helical content decreased significantly with increase in temperature above 25°.

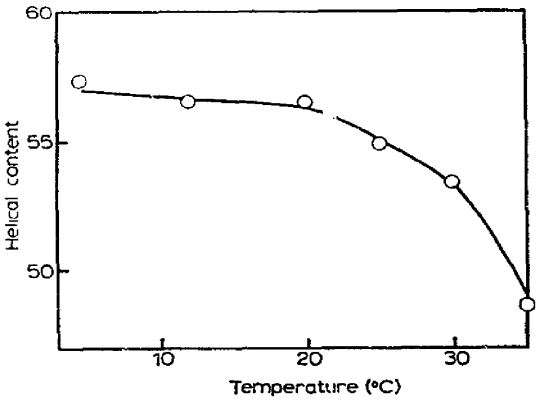

Fig. 6. Temperature-dependence of helical content (in %) of myosin A. 0.6 M KCl, myosin A No. 85, 4.72 mg/ml (pH 7.0).

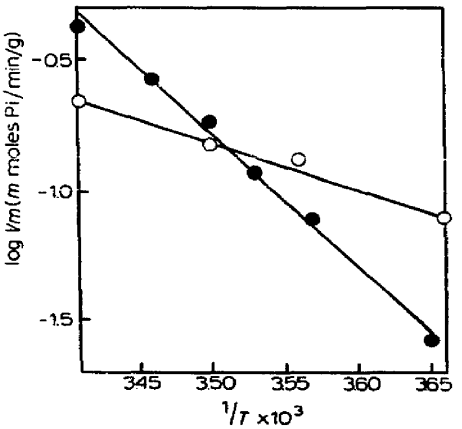

Fig. 7. Maximum velocities of ATPase (V_m) as functions of temperature in presence and absence of 3 moles PCMB/10^5 g protein. ATPase activity was measured in 7 mM Ca^{2+}, 2 mM ATP and 10 mM Tris–maleate buffer (pH 7.0). Myosin A No. 96, 0.1–0.4 mg/ml. O—O, control; ●—●, 3 moles PCMB/10^5 g.

As has been already reported[3,4,15] the ATPase activity exhibits a marked increase when about one half the sulfhydryl groups have been titrated with PCMB and completely disappears when all the SH groups have reacted. Fig. 7 represents temperature dependence of the maximum velocity, V_m, of ATPase in the presence and the absence of 3 moles PCMB/10^5 g; at 20° V_m increased from 0.22 to 0.44 moles P_i/min/g. The Michaelis constant, K_m, increased from $1.3 \cdot 10^{-4}$ to $1.5 \cdot 10^{-4}$ M on the addition of 4 moles PCMB/10^5 g. The apparent activation energies of V_m and K_m in the absence of PCMB were 8.2 and 5.0 kcal/mole, respectively, and those in the presence of PCMB were 23.2 and 6.2 kcal/mole, respectively. Thus, dioxane[6] and PCMB increased V_m, K_m, and especially the temperature dependence of V_m.

A typical example of the effect of PCMB on the optical-rotatory dispersion of myosin A is shown in Fig. 8 and several results obtained are listed in Table I. The helical content was calculated from the b_0 term measured 30–60 min after the addition of PCMB. At all the temperatures measured the helical content increased by 1–7%

on adding 4 moles PCMB/10^5 g, while in the presence of 8–9 moles PCMB/10^5 g it was lower than in the presence of 4 moles PCMB and mostly lower by 2–4% than that of the control. Several percent increase in the helical content by 4 moles PCMB/10^5 g was also observed at pH 5.6 and 10.6.

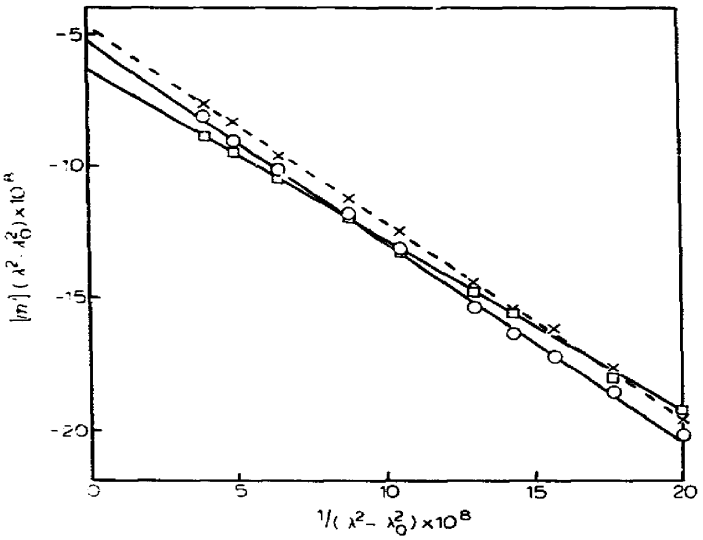

Fig. 8. MOFFITT–YANG plots of dispersion data in presence of various amounts of PCMB. 0.6 M KCl, myosin A No. 84, 5.1 mg/ml, at 20° (pH 7.1). ×—×, control; ○—○, 4 moles PCMB/10^5 g; □—□, 8 moles PCMB/10^5 g.

In the presence of 4 moles PCMB/10^5 g the optical power remained unchanged with time, but in the presence of 8 moles PCMB the $-b_0$ term decreased gradually. Furthermore, it was found that the optical rotatory dispersion curve of myosin A recovers to the original one by the treatment with 140 moles β-mercaptoethanol/10^5 g after the treatment with 7 moles PCMB/10^5 g*.

After successive addition of 1 mM Mg^{2+}-PP_i and 4 moles PCMB/10^5 g, the helical content of myosin A was observed to be nearly equal to that of the original myosin A, while it decreased by several percent on adding PP_i alone[2]. The helical content of myosin A increased by several percent on adding 3 mM Mg^{2+}-ATP (see ref. 2), while on adding 8 moles PCMB/10^5 g in the presence of ATP it fell in between those in the presence of either of the two. Several percent increase in the helical content was also observed in 0.6 M NaCl either by ATP or by 4 moles PCMB/10^5 g. Contrary to the case of the absence of ATP, the helical content in the presence of ATP decreased by several percent on adding 4 moles PCMB/10^5 g.

* One of the present authors[16] has demonstrated that the "intrinsic" Ca^{2+} is removed by the treatment of myosin A with PCMB and then with cysteine or β-mercaptoethanol. Actomyosin, which was composed of Ca^{2+}-free myosin A and F-actin, superprecipitated without showing the "clearing response" immediately after the addition of a high concentration of ATP, though the ATPase activity of this modified myosin A was the same as that of the original myosin A and this modification produced no change in the viscosity, the sedimentation, and the extent of binding to F-actin. These results, together with the one described here, suggest that the molecular structure of myosin A does not change on this modification and that the combination of a relaxing factor (high ATP etc.) to the "intrinsic" Ca^{2+} is necessary to the "clearing response" of actomyosin.

TABLE I

EFFECT OF PCMB ON HELICAL CONTENT OF MYOSIN A*

Preparation No.	*84*	*85*		*87*		*86*
PCMB (moles/10⁵ g)	*Temperature*					
	20	*4.5*	*12*	*5*	*20*	*4.5*
0	61.3	57.3	56.5	60.4	56.5	57.6
4.0	62.5	64.6	61.6		58.2	61.2
4.7				61.2		
8.0	53.5	59.7	55.8		54.1	
9.4				56.8		55.3

* Helical content was estimated from the b_0 term in 0.6 M KCl and at pH 7–7.3.

DISCUSSION

On adding 10% dioxane the helical content of myosin A estimated from the b_0 term increased firstly and then decreased gradually. The effect of dioxane on the optical rotation of myosin A could not be ascribed to the change in the environment of amino acid residues by the absorption of dioxane to myosin A, since not only the a_0 term but also the b_0 term changed in a similar way to the ATPase activity, the radius of gyration, and the viscosity of myosin A. Furthermore, remarkable activation and inhibition of ATPase by dioxane were observed concomitantly with slight increase and decrease in helical content, respectively. ELÖDI's recent investigations[17,18] have also shown that organic solvents produce significant activation and inhibition of the activities and marked increases in the viscosity without pronounced changes in optical-rotatory power of phosphoglyceraldehyde dehydrogenase and ribonuclease.

BLUM[4] has suggested from his kinetic study on the PCMB-activation a structural change in the myosin A molecule by its binding with PCMB. We[15] have also deduced the same conclusion from the observations that ATPase is more readily inactivated by urea in the presence than in the absence of PCMB. In the present paper, it has been demonstrated that 4 moles PCMB/10^5 g induces an increase in the helical content by a few percent and a remarkable activation of ATPase, while 8 moles PCMB/10^5 g induce a decrease in the helical content by a few percent and a complete inhibition of ATPase*. Furthermore, both PCMB and dioxane increased V_m, K_m and the temperature dependence of V_m and removed the neutral depression of the pH activity curve[6,15]. The ATPase activity was generally measured at 20° and in the presence of Ca^{2+}, while the optical rotatory measurements were made in the absence of Ca^{2+}. Then it must be said that the change in helical content is closely related to the change in properties of ATPase and not to the ATPase activity itself.

It seems, however, to be very difficult to clarify the relationship between enzymic

* Optical-rotatory dispersion curve was measured in the absence of ATP, while the ATPase activity was measured of course in the presence of ATP. Therefore, it may be desirable to determine effects of medium and reagent on the optical rotation in the presence of ATP. Such measurements are, however, very difficult and remain to a further research, since both ATP and ADP are optically active[19], and since ATP is hydrolyzed rapidly because of high concentration of myosin A.

activity and helical structure, as the active site makes up a very small fraction of the enzyme molecule and optical rotation can show only a net increase or decrease of helical content but does not reveal other conformation changes. The apparently close relation between remarkable activation and inhibition in ATPase activity by dioxane and PCMB and slight increase and decrease in helical content may be due to a coincident change in ATPase activity and the net change in helical content of myosin A molecule as a whole. However, a possible explanation of the present results is as follows; the structure of the active site is helical, the structure around the active site is very susceptible to the influence of medium and reagent, and net changes in the helical content of polypeptide chain of other part than the active site is not large*. This assumption seems to be supported by the results that on adding dioxane to the alkaline-inactivated myosin A[20] no change in the helical content was observed and that in the presence of PP_i, which binds to the active site of myosin A ATPase, the shift in the helical content with time on adding dioxane was not observed any more. The result that the helical content after addition of ATP or PP_i and PCMB fell in between the values in the presence of either of the two seems also to support the above assumption, since PCMB and ATP or PP_i are known to bind to myosin A competitively[22]. Our investigations[20,23] on the heat, acid, alkaline and salt inactivations of ATPase have also revealed that the secondary structure of the active site is particularly sensitive to these treatments.

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* The change in helical content reported in this paper can not be attributed to impurities present in our myosin A preparation, since the change in helical content was not affected by further purification of myosin A by the DEAE-cellulose column chromatography, and since the amount of N-terminal amino acid was 0.062 moles per 10^5 g protein, which is much smaller than that reported by BAILEY[21] (0.13 mole/10^5 g protein).

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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.