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EFFECTS OF PRESSURE ON ATPase OF MYOSIN A, HEAVY MEROMYOSIN, AND SUBFRAGMENT 1

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SUMMARY

The effect of pressure on the ATPase of myosin A, heavy meromyosin, and Subfragment 1 was investigated[1] over a pressure range from 1 atm to 2200 kg/cm^2. In the presence of Ca^{2+}, the ATPase activity of myosin A, heavy meromyosin, and Subfragment 1 was first enhanced and then depressed by the increase in pressure, having a maximum at about 1000 kg/cm^2. The optimal pressure for activation and the extent of the activation increased with increasing salt concentration[2]. When the pH of a solution containing Ca^{2+} was changed, the ATPase activity of heavy meromyosin and Subfragment 1 at 1300 kg/cm^2 had a maximum around pH 8 in Tris–HCl and borate–KOH buffers. After the correction of the pH of the buffers under pressure by use of pH indicators (0.3 pH unit for Tris–HCl and −0.2 unit for borate–KOH buffer at 1300 kg/cm^2), the maximal acivation was found at pH 8.1 in both buffers[3]. In contrast with the activation of the Ca^{2+}-activated ATPase, the EDTA-activated ATPase of heavy meromysin and Subfragment 1 was depressed by pressure in the same way as it was depressed by *p*-chloromercuribenzoate[4]. These effects of pressure, especially depression, were not due to any irreversible process, because the original activity was recovered after the release of pressure[5]. The pressure effects on the ATPase were analysed according to the Michaelis–Menten equation. As a result, it is inferred that pressure has effects on both the binding of the substrate and the splitting of ATP.

INTRODUCTION

Many investigations using physico-chemical methods have been carried out on the structure and function of myosin A[1], the enzyme that plays an important role in muscle contraction. In order to elucidate the relationship between the structure and function of this enzyme, pressure experiments may be of interest. They should yield information concerning the effect of pressure on the enzymatic activity associated with the change in the structure as well as the interaction with substrate molecules. Several investigations of the effect of pressure on the ATPase of myosin have been

Abbreviations: EGTA, glycol ether diamine tetraacetic acid; PCMB, *p*-chloromercuribenzoate.

reported[2-5] although the proteins used in some of the early experiments were not characterized as myosin A or B[2,3].

It is necessary, however, to distinguish between myosin A and B because the ATPases of these proteins have different features, *e.g.* their dependence on Ca^{2+} and Mg^{2+}, and also pressure has different effects on the proteins[5]. In this study, the effects of pressure on the ATPase activity of myosin A, heavy meromyosin and Subfragment 1 have been measured. The ATPase of myosin A is activated by Ca^{2+} but not by Mg^{2+}. Interestingly, versenate further activates the Ca^{2+}–ATPase of myosin, and the activated activity is depressed by *p*-chloromercuribenzoate (PCMB)[14]. Therefore, both Ca^{2+}- and EDTA-activated ATPases were measured throughout this study.

EXPERIMENTAL

Materials

Myosin A, heavy meromyosin and Subfragment 1 were prepared according to the method described in the previous paper[5].

Equipment

The pressure apparatus was the one described in the previous paper[5]. A pressure cell with two quartz windows was used for the measurement of pH under pressure, and a cell without windows was used for ATPase measurements.

Measurement of pH

The pH of the buffer solution under pressure was measured with the pH indicators, bromothymol blue and cresol red. The indicators were prepared in the following way: 0.1 g of each indicator was dissolved in 8 ml of 0.02 M NaOH solution, and diluted with water to 250 ml to give a stock solution. Before the experiments, 0.1 ml of stock solution was added to 20 ml of 20 mM buffer solution. Pressure was applied to the solution and absorbance under pressure was measured with a Hitachi EPU-2A spectrophotometer at 617 nm for bromothymol blue and 572 nm for cresol red. Since Tris–HCl and borate–KOH buffers were used for the study of the pH dependence of ATPase activity under pressure, the pressure-induced change in absorbance of the indicators was measured for both buffers.

ATPase activity

ATPase under pressure was measured by the method described in the previous paper[5]. The temperature was controlled at 25° throughout the experiments.

Whether or not the pressure produced any irreversible change in the structure of the proteins could be detected by measuring the rate of inorganic phosphate liberation before and after the application of pressure. This method determined the enzymatic activity under pressure[5].

The substrate (ATP) concentration was either 2 mM or 4 mM. Reagents were of analytical grade when available.

RESULTS

As shown in Fig. 1, the ATPase activity of myosin A in the presence of Ca^{2+} was enhanced by pressure within the range of 1 atm to 2160 kg/cm^2. The curve formed

a bell shape, having a maximum dependent on the salt concentration. The optimal pressure increased with increasing salt concentration; 1200 kg/cm² in 2.5 mM KCl and 1800 kg/cm² in 0.5 M KCl. The activity at the optimum pressure was 4 times as great as that at 1 atm in 2.5 mM KCl and 13 times in 0.5 M KCl. The inhibitory effect of pressures greater than the optimum was not due to an irreversible loss in the activity, because the rate of the inorganic phosphate liberation after the release of pressure was almost the same as that before the application of pressure. The extent of activation was greater at a lower KCl concentration (2.5 mM) when the pressure was lower than 700 kg/cm² as LAIDLER AND BEARDELL[2] observed, whereas, at pressures higher than 700 kg/cm², the extent of activation decreased for the lower KCl concentration and increased for the higher KCl concentration (0.5 M).

The effect of pressure on the Ca^{2+}-activated ATPase activity of heavy meromyosin and Subfragment 1 is shown in Figs. 2 and 3. Activation effects and maxima similar to those for myosin A were observed, but the extent of the activation always decreased with decreasing KCl concentration, even at pressures less than 700 kg/cm². The optimal pressure for the ATPase activity of heavy meromyosin and Subfragment 1 increased with the increase in salt concentration: 900 kg/cm² for 5 mM KCl, 1200 kg/cm² for 0.15 M KCl, and 1800 kg/cm² for 0.5 M KCl, repectively. The extent of the activation also increased with the increase in salt concentration: approx. 2.5-fold for 5 mM KCl, approx. 4-fold for 0.15 M KCl, and approx. 7-fold for 0.5 M KCl at the optimum pressure. These values were smaller than those for myosin A. Depression of the activation observed beyond the optimal pressure was also due not to irreversible denaturation but to a reversible pressure effect as for myosin A, since the same activity was recovered after the release of pressure.

Fig. 4 shows the pH dependence of the Ca^{2+}-activated ATPase of heavy

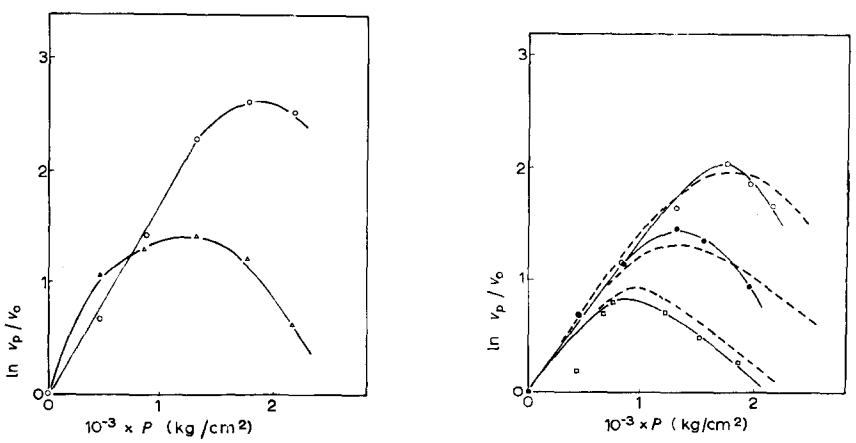

Fig. 1. The activation of Ca^{2+}-activated ATPase of myosin A by pressure. The ordinate is the natural logarithm of the relative activity of ATPase under pressure to activity at atmospheric pressure. △, 2.5 mM KCl; ○, 0.5 M KCl. Myosin A concentration, 0.1 mg/ml; 5 mM $CaCl_2$; 18 mM Tris–HCl (pH 7.6); 4 mM ATP.

Fig. 2. Pressure activation (as shown in Fig. 1) for heavy meromyosin. □, 5 mM KCl; ●, 0.15 M KCl, , ○ 0.5 M KCl. Heavy meromyosin concentration, 0.06 mg/ml; 5 mM $CaCl_2$; 20 mM Tris–HCl (pH 7.6); 2 mM ATP. Broken lines represent three theoretical curves, from top ($\Delta V^* = -40$ ml/mole, $\Delta V = -60$ ml/mole, $K_m/[S] = 0.03$) ($\Delta V^* = -45$ ml/mole, $\Delta V = -63$ ml/mole, $K_m/[S] = 0.08$), and ($\Delta V^* = -50$ ml/mole, $\Delta V = -65$ ml/mole, $K_m/[S] = 0.2$).

meromyosin under pressure and at 1 atm. The experiments were performed at 1300 kg/cm^2 which is around the optimal pressure for the KCl concentration used (0.07 M) and the pH of the solution was controlled by Tris–HCl or borate–KOH buffer. The pressure-activated ATPase had a maximum near pH 8, while, at 1 atm, such a peak was not observed. Although the extent of ATPase activation by pressure in the presence of borate–KOH buffer was lower than that in the presence of Tris–HCl buffer, the optimum pH values for the activation were the same for both buffers (see below).

Similar results were obtained for Subfragment 1 (Fig. 5). The optimum pH for the Ca^{2+}-activated ATPase activity of Subfragment 1 under 1300 kg/cm^2 was around pH 8 in Tris–HCl and borate–KOH buffer. The activity observed was several times greater than that for heavy meromyosin.

Since the pH of a buffer solution is expected to be altered by pressure, the pH of Tris–HCl and borate–KOH buffers was measured with cresol red under pressure. Ionization of salts increases on application of pressure in order to reduce the total volume of the solution by compact hydration due to electrostriction. Since HCl and KOH will dissociate completely into ions in the present pH region (pH 6.0–9.0), the pressure dependence of the ionization of Tris or borate is the main factor determining the pH. When the ionization of Tris proceeds, the pH will increase, and when borate ionizes, the pH will decrease. Actual measurements of absorbance (A) of cresol red against pressure showed an increase in absorbance with pressure in the Tris–HCl buffer, indicating that a shift to a higher pH had occurred. On the other hand, the pH decreased in the borate–KOH buffer. The extent of the pH shift for Tris or borate was calculated from the curve of *A vs.* pH, assuming a linear relation. The shifts were approx. 0.4 pH unit at 1300 kg/cm^2 and approx. 0.6 unit at 2160 kg/cm^2 for the Tris–HCl buffer, and approx -0.3 unit at 1300 kg/cm^2 and approx. -0.5 unit at 2160 kg/cm^2 for the borate–KOH buffer, respectively. When bromothymol blue was

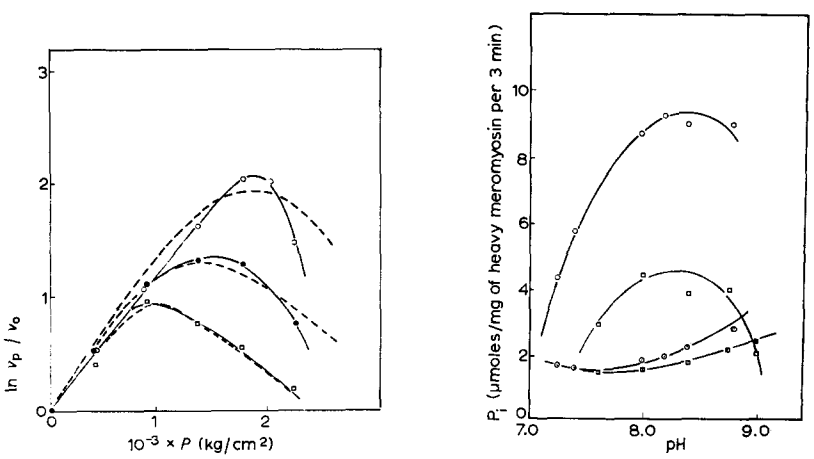

Fig. 3. Pressure activation (as shown in Fig. 1) for Subfragment 1. □, 5 mM KCl, ●, 0.1 M KCl, ○, 0.5 M KCl. Subfragment 1 concentration, 0.12 mg/ml; 5 mM $CaCl_2$; 20 mM Tris–HCl (pH 7.6); 4 mM ATP. The broken lines are the theoretical curves shown in Fig. 2.

Fig. 4. The pH dependence of the Ca^{2+}-activated ATPase of heavy meromyosin under pressure and at 1 atm. ○, Tris–HCl, 1300 kg/cm^2; ⊙, Tris–HCl, 1 atm; □, borate–KOH, 1300 kg/cm^2; ⊡, borate–KOH, 1 atm. heavy meromyosin concentration, 0.04 mg/ml; 73 mM KCl; 4 mM $CaCl_2$; 18 mM buffer; 4 mM ATP.

used as an indicator, similar values for the shifts in pH of the buffers were obtained, *e.g.* 0.2 unit at 1300 kg/cm² for Tris–HCl buffer. Taking the mean value of both experiments, the increase in pH of the Tris–HCl buffer under 1300 kg/cm² was estimated as 0.3 unit. The volume decrease per mole of hydrogen ion was calculated to be 28 ml

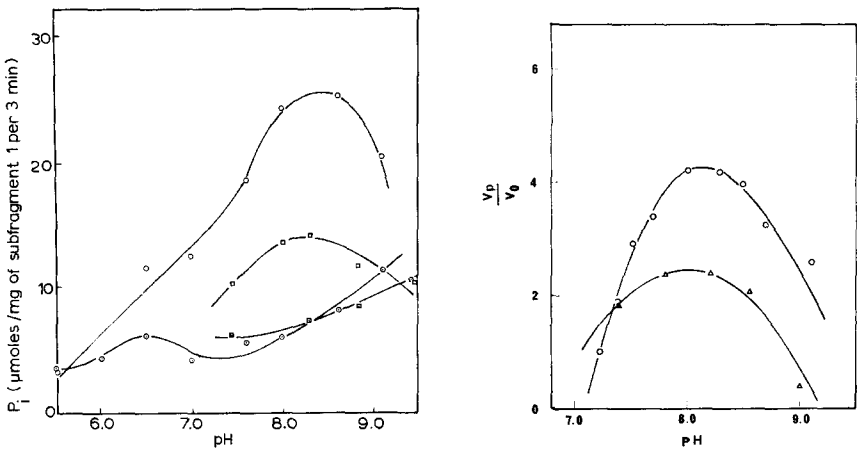

Fig. 5. The pH dependence of the Ca^{2+}-activated ATPase of Subfragment 1 under pressure and at 1 atm. ○, Tris–malate, and Tris–HCl above pH 7.6, at 1300 kg/cm²; ⊙, Tris–HCl at 1 atm, □, borate–KOH at 1300 kg/cm², ⊡, borate–KOH at 1 atm. Subfragment 1 concentration, 0.05 mg/ml; 75 mM KCl; 4 mM $CaCl_2$; 18 mM buffer; 4 mM ATP.

Fig. 6. The ratio of the activity of the ATPase of heavy meromyosin under pressure to the activity at atmospheric pressure was plotted against pH after the correction of the pH shift under pressure. ○, Tris–HCl; pH correction was 0.3 pH unit at 1300 kg/cm²; △, borate–KOH; pH correction was −0.2 pH unit at 1300 kg/cm².

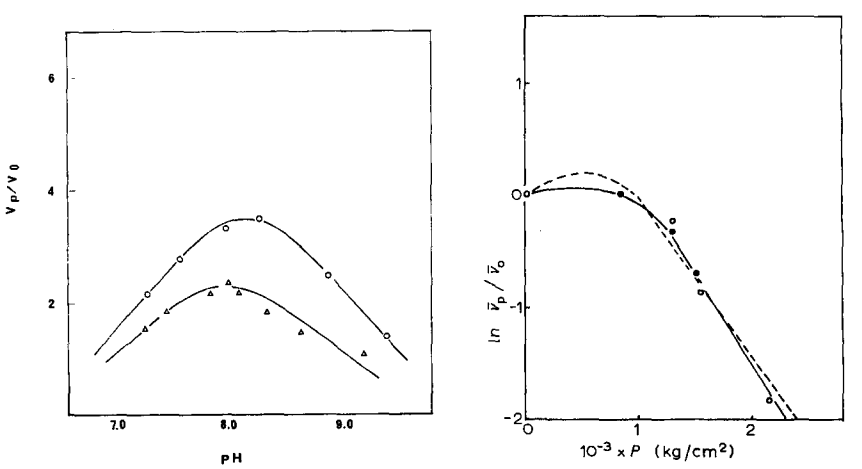

Fig. 7. The ratio of the activity of the ATPase of Subfragment 1 under pressure to the activity at atmospheric pressure was plotted against the corrected pH. pH correction was the same as in Fig. 6. ○, Tris–HCl; △, borate–KOH.

Fig. 8. The depression of the EDTA-activated ATPase of heavy meromyosin (●) and Subfragment 1 (○). The broken line represents a theoretical curve for $\Delta V^* = -40$ ml/mole, $\Delta V = -80$ ml/mole, and $K_m/[S] = 0.2$. Heavy meromyosin concentration, 0.18 mg/ml; 0.5 M KCl; 2 mM Tris–HCl (pH 7.6); 1 mM EDTA; 2 mM ATP.

from a thermodynamical equation, the value which corresponds to the protonation of amino groups[6]. The average pH shift for the borate–KOH buffer was −0.2 unit at 1300 kg/cm².

Using the shift in pH of the buffers at 1300 kg/cm² (0.3 pH unit for Tris–HCl and −0.2 unit for borate–KOH), ratios of the ATPase activity under pressure to that at 1 atm for heavy meromyosin and Subfragment 1 were plotted against the corrected pH. As shown in Figs. 6 and 7, the activation of both proteins has a maximum at the same pH, 8.1, in both Tris–HCl buffer and borate–KOH buffer. That is, application of pressure gives rise to maximal ATPase activity at pH 8.1. The difference in the extent of activation between the two buffers seems to be due to the difference in salts.

In contrast with the pressure activation for the Ca^{2+}-activated ATPase of heavy meromyosin and Subfragment 1 described above, the EDTA-activated ATPase activity was depressed by pressure as shown in Fig. 8. Again, the depression was not due to an irreversible loss of activity, since heavy meromyosin and Subfragment 1 recovered their ATPase activity after release of the pressure.

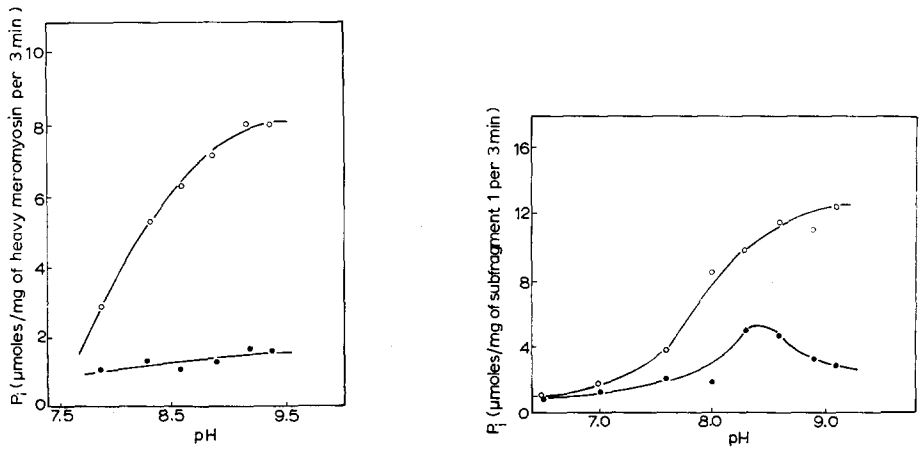

Fig. 9. The pH dependence of the depression of EDTA-activated ATPase of heavy meromyosin by pressure. ○, 1 atm; ●, 1780 kg/cm². Heavy meromyosin concentration, 0.08 mg/ml; 0.8 mM EDTA; 0.4 M KCl; 18 mM Tris–HCl; 4 mM ATP.

Fig. 10. The pH dependence of the depression of the EDTA-activated ATPase activity of Subfragment 1 by pressure. ○, 1 atm; ●, 1730 kg/cm². Subfragment 1 concentration, 0.07 mg/ml; 0.8 mM EDTA; 0.4 M KCl; 18 mM Tris–HCl (Tris–malate for pH 6.5 and 7.0); 4 mM ATP.

TABLE I

ATPase ACTIVITY OF HEAVY MEROMYOSIN WITH AND WITHOUT EGTA

Pressure (kg/cm²)	*EGTA (mM)*	*v (μmoles/mg of heavy meromyosin per min)*
1	1	0.10
1	0	0.09
1300	1	0.23
1300	0	0.24
1730	1	0.16

When the pH of a heavy meromyosin solution in the presence of EDTA was varied from 7.7 to 9.5, the ATPase activity under pressure was always lower than that at 1 atm (Fig. 9). That is, the activity at 1 atm increased with an increase in pH, whereas the activity at 1780 kg/cm^2 remained almost constant. This is a completely different result from that for the Ca^{2+}-activated ATPase. The ATPase activity of Subfragment 1 at 1730 kg/cm^2 in the presence of EDTA was always lower than at 1 atm in the pH region from 6.5 to 9.1 as shown in Fig. 10. The activity under a pressure of 1730 kg/cm^2 has a small maximum around pH 8.3 (without pH correction).

If the effect of EDTA is due to chelation of Ca^{2+}, glycol ether diamine tetraacetic acid (EGTA) might have the same effect on the activation of ATPase by pressure as EDTA. As shown in Table I, the ATPase activity of heavy meromyosin in the presence of EGTA was almost the same as that in the absence of EGTA at 1 atm, *i.e.* EGTA could not activate the heavy meromyosin ATPase activity at 1 atm. The ATPase activity in the presence of EGTA at 1300 kg/cm^2 was almost the same as that in the absence of EGTA but was higher than that at 1 atm. The activity at 1730 kg/cm^2 was lower than at 1300 kg/cm^2. These results are regarded as similar to those observed for the effect of pressure on the Ca^{2+}-activated ATPase activity.

DISCUSSION

The present experiments indicate the pressure-induced activation of Ca^{2+}-activated ATPase for myosin A and its derivatives at pressures of 1 atm to 2000 kg/cm^2. The difference in materials (*i.e.* heavy meromyosin or Subfragment 1 obtained by fragmentation with tryptic hydrolysis of myosin A) did not alter the general features of the pressure dependence of the ATPase activity, although the extent of activation was different. This result suggests that the active site for the ATPase of heavy meromyosin and Subfragment 1 would have a similar structure to myosin A despite the removal of some peptides from the molecule. Some specific interaction of the ions with proteins would be expected, since dependence of activation on the kind of buffer used (Tris–HCl or borate–KOH) was observed; that is, as shown in Figs. 5 and 6, the extent of the activation is different for the two buffers.

The curves of the pressure dependence of the activation of ATPase have maxima dependent upon salt concentration. These reversible activation phenomena may be due to some change in the structure or to pressure effects on a reaction mechanism of the enzyme. The simplest way to interpret the results is to analyse the reaction with the well-known Michaelis–Menten equation

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

$$v = k_2 [E_0]/(1 + K_m/[S]) \qquad (1)$$

where v represents the rate of the reaction in the steady state, k_1 is the rate constant for the formation of an enzyme–substrate complex, ES; k_{-1} is the rate constant for the opposite direction of k_1; k_2 is the rate constant for the breakdown of ES to the enzyme and the product, $E + P$; K_m is the Michaelis constant, and $[E_0]$ and $[S]$ are concentrations of the enzyme and the substrate, respectively. When this reaction takes place under pressure, the equation may be rewritten as follows

$$v_p = k_2 \exp(-p\Delta V^*/RT)[E_0]/1 + (K_m \exp(-p\Delta V/RT)/[S]) \qquad (2)$$

where ΔV^* represents the volume change from the activated state of ES to $E + P$, and ΔV is the volume change associated with K_m, or the volume change accompanied by dissociation of the ES complex to $E + S$ under steady state. When k_2 is much less than k_1, ΔV may be regarded as the volume change of dissociation of the ES complex into $E + S$ in equilibrium. By the use of Eqns. 1 and 2, the following relation may be obtained[7]

$$\ln (V_p/V_0) = -p\Delta V^*/RT + \ln ((1 + K_m/[S])/(1 + K_m/[S] \exp (-p\Delta V/RT))) \quad (3)$$

The last term will be small at a low pressure, so that the initial slope of a curve of $\ln v_p/v_0$ *vs.* pressure would give the volume change of activation, ΔV^*. When the pressure is increased, the second term will approach $-p\Delta V^*/RT$ in magnitude, thus we have the downwards slope observed at higher pressures, giving a resultant of ΔV^* and a volume change associated with dissociation of ES comples to $E + S$, ΔV.

Since experimental values[8,9] for K_m are in the order of 10^{-4} M and the value of $[S]$ in the present experiment is 10^{-3} M, $K_m/[S]$ may be around 10^{-1}. Therefore, one can estimate ΔV and ΔV^* so that the curves calculated from Eqn. 3 fit the experimental ones. The dashed lines in Figs. 2 and 3 represent the calculated curves, where ΔV^* is assumed to be -40 to -50 ml/mole and ΔV, -60 to -65 ml/mole. The increase in activation with the increase in salt concentration seems to be due to a decrease in ΔV^* and in K_m.

It should be noted that the scheme in Eqns. 1 and 2 does not include explicitly the volume change associated with the interaction of the substrate, ATP, with Ca^{2+}. The values of ΔV obtained above, therefore, must include the volume change in dissociation of Ca–ATP to Ca^{2+} + ATP, about -20 to -30 ml/mole (-20 ml/mole[4] for dissociation of Mg–ATP), so that the net volume change of ES complex to $E + S$ would be approx. -30 to -40 ml/mole. The differences in the values of ΔV and ΔV^* from those obtained by LAIDLER AND BEARDELL[2] may arise from the pressure range measured, *i.e.* below 700 kg/cm^2 in the latter experiments and 2200 kg/cm^2 in ours.

The depression of activity in the presence of EDTA may be represented by Eqn. 3, taking appropriate values for parameters, ΔV^*, ΔV and $K_m/[S]$. The values of -40 ml/mole, -80 ml/mole and 0.2 for ΔV^*, ΔV and $K_m/[S]$, respectively, give rise to the best fit of the curve demonstrated by the dashed line in Fig. 8. When the analysis holds true, the principal origin of the depression in activity under pressure seems to be a large decrease in ΔV, suggesting the presence of an additional factor making a large volume change. Since EGTA did not show the peculiar activation induced by EDTA and also has little pressure dependence, the EDTA-activated ATPase is not explained by such a simple mechanism as chelation of Ca^{2+}. Furthermore, direct binding of EDTA to the protein molecule would not be plausible, although such binding could explain the pressure effect on the EDTA-activated ATPase. Presumably, some complicated action of various ions, especially divalent cations including Mg^{2+} with the molecule may be involved in the EDTA-activated ATPase, since a number of reagents activate myosin ATPase[16].

The pH dependence of the pressure effects on heavy meromyosin and Subfragment 1 ATPase activity shows a maximum around pH 8.1 after the pH correction under pressure (Figs. 6 and 7). Since neither Eqns. 1 nor 2 are represented as a function of pH, the result may be interpreted qualitatively in terms of the pH dependences of

ΔV^*, K_m and ΔV. Of course, an equation could be derived on the basis of a scheme taking into account the effect of H^+ as GUTHE AND BROWN did[13]. We, however, have to postulate at least two pH-dependent terms in the scheme in order to show the existence of pH maximum for the pressure activation. This implies that the scheme becomes complicated and that analysis by the above equations might not be meaningful. The simplest method of analysis, therefore, is to assume that ΔV^* and ΔV are not significantly pH dependent and K_m is a function of pH[8]. This is equivalent to the mechanism taking the pH dependence into consideration explicitly.

The curves in Figs. 6 and 7 are considered to represent the profile of the pH dependence of K_m with a maximum at pH 8.1 on the basis of the above assumption. The resemblance of the present results to the pH dependence of K_m at 1 atm obtained by PELLETIER AND QUELLET[12] suggests the possible validity of the assumption. If the interpretation is valid, there must be two ionizable groups with different pK values, approx. 7.4 and 8.6 (ref. 11) responsible for the pH dependence of K_m, so that one can obtain a maximum around 8.1. The ionizable group of pK 8.6 would be cysteine[10,11]. Pressure seems to change the environment of the cysteine residues, since the effect of pressure on the EDTA-activated ATPase activity is similar to the effect of PCMB blocking of thiol groups[14].

It should be noted that the above interpretation is not the only one, but could be explained by combination of the changes in K_m and ΔV and more exactly ΔV^*. The data given in the present experiments are not sufficient to give the conclusive mechanism for pH dependence under pressure.

The analyis using the Michaelis–Menten equation described above shows that pressure has two effects; one weakening the substrate binding to the protein molecule and the other accelerating the splitting of ATP. Since the former results in depression of the activity and the latter activates the ATPase, the activity under pressure may depend on the magnitude of both effects. The reaction mechanism of the myosin ATPase, however, would be more complicated than this. Because the reaction is particularly sensitive to pressure, further pressure experiments carried out in more detail by selecting several experimental conditions (*e.g.* temperature and reagents) may help to elucidate the reaction mechanism.

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