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The Effects of Pressure on Actomyosin Systems*

Takamitsu Ikkai† and Tatsuo Ooi

ABSTRACT: The interactions of actin with myosin, heavy meromyosin, and subfragment 1 were investigated under pressure from 1 atm up to 4000 kg/cm². The results obtained by the change in the intensity of transmitted light through a pressure cell showed that, in the absence of adenosine triphosphate, turbid solutions of myosin B, actomyosin, and heavy actomeromyosin became transparent with increasing pressure finally to give constant intensities of transmitted light at *ca.* 2000 kg/cm², indicating some change in the state of molecules in a solution under pressure. When dissociation of actomyosin into actin and myosin under pressure is assumed, pressure effects on actomyosin should be the same as on the individual component. However, experiments for denaturation of F-actin, heavy meromyosin, and heavy actomeromyosin by pressure demonstrated that heavy meromyosin plus pressure-treated F-actin (at 3500 kg/cm²) had less adenosine triphosphatase activity than F-actin plus pressure-treated heavy meromyosin and pressure-treated heavy actomeromyosin, suggesting that heavy meromyosin protected denaturation of F-actin. In the presence of adenosine triphosphate, high magnesium adenosine triphosphatase activity of heavy actomeromyosin, and actosubfragment 1 decreased sharply to magnesium adenosine triphosphatase of heavy meromyosin, and subfragment 1 in the pressure range from 1 atm to 700 kg/cm². Calcium-activated adenosine triphosphatase of heavy actomeromyosin and actosubfragment 1, on the other hand, showed an increase in activity by pressure, the increase which was due to the pressure effect for adenosine triphosphatase of heavy meromyosin, and subfragment 1. Summarizing those results, the pressure effects on actomyosin systems are accounted for in terms of depolymerization or dissociation of protein components, and adenosine triphosphate plays an important role in interactions of actin and heavy meromyosin.

Many investigations have been carried out on interactions between actin and myosin, which would be a key reaction in muscle contraction. Characteristic properties of actomyosin systems, *e.g.*, Mg-activated ATPase activity, superprecipitation, and dissociation of actin and myosin on the addition of ATP, etc., may originate from these interactions. Measurements of volume changes seem to be useful for the elucidation of the nature of the interactions.

Baskin (1964), using dilatometer, found that a volume of a suspension of myofibrils was decreased or increased on the addition of a low or a high concentration of ATP, the result which was confirmed by Hotta and Terai (1966). However, any definite result which indicates a volume change arises from the interactions between actin and myosin has not been reported so far. This kind of experiment was tried once by Szent-Györyi (1951). Noguchi *et al.* (1964) who intended to measure directly a volume change accompanied with the interaction between myosin B and ATP could not find any definite change. We have tried to measure a volume change induced by mixing of actin and heavy meromyosin with dilatometers of the same type as Noguchi *et al.* used, but any conclusive result could not be obtained either. The accuracy of our apparatus was *ca.* 100 ml/mole of heavy meromyosin, and when the volume change be smaller than this value, it could not be detected.

Compared with direct measurements of a volume change with dilatometry, pressure experiments are much more sen-

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sitive to a change in the volume. When we could measure a degree of dissociation as a function of pressure for the system which undergoes association–dissociation equilibrium, a volume change for the dissociation may be calculated by the pressure dependence of equilibrium constant, K, *i.e.*, $\Delta V = -RT\mathrm{d} \ln K/\mathrm{d}p$. Such examples are illustrated by depolymerization of F-actin under pressure (Ikkai and Ooi, 1966) and dissociation of myosin polymer (Josephs and Harrington, 1967). For myosin B systems, gel–sol transformation was reported under pressure (Marsland and Brown, 1942), the result being analyzed by the above-mentioned method. The interesting results on pressure effects for superprecipitation of myosin B systems were obtained by Rainford *et al.* (1965); they employed an ingenious device to mix ATP with a myosin B solution under pressure, and found depression of superprecipitation. The volume change was also calculated by the equation.

Many workers studied the dissociation of actin and myosin by viscometry or light-scattering technique, and the latter can easily be applied under pressure. Another method, ATPase activity under pressure, may be applied for the present purpose, since Yagi *et al.* (1965) reported that the ATPase activity of heavy meromyosin decreased with the addition of F-actin in the presence of Ca^{2+} but increased in the presence of Mg^{2+}. Therefore, we have used both methods to detect the change in a state of actomyosin systems under pressure.

Experimental Section

Materials

Myosin B extracted from rabbit skeletal muscle with the Weber–Edsall solution was diluted with 0.5 M KCl and purified on the basis of solubility of myosin B to take precipitates at a low ionic strength and supernatants at a high ionic strength by centrifugation (Szent-Györgyi, 1951).

Myosin A was prepared according to the following procedure. After extraction of crude myosin A from minced muscles with the Guba–Straub solution for 10 min at 0°, the residue was removed by a Marusan centrifuge at 1450*g*. The supernatant was further clarified by filtration through gauze. Myosin A was precipitated by the addition of 12 volumes of cold water, and the collected precipitate was dispersed in 0.3 M KCl stirring with a magnetic stirrer for 1 hr and the solution was centrifuged in the no. 21 rotor of a Hitachi Model 40 P ultracentrifuge for 2 hr at 44,000*g*. To the supernatant, 6.5 volumes of cold water was added and the aggregate was spun down at 1450*g*. The precipitate was dispersed again in 0.3 M KCl and aggregates not dissolved were removed by centrifugation for 1 hr in the no. 30 rotor at 77,000*g*. Then 3 M KCl solution was added to the supernatant and the ionic strength was raised from 0.3 to 0.5 M to obtain clear myosin A solutions.

Actin. The acetone dry muscle prepared after extraction of myosin A was suspended in cold water for 1 hr and the extracted G-actin was polymerized with $MgCl_2$ or KCl. The purification was performed according to the procedure described previously (Ikkai and Ooi, 1966).

Heavy Meromyosin. Freshly prepared myosin A, in 0.5 M KCl–20 mM Tris-malate buffer (pH 7.0), was digested with trypsin for 10 min at room temperature (Szent-Györgyi, 1953). The weight ratio of myosin A to trypsin was 200 according to the method of Lowey and Cohen (1962). Digestion was stopped by the addition of soybean trypsin inhibitor twice of trypsin in weight. After dialysis of the reacted solution against ten volumes of 0.02 M neutral Tris buffer, the aggregate (light meromyosin) was removed by centrifugation in the no. 30 rotor at 77,000*g* for 1 hr. The precipitate produced by the addition of $(NH_4)_2SO_4$ to the supernatant (42% saturation) was removed by centrifugation at 16,000*g* for 10 min. Then, heavy meromyosin was precipitated by further addition of $(NH_4)SO_4$ to 60% saturation, and centrifuged down at 16,000*g*. The collected precipitate was dissolved in 0.02 M Tris buffer (pH 7.0) and the solution was dialyzed extensively against the buffer. When the heavy meromyosin solution thus obtained was not used immediately, sucrose was added to a final concentration of 0.1 M, and it was stored in a cold room after lyophilization. Before experiments, the lyophilized heavy meromyosin was dissolved in a desirable buffer solution and dialyzed against the buffer overnight.

Subfragment 1. The heavy meromyosin in 0.1 M KCl solution was digested with trypsin of a weight ratio $^1/_{150}$ to heavy meromyosin at room temperature. The time course of digestion was followed by a pH-Stat (Radiometer, TTT 1) for 1 hr and the reaction was stopped by the addition of soybean trypsin inhibitor. The two-step fractionation of gel filtration and DEAE-cellulose column similar to the method by Tokuyama *et al.* (1966) was employed. The digested materials were immediately applied on a column of Sephadex G-200 (4 × 35 cm) equilibrated with 0.1 M Tris-HCl (pH 7.6) according to Young *et al.* (1965). The column was developed at 6° with the same solvent at a flow rate of 15 ml/hr at a pressure of water head 17 cm and fractions were collected by 3 ml in test tubes of a fraction collector. After reading optical densities of the collected solutions at 280 mμ, fractions of peak 2 (Young *et al.*, 1965) were combined and the solution was applied on a DEAE-cellulose column (2 × 20 cm) equilibrated with 0.1 M KCl–0.02 M Tris buffer (pH 7.6) according to the methods of Mueller and Perry (1961). The column was developed with the same solvent at a flow rate of 20 ml/hr in a cold room, and fractions were collected by 3 ml. The peak eluted at the front contained purified subfragment 1. The subfragment 1 thus prepared had a single peak in a sedimentation pattern obtained with a Spinco Model E analytical centrifuge at 59,780 rpm.

Reagents. All reagents were of analytical grade and deionized water was used throughout the experiments.

ATP, trypsin in twice-crystallized form (lot 36B-1430), and soybean trypsin inhibitor (lot 46B-0480) were purchased from Sigma Chemicals Co.

Protein Concentration. Concentrations of myosin B, myosin A, and actin were determined by the biuret reaction (Gornall *et al.*, 1949) and those of heavy meromyosin and subfragment 1 were measured by the method of Young *et al.* (1965).

ATPase Activity. Substrate concentrations of higher than 0.002 M ATP were chosen in order to avoid the effect of pressure on the binding between enzyme and substrate (Brown *et al.*, 1958), because the preliminary experiments showed the same results in the concentration range of 0.0005–0.004 M ATP. ATPase activity under pressure could not be measured directly, so that the following device was employed to measure ATPase under pressure. Immediately after the addition of ATP to an enzyme solution, pressure was applied for 3–10 min on the solution sealed in a polyethylene bag, and after release of pressure, the solution was transferred with a pipet

into a test tube, which was immersed in a water bath at 25° to keep temperature constant. The manipulating time needed for the application of pressure after the addition of ATP and for the removal of the reacted solution from a polyethylene bag, was approximately 1 min. At an appropriate time interval, 0.5 ml of the reacting solution was withdrawn from the test tube and the reaction was stopped by addition of 4 ml of 12.7% trichloroacetic acid into the solution. The amount of P_i liberated was determined by the method of Taussky and Shorr (1953). In this way, the amount of P_i liberated under pressure could be measured (Figures 7 and 9). In this experiment, P_i liberation was regarded as a zero-order reaction.

Equipment

Pressure Apparatus. Turbidity under pressure was measured with a modified apparatus originally constructed by Suzuki *et al.* (1963). The pressure cell equipped with two quartz windows, 0.8 cm in thickness and 1.2 cm in diameter (the optical path in the cell was 1.4 cm), was made in a cubic form having a side length of 10 cm from SNCM (steel–nickel–chrome–molybdenum) block. The capacity of solution in the cell was *ca.* 20 ml. The pressure cell was set in the middle of an optical path of a Hitachi EPU-2A spectrophotometer and the intensity of the transmitted light was read at 420 mμ. Since a reference solution could not be inserted, relative values to that at 1 atm were measured. In order to get approximate absorbance, water was used as a blank. The maximal change in transmittance in the present experiments was *ca.* 20%, which corresponded to the optical density of 0.1. Application of pressure was done by a hydraulic press as described before (Ikkai and Ooi, 1966). The pressure cell used for measurements of ATPase was the one without windows described in the previous paper (Ikkai and Ooi, 1966). Temperature was controlled by circulating temperature-controlled water through a jacket outside of the cell at 25°.

Results

When pressure was applied on a myosin B solution (0.5 M KCl, pH 7.0), the intensity, T, began to increase with increasing pressure beyond 500 kg/cm² and reached a constant value at around 2000 kg/cm² as shown in Figure 1. On the other hand, when Mg-ATP was added before the application of pressure, any change in the T was not observed. Myosin B contains other proteins than actin and myosin (Ebashi and Ebashi, 1964), so that similar experiments on synthetic actomyosin systems were performed to avoid effects of the contaminations.

Myosin and actin were mixed with the ratio of 3.8:1 and 1.3:1. Figure 2 shows the change in intensity by pressure for those actomyosin solutions (0.5 M KCl, pH 7.0). In the case of M/F ratio 3.8, T began to increase at 500 kg/cm² and saturated at *ca.* 2300 kg/cm². When pressure was decreased slowly, intensity decreased again with decreasing pressure. The experimental points did not lie on the curve obtained by raising pressure. When the M/F ratio was decreased to 1.3, the extent of the change in T was decreased. That is, when the amount of myosin bound to actin was greater, the change in the intensity of the transmitted light became greater. The final extent of the change in the intensity of the transmitted light increased linearly with the M/F ratio from 0.6 until the ratio *ca.* 3, and above this value no appreciable increase was observed as shown in Figure 3.

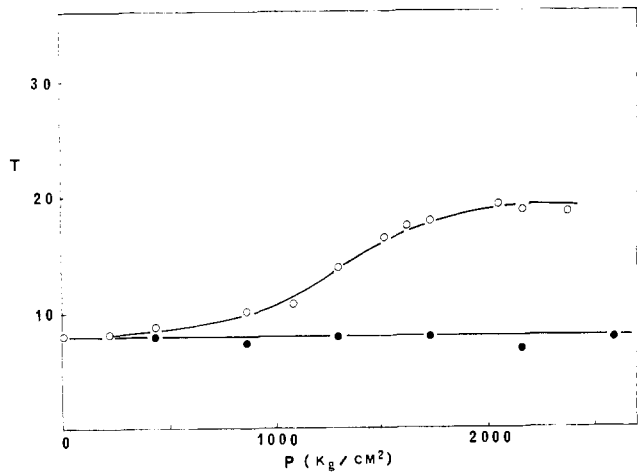

FIGURE 1: Changes in turbidity of myosin B solutions under pressure expressed by intensity of transmitted light, T (arbitrary scale). (○) Myosin B: protein concentration, 0.66 mg/ml; 0.5 M KCl and 0.02 M Tris-malate buffer (pH 7.0). (●) Myosin B: protein concentration, 0.83 mg/ml; 0.6 M KCl, 0.02 M Tris-malate buffer (pH 7.0), and 0.005 M Mg-ATP.

T of heavy actomeromyosin suspension also showed an increase under pressure at 300 kg/cm² and reached a constant value at approximately 2800 kg/cm² (Figure 4), the pressure which was higher than that for actomyosin by 500 kg/cm². When pressure was decreased in order to examine reversibility, turbidity increased again, but the curve did not coincide the one obtained on raising pressure. At an atmospheric pressure, the pressure-treated solution was less turbid than the starting solution. When pressure was applied to the solution once more, T decreased as was observed for first ap-

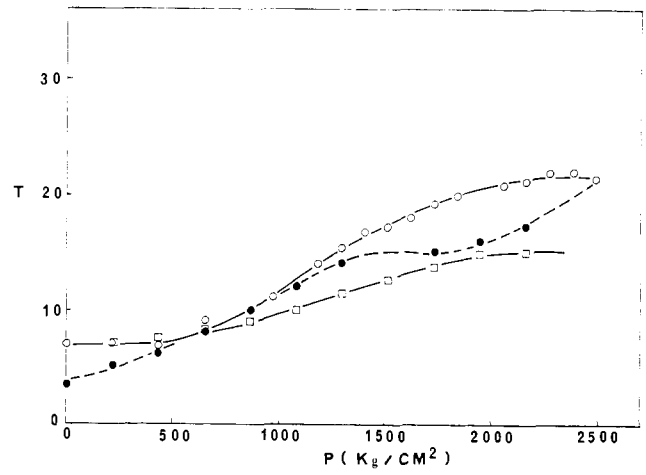

FIGURE 2: Changes in T (arbitrary scale) of actomyosin solutions under pressure and recovery of intensity with decreasing the pressure. (○) Pressure increased; (●) pressure decreased. F-actin (protein concentration 0.4 mg/ml), myosin A (protein concentration 1.5 mg/ml), and 0.5 M KCl and 0.02 M Tris-malate buffer (pH 7.0). (□) F-actin (protein concentration, 0.78 mg/ml), myosin A (protein concentration, 1.02 mg/ml), and 0.5 M KCl and 0.02 M Tris-malate buffer (pH 7.0).

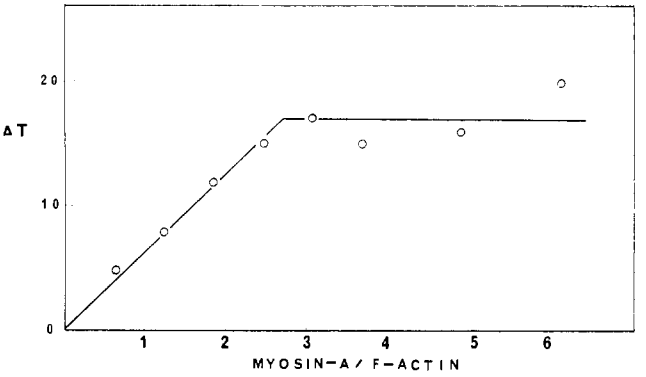

FIGURE 3: Plots of the extent of maximal changes in T (arbitrary scale) *vs.* weight ratio of myosin A to F-actin; 0.5 M KCl and 0.02 M Tris-malate buffer (pH 7.0); pressure *ca.* 2800 kg/cm²; F-actin (protein concentration, 0.4 mg/ml).

plication of pressure, and the final extent of the change in the intensity of the transmitted light was smaller than that for the first application. Although completely reversible curves were not obtained, the change in intensity of heavy actomeromyosin as well as actomyosin by pressure may be regarded to be reversible, because a similar curve was obtained on the second application of pressure.

Since actomyosin solutions become turbid at salt concentrations lower than 0.5 M KCl at neutral pH, experiments were limited around 0.6 M KCl. However, there is no limitation for heavy actomeromyosin solutions, so that the effect of salts on the intensity change by pressure could be measured as shown in Figure 5. In the range of a salt concentration of 0.09–0.41 M, the curves of T *vs.* pressure were almost the same. The result suggests that the state of heavy actomeromyosin modified by pressure is almost independent of the salt concentration.

When the dissociation of heavy actomeromyosin is forced by pressure, ATPase of heavy actomeromyosin under pressure

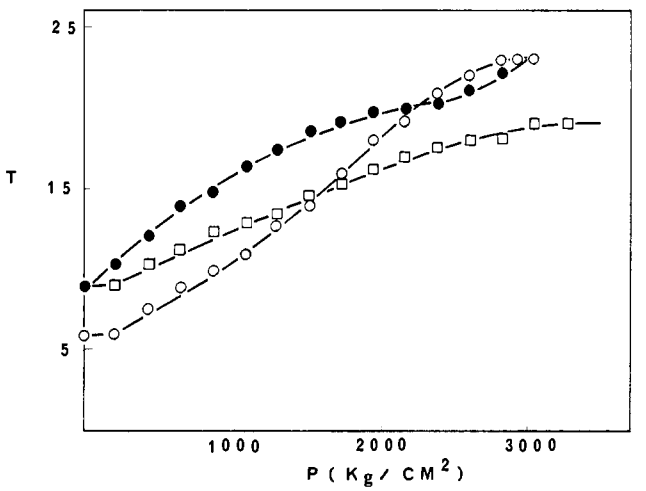

FIGURE 4: Changes in T of an heavy actomeromyosin solution under pressure and the reversibility. F-actin (0.43 mg/ml) plus heavy meromyosin (0.72 mg/ml); 0.1 M KCl and 0.02 M Tris-malate buffer (pH 7.0). (○) Initial increase, (●) decrease, and (□) second increase.

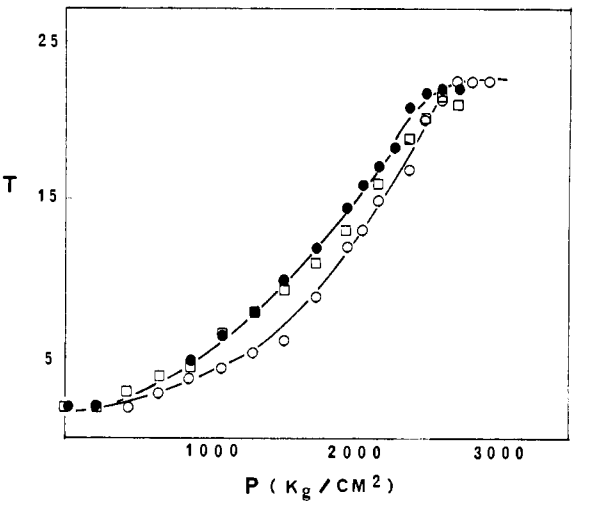

FIGURE 5: Salt effects on the increase in intensity (arbitrary scale) for heavy actomeromyosin solutions. F-actin (0.3 mg/ml) plus heavy meromyosin (0.97 mg/ml); 0.02 M Tris-malate buffer (pH 7.0); (●) 0.41 M KCl, (○) 0.22 M KCl, and (□) 0.09 M KCl.

would be different from that at 1 atm. To begin with, the effects of pressure on ATPase of heavy actomeromyosin, not under pressure, were investigated for three cases. First, pressure was applied on heavy meromyosin, second on F-actin, and third on heavy actomeromyosin for 5 min. After release of pressure, the heavy meromyosin was mixed with F-actin on which pressure was not applied, and the F-actin was mixed with heavy meromyosin on which pressure was not applied; that is, we have three types of heavy actomeromyosin, pressure-treated heavy meromyosin plus F-actin, heavy meromyosin plus pressure-treated F-actin, and pressure-treated heavy actomeromyosin. The results on Mg-activated ATPase for each heavy actomeromyosin were shown in Figure 6. For all materials, there was not appreciable decrease in ATPase activity until 2000 kg/cm². When pressure was increased above 2000 kg/cm², the difference in the treatment appeared. Heavy meromyosin plus pressure-treated F-actin showed a greater decrease in the activity, 85% of the initial value at 3500 kg/cm², and lost Mg-activated ATPase at 4000 kg/cm². On the other hand, the rest of two retained 80% of the initial ATPase activity at 3500 kg/cm². If heavy actomeromyosin dissociates into actin and heavy meromyosin under pressure, the ATPase activity should be equal to or lower than that of heavy meromyosin plus pressure-treated-F-actin. The results show that this is not the case. Since the results shown in Figure 6 were obtained on loss in Mg-activated ATPase, the pressure range where effects of pressure are dominant is higher than that for turbidity measurements, that is, 3500 kg/cm² for ATPase compared with 1500 kg/cm² for turbidity. The results show that some interactions between actin and heavy meromyosin must be present to prevent denaturation of F-actin. From these results, however, it is not easily concluded whether dissociation occurs or not under pressure in the absence of ATP.

It has been known that the subfragment 1 obtained by Mueller and Perry from the tryptic digest of heavy meromyosin is able to bind with F-actin. Therefore, Mg-activated ATPase of heavy actomeromyosin and actosubfragment 1 were studied under pressure. Figure 7 shows the time course

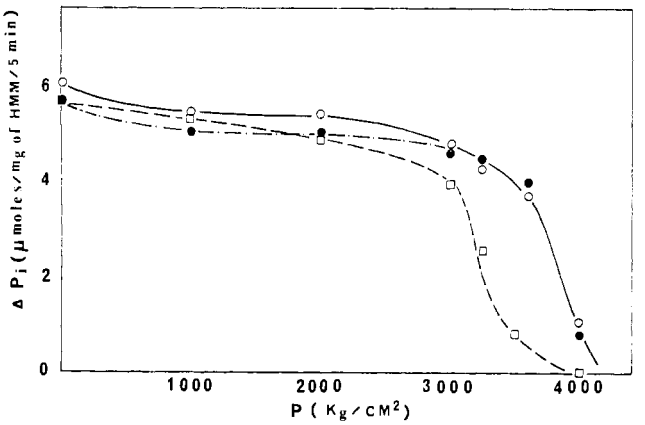

FIGURE 6: Inactivation of Mg-activated ATPase of heavy actomeromyosin by pressure. Pressure was applied for 5 min at 25°, and after release of pressure, ATPase was measured; 0.005 M KCl, 0.02 M Tris-malate buffer (pH 7.0), and 0.002 M $MgCl_2$. (□) Pressure-treated F-actin (0.36 mg/ml) plus heavy actomeromyosin (0.12 mg/ml); (○) F-actin (0.36 mg/ml) plus pressure-treated heavy actomeromyosin (0.12 mg/ml); and (●) pressure-treated heavy actomeromyosin, F-actin (0.18 mg/ml), heavy meromyosin (0.06 mg/ml). A control of Mg-ATPase of heavy meromyosin was 0.2 μmode/mg per 5 min.

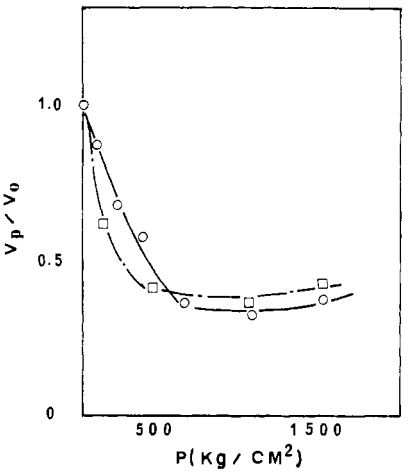

FIGURE 8: The effect of pressure on the Mg-activated ATPase of heavy actomeromyosin (○) and actosubfragment 1 (□) expressed by the ratio of the rate of P_i liberation (V_p) to that at 1 atm (V_0). Heavy actomeromyosin: F-actin, 0.82 mg/ml; heavy meromyosin, 0.23 mg/ml. Actosubfragment 1: F-actin, 0.82 mg/ml; subfragment 1, 0.28 mg/ml. 0.01 M KCl, 0.024 M Tris-HCl (pH 7.6), 0.0024 M $MgCl_2$, and 0.004 M ATP.

of Mg-activated ATPase of heavy actomeromyosin under pressure. The interpolation of the curve after release of pressure gave the P_i value liberated for 10 min under pressure. When the time interval under pressure was 5 min, the interpolated point fell on the dashed line in the figure, indicating that the rate of P_i liberation under pressure was constant and smaller than that at 1 atm. After release of pressure, the initial ATPase activity was recovered and liberation of P_i proceeded. The ATPase activity under pressure thus obtained, of heavy actomeromyosin and actosubfragment 1 in the presence of Mg^{2+}, decreased sharply with increasing pressure, and became constant approximately 35% of the initial activity at pressure of *ca.* 700 kg/cm². This result is consistent with those obtained by Rainford *et al.* (1965). When pressure was increased further, a slight increase of the ATPase activity was observed. There was no essential difference between heavy actomeromyosin and actosubfragment 1 as shown in Figure 8. The activity of higher pressures than 700 kg/cm² is almost the same as that of heavy meromyosin or subfragment 1 in the presence of Mg^{2+}.

To examine the effect of pressure on the ATPase activity

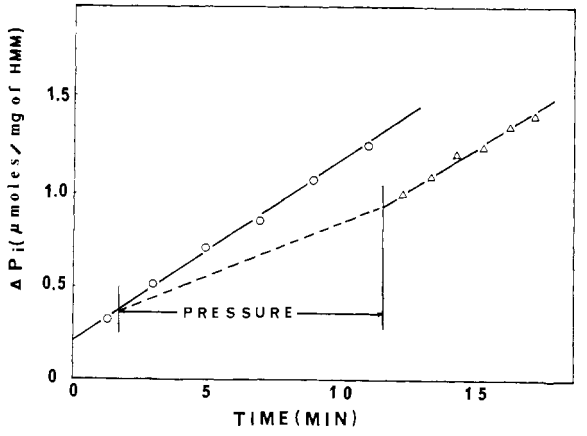

FIGURE 7: Time courses of P_i liberation by Mg-activated ATPase of heavy actomeromyosin at 1 atm and under pressure. F-actin, 0.82 mg/ml; heavy meromyosin, 0.23 mg/ml; 0.01 M KCl, 0.024 M Tris-HCl buffer (pH 7.6), 0.0024 M $MgCl_2$, and 0.004 M ATP. (○) 1 atm and (△) 390 kg/cm².

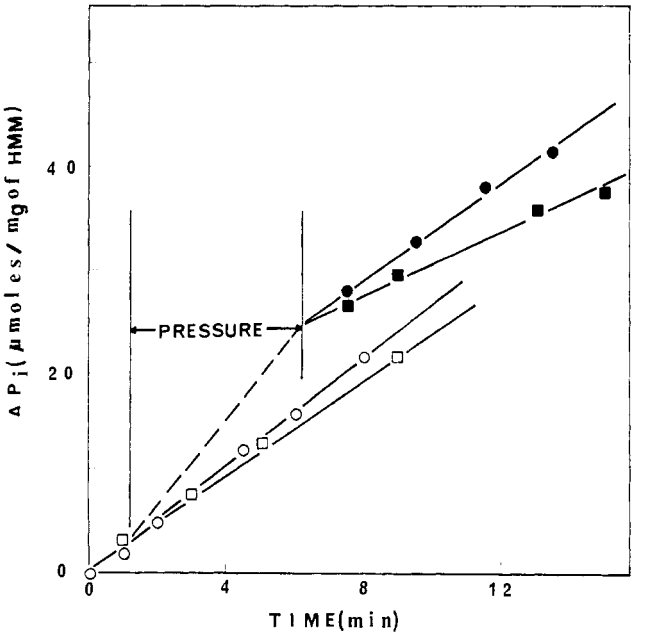

FIGURE 9: Time courses of P_i liberation by Ca-activated ATPase of heavy actomeromyosin and heavy meromyosin at 1 atm and under pressure. F-actin, 0.09 mg/ml; heavy meromyosin, 0.03 mg/ml; 0.005 M KCl, 0.02 M Tris-malate buffer (pH 7.0), 0.005 M $CaCl_2$, and 0.002 M ATP, heavy actomeromyosin: (□) at 1 atm and (■) at 1000 kg/cm²; heavy meromyosin: (○) at 1 atm and (●) at 1000 kg/cm².

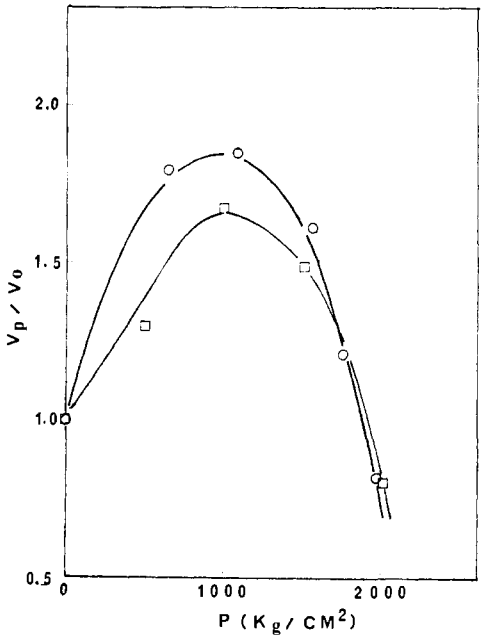

FIGURE 10: Pressure dependence of Ca-activated ATPase of heavy actomeromyosin and actosubfragment 1. Heavy actomeromyosin: F-actin 0.09 mg/ml; heavy meromyosin, 0.03 mg/ml (○). Actosubfragment 1: F-actin, 0.37 mg/ml; subfragment 1 0.12 mg/ml (□); 0.005 M KCl, 0.01 M Tris-malate (pH 7.6), 0.005 M $CaCl_2$, and 0.004 M ATP.

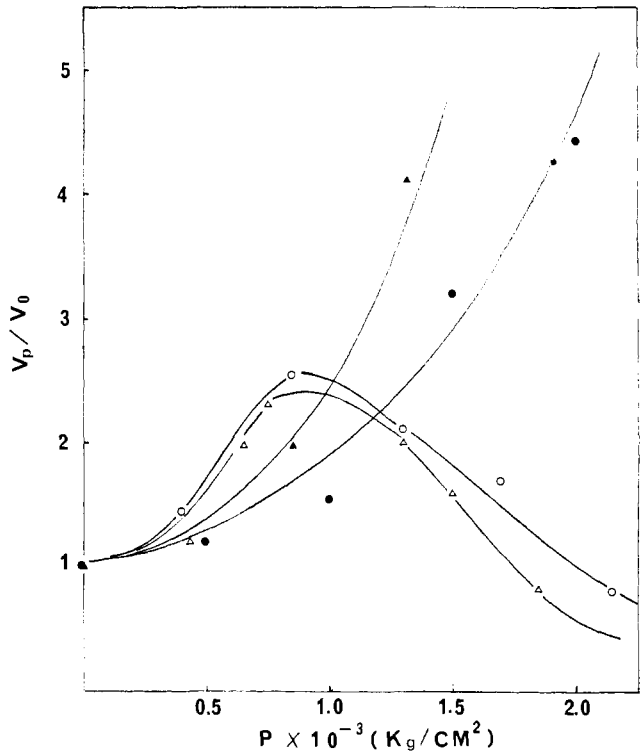

FIGURE 11: Pressure dependence of Ca- and Mg-ATPase of heavy meromyosin and subfragment 1 expressed by relative rate to that at 1 atm. Because Mg-ATPase is low, the experimental points are scattered when expressed in relative rate. (○) Heavy meromyosin (0.06 mg/ml): 0.005 M $CaCl_2$, 0.002 M ATP, 0.005 M KCl, and 0.020 M Tris-HCl (pH 7.6). (●) Heavy meromyosin (0.5 mg/ml): 0.005 M KCl, 0.001 M GEDTA, 0.002 M $MgCl_2$, 0.02 M Tris-malate (pH 7.0), and 0.004 M ATP. (△) Subfragment 1 (0.12 mg/ml): 0.005 M $CaCl_2$, 0.005 M KCl, 0.020 Tris-HCl (pH 7.6), and 0.004 M ATP. (▲) Subfragment 1 (1.1 mg/ml): 0.001 M $MgCl_2$, 0.15 M KCl, 0.021 M Tris-HCl (pH 7.6) and 0.002 M ATP.

of heavy actomeromyosin and actosubfragment 1 in the presence of Ca^{2+}, similar experiments in Figure 9 were carried out under a pressure of 1000 kg/cm². At an atmospheric pressure, the ATPase activity (the slope of P_i liberation in Figure 9) of the heavy actomeromyosin was slightly lower than that of heavy meromyosin, but interpolated values of P_i under 1000 kg/cm² for 5 min were the same, indicating that the ATPase activity of heavy actomeromyosin was identical with that of heavy meromyosin under 1000 kg/cm². Furthermore, the extent of P_i liberation under pressure was higher than that at 1 atm, *i.e.*, activation of ATPase activity under pressure. The ATPase activity of heavy actomeromyosin and actosubfragment 1 in the presence of Ca^{2+} were measured as a function of pressure as shown in Figure 10. The ATPase activity of heavy actomeromyosin has a maximum 1.7-fold of that at 1 atm, at around 1000 kg/cm², and the ATPase activity of actosubfragment 1 has a maximum value, 1.9-fold at 1000 kg/cm². The dependence of ATPase activities upon pressure is quite similar to those for heavy meromyosin and subfragment 1 described below.

The activations of heavy meromyosin and subfragment 1 ATPase by pressure in the presence of Mg^{2+} or Ca^{2+} are shown in Figure 11. The Mg-activated ATPase activity for both heavy meromyosin and subfragment 1 showed monotonous increase under pressure, whereas Ca-activated ATPase has a maximal value for activation at around 1000 kg/cm².

These results suggest the dissociation under pressure of heavy actomeromyosin or actosubfragment 1 in the presence of ATP, because the ATPase of heavy actomeromyosin or actosubfragment 1, which is activated by F-actin in the presence of Mg^{2+} and is inhibited in the presence of Ca^{2+} at 1 atm, becomes identical with that of heavy meromyosin or subfragment 1 under pressure.

Discussion

In pressure experiments, a change in pH by pressure might have serious effects on the results, so that it is necessary to examine the effects by using different buffers such as Tris buffer and borate buffer. The pH does alter by *ca.* 0.3 pH unit at 1300 kg/cm² (T. Ikkai and T. Ooi, to be published). However, significant effects were not observed on the results described previously.

Turbidity experiments on actomyosin systems show that the turbidity of solutions in the absence of ATP decreases on application of pressure, the results which indicate the change in the state of molecules under pressure, *e.g.*, dissociation of actomyosin to actin plus myosin, disaggregation of $(actosin)_n$ to n(actomyosin), or conformational change in actinmyosin molecules, etc.

If the dissociation of heavy actomeromyosin to actin plus heavy meromyosin under pressure is assumed, the results shown in Figure 6 on the loss in Mg-activated ATPase activity by application of pressure seem to be inconsistent with the dissociation of heavy actomeromyosin molecules, *i.e.*, if the dissociation into actin and heavy meromyosin occurs under pressure, pressure should give the same effect on heavy actomeromyosin molecule and on actin, but pressure-treated F-actin plus heavy meromyosin has less activity than pressure-

treated heavy actomeromyosin at 3500 kg/cm². Therefore, possible mechanisms for the effect of pressure on heavy actomeromyosin systems in the absence of ATP would be such that the change in a state of heavy actomeromyosin molecules may occur to give turbidity decrease and some interaction between actin and heavy meromyosin protects denaturation of F-actin. Hence, the dissociation is not probable for the mechanism.

When aggregations of molecules are present in original solutions, the solutions become turbid by aggregates. A typical example is a myosin A solution at an ionic strength of 0.1 M KCl; we have observed a sharp decrease in turbidity under pressure at less than 500 kg/cm². The calculated volume change for this case is *ca.* 180 ml/mole of myosin A. If disaggregation is assumed for actomyosin systems under pressure, the extent of the association of molecules in the systems should be nearly proportional to M/F ratio; that is, the number of myosin bound to F-actin relates to the degree of the association, because the turbidity change under pressure is linearly dependent on M/F ratio (Figure 3). This might be one of the possible explanations.

It must be noted that at the pressure range where turbidity changes occur, F–G transformation of actin also takes place and the curves of pressure dependence of the turbitity are similar to those of depolymerization of F-actin. Therefore, another possible mechanism to interpret the results is the following. F-actin molecules in heavy actomeromyosin dissociate under pressure to depolymerized actin which still interacts with heavy meromyosin to form G-heavy actomeromyosin, protecting pressure denaturation. This is probable when actin–actin interactions are weaker than actin–heavy meromyosin interactions. If the mechanism described above is correct, we can estimate a volume change due to a change in the state by pressure, because depolymerization of F-actomyosin to G-actomyosin is a similar phenomenon to F–G transformation of actin (Ikkai and Ooi, 1966). Values of −70 ml/mole for actomyosin and −50 ml/mole for heavy actomeromyosin are evaluated from slopes of the lines obtained by plots of $\ln \alpha/(1-\alpha)$ (α, fraction of turbidity change) against pressure calculated from the curves in Figures 2 and 5. These values are of the same order of the volume change for F–G transformation of actin, −84 ml/mole.

In the presence of ATP, a change in the state of actomyosin molecules under pressure is deduced from Mg- and Ca-activated ATPase. Mg-activated ATPase of heavy actomeromyosin decreases to Mg-ATPase of heavy meromyosin on application of pressure, and Ca-activated ATPase of heavy actomeromyosin exhibits pressure induced activation, the activation which is due to Ca-activated ATPase of heavy meromyosin (Figure 9), so that the results may be interpreted by dissociation of actomyosin to actin plus myosin on the basis of ATPase activity. However, another important factor to be considered is the dissociation of Mg-ATP complex to Mg^{2+} and ATP^{4-} under pressure as was pointed by Rainford *et al.* (1965). Probably, both effects, the dissociations of heavy actomeromyosin and Mg-ATP would account for the reduction of Mg-activated ATPase under pressure.

When only the dissociation of heavy actomeromyosin is assumed to be responsible for pressure effects as the extreme case, a volume change for the dissociation to actin and heavy meromyosin in the presence of ATP is estimated by the curves in Figure 8. Since a decrease in Mg-activated ATPase is proportional to the amount of dissociated heavy actomeromyosin according to the assumption, the curve is regarded to represent the extent of the dissociation as a function of pressure. The equilibrium constant, K, for this case is expressed as $K = [AM]/[A][M]$ (M represents heavy meromyosin here), so that the corresponding volume change is calculated by the slope of plots of ln K *vs.* pressure. An estimated value was *ca.* −150 ml/mole. This is apparently overestimated, because the dissociation of Mg-ATP was neglected in the above calculation. Presumably, a volume change of around −100 ml/mole would take place for the dissociation of actin and heavy meromyosin, because complex formation of Mg-ATP accompanies a volume change of 21.9 ml/mole (Rainford *et al.*, 1965).

Levy and Ryan (1966) discussed from the kinetic analysis of ATPase activity of superprecipitation that actin and myosin interact at two sites, one of which is responsible for ATPase activity. Perry *et al.* (1966) have shown that when viscosity and turbidity is low, *i.e.*, apparent dissociation occurs, the ATPase activity at the initial state is high. A kinetic analysis of actin activation for ATPase activity of heavy actomeromyosin has shown significant effects of ATP on dissociation of heavy actomeromyosin (Eisenberg and Moos, 1968). Therefore, present experiments together with the above-mentioned results suggest that interactions between actin and myosin in the presence of ATP seems to be different from those in the absence of ATP and the role of ATP is remarkable in modification of interaction between actin and myosin: *i.e.*, the dissociation of actin and heavy meromyosin is unlikely in the absence of ATP (the turbidity experiments), but quite possible in the presence of ATP (the ATPase measurements) under pressure.

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Formation of the Thiazoline Ring in Pantetheine*

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ABSTRACT: In a study of the possible thiazoline formation in coenzyme A, pantetheine was used as a model compound. The thiazoline ring in pantetheine was formed by heating in strong acid and was measured by absorption at 266 mμ. The rate was proportional to both acid and pantetheine concentration in the time period studied and was temperature dependent. The pK for the conversion of thiazoline into the protonated thiazolinonium form was found to be between pH 2 and 3. The ring was hydrolyzed readily at very acid pH values but was stable in neutral and basic solution. The pantetheine thiazoline was located as a distinct spot on paper chromatography.

The formation of the thiazoline ring in certain biologically important compounds has been proposed several times. Linderstrom-Lang and Jacobson (1941) studied the properties of 2-methylthiazoline and postulated that the thiazoline structure might explain the unreactivity of some sulfhydryl groups in native proteins, and their appearance upon denaturation. Calvin (1954) observed the production of a new absorption peak at 268 mμ when glutathione was heated in strong acid and proposed that thiazoline formation could account for this observation. Further studies on the cyclization of glutathione in acid solution have subsequently been carried out in several laboratories (Préaux and Lontie, 1958; Garfinkel, 1958; Martin and Edsall, 1958; Goodman and Salce, 1965; Jocelyn, 1967).

Basford and Huennekens (1955) made a study of the various forms of CoA in a commercial preparation. They proposed that one of the compounds was the thiazoline form of CoA. In a study of thiazoline formation in compounds related to CoA, they observed the expected peak with β-alanylcysteine but not with pantetheine. In an attempt to study cyclization in CoA analogs we have observed the characteristic absorption peak at 266 mμ with pantetheine and therefore, based on previous studies, thiazoline ring formation was assumed. This report describes our observations concerning the probable thiazoline ring formation in pantetheine.

Experimental Section

Chemicals. D-Pantethine (bis(*N*-pantothenylamidoethyl)-disulfide) was obtained from Sigma Chemical Co. Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid), was obtained from Aldrich Chemical Co.

Reduction of Pantethine. Pantetheine was obtained by the reduction of pantethine with 0.5% sodium amalgam which was prepared by reacting 0.5 g of clean sodium metal with 100 g of mercury under toluene; 2 ml of pantethine of the desired concentration was incubated over 0.2 ml of the sodium amalgam with occasional shaking for 30 min at 37°. The solution was neutralized to pH 7.0 with HCl.

Determination of SH. The SH concentration was determined by the use of Ellman's (1959) reagent, 5,5′-dithiobis-(2-nitrobenzoic acid). To 0.5 ml of the properly diluted solution was added 3.0 ml of 5,5′-dithiobis(2-nitrobenzoic acid), 1.5×10^{-3} M (dissolved in 6×10^{-3} M glycyl-glycine, pH 8.0). The yellow color was allowed to develop for 2 min and then the solutions were read against a blank at 412 mμ in a Beckman D.U. spectrophotometer. The absorbance values were converted into micromoles of SH by use of a standard curve determined with glutathione.

Ultraviolet Spectra. The ultraviolet spectra were obtained on a Bausch & Lomb Spectronic 505 spectrophotometer. The samples were read against a water blank in 1-cm, 1-ml quartz cuvets.

Preparation of Pantetheine Thiazoline. The usual method for preparation of the thiazoline was as follows: 0.5 ml of pantetheine solution (concentration varied depending upon experiment) and 0.5 ml of concentrated HCl were heated at 100° for 8 min. The solution was lyophilized to dryness thus removing most of the HCl. The material was either stored as powder or redissolved in H_2O and the pH was adjusted to the desired value. The material was rather stable as a powder but decomposed rapidly when in weakly acidic solution.

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