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The Effects of Pressure on F–G Transformation of Actin*

Takamitsu Ikkai and Tatsuo Ooi

ABSTRACT: The effects of pressure on F- and G-actin were measured in a range up to 4000 kg/cm^2 at room temperature. F-actin in the absence of adenosine triphosphate (ATP) started to undergo irreversible denaturation at a pressure of *ca.* 1500 kg/cm^2, and complete denaturation at 3000 kg/cm^2, whereas in the presence of ATP the pressure where denaturation began was 2500 kg/cm^2, showing a significant protective effect of ATP *vs.* pressure denaturation of F-actin. The addition of EDTA to the system had no effect on the pressure denaturation curve of F-actin in the absence of ATP. On the other hand, for the system containing both ATP and EDTA, the pressure denaturation curve became almost the same as that without ATP (*i.e.*, irreversible denaturation of F-actin began at *ca.* 1500 kg/cm^2 of applied hydrostatic pressure), suggesting that reversible depolymerization occurred in the presence of ATP, since G-actin is known to denature easily in the presence of EDTA, while F-actin does not. Repolymerization of the depolymerized actin after the release of pressure could be observed under suitable conditions, a result which indicates that the transformation of F- to G-actin had occurred under applied pressure. Therefore, it is inferred that the denaturation curve without ATP would represent a reversible F–G transformation caused by pressure. A volume change estimated from the pressure denaturation curve is calculated as −84 ml/mole of monomer, indicating that a volume increase occurs on polymerization of G-actin, presumably due to hydrophobic bonding between monomers. The effect of ATP to protect F-actin *vs.* pressure denaturation is significant compared with the effects of ADP, Mg^{2+}, and Ca^{2+}, suggesting that the protein's structure is stabilized by an appropriate sterical arrangement of a nucleotide binding site fitted for an ATP molecule. Similar experiments performed on G-actin showed that G-actin which requires low salt solutions is, nevertheless, less stable *vs.* pressure denaturation than is F-actin. The concentration dependence for pressure denaturation of F-actin showed a linear but nonproportional relationship, that is, the amount of protein denatured by pressure is dependent upon the initial protein concentration.

Many studies have been reported on the G–F transformation and on the molecular structure of actin. Recent experiments show that an actin molecule composed of two subunits has a rigid hard core (Mihashi and Ooi, 1965a), and that some structural change of the molecule has occurred in the course of the G–F transformation as judged from observations of the difference spectrum between G- and F-actin (Higashi and Oosawa, 1965). Therefore, on the addition of salts, the G-actin which existed in salt-free solution undergoes a specified change in its conformation necessary for polymerization into the double-stranded helical structure shown by electron microscopic studies (Hanson and Lowy, 1963).

Another important feature of actin is the role played by the divalent cations, Ca^{2+} and Mg^{2+}, and nucleo-

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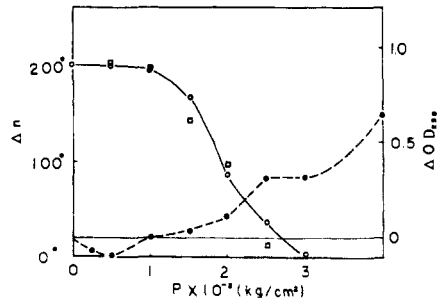

FIGURE 1: Curves of pressure denaturation for F-actin in the absence of ATP represented by flow birefringence, Δn, and optical density of difference spectrum at 258 mμ, ΔOD_{258}, as a function of pressure. Each measurement was carried out after release of the pressure which was applied for 10 min at 25°; protein concentration, 3.3 mg/ml; KCl, 0.06 M; Tris–HCl buffer, 0.005 M, pH 8.1; ○, Δn in the absence of EDTA; □, Δn in the presence of 0.001 M EDTA; ⊙, ΔOD_{258} in the absence of EDTA.

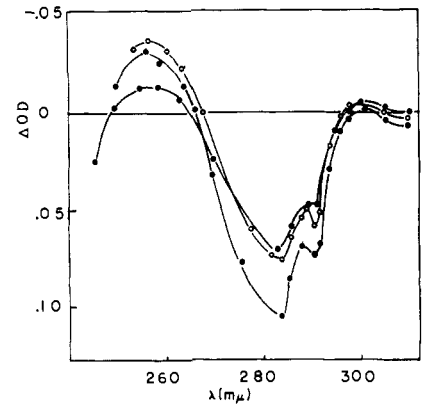

FIGURE 2: Difference spectra for pressure denaturation of F-actin. Pressure was applied for 10 min at 25°; protein concentration, 3.3 mg/ml; KCl, 0.06 M; Tris–HCl buffer, 0.005 M, pH 8.1. The difference spectrum was measured by taking the same solution without pressure treatment as a reference; ⊙, 2000 kg/cm²; ●, 2500 kg/cm²; ○, 3000 kg/cm².

tides, such as ATP[1] and ADP, on the G–F transformation (Szent-Györgyi, 1951, Bárány *et al.*, 1954). Native actin has a bound Ca^{2+} and nucleotides, ATP on G-actin and ADP on F-actin (Szent-Györgyi, 1951). Without these constituents, actin loses its polymerizability in aqueous solutions. Concerning this point, isotopic exchange experiments demonstrated that both Ca^{2+} and ATP on G-actin were easily exchangeable for those added to the solvent, but on the other hand, exchange of Ca^{2+} and ADP did not occur in the case of F-actin (Martonosi *et al.*, 1960; Strohman, 1959), suggesting involvement of these small molecules in the architecture of F-actin.

Because of the strong interaction between nucleotides and F-actin, splitting of ATP takes place in F-actin solutions under such conditions as high temperature (H. Asai and K. Tawada, to be published) and in a sonic field (Asakura, 1962); in other words, F-actin is a kind of ATPase.

The properties mentioned above are found at normal atmospheric pressure. Therefore, it is of interest to carry out experiments on the effect of pressure on actin molecules in order to obtain further knowledge about the structure and properties of actin.

Experimental Section

Materials. Actin extracted from acetone-dried rabbit skeletal muscle with water was purified according to a modification of Mommaerts' procedure (Mihashi and Ooi, 1965a). Special care was taken to remove α-actinin by washing with water in the step removing sodium bicarbonates (Ebashi and Ebashi, 1965; Ebashi and Maruyama, 1965). During the purifying process to remove free nucleotides and metal ions, F-actin was repeatedly dispersed in 0.06 M KCl (0.005 M Tris–HCl buffer, pH 8.0), and then sedimented at 100,000*g* in a Spinco Model L ultracentrifuge. G-actin solutions were prepared by dialyzing the solution of an F-actin pellet in water (ATP, 500 μM, Tris–HCl buffer, 0.002 M, pH 8.0), against water containing 25 μM ATP, then clarifying by centrifugation at 140,000*g* after dialysis.

ATP and ADP were obtained from Sigma Chemical Co. Other chemicals of reagent grade were purchased from Katayama Chemical Co. ^{45}Ca was obtained from the Oak Ridge National Laboratory.

Protein concentration was determined by the Biuret reaction after calibration (Mihashi and Ooi, 1965a). Concentrations of F-actin was measured by the degree of flow birefringence after calibration, since the amount of F-actin is proportional to the degree of the birefringence (Oosawa *et al.*, 1959).

Polymerizability of G-actin was determined by the amount of F-actin formed after the addition of KCl, the final solvent conditions being 0.06 M KCl, 0.005 M Tris–HCl buffer, pH 8.1.

Pressure Equipment. A cell for application of pressure was fabricated from an SNCM (steel–nickel–chrome–molybdenum) block having a dimension of 10 cm in diameter and 15 cm in height with a hole of 1-cm diameter at the center (Suzuki and Suzuki, 1962). The hole was filled with water and a plastic bag (sealed with a soldering iron after filling with a protein solution) was dipped into the hole. Pressure was applied by pressing down upon a bar fitted in the hole using hydropressure alignment. The maximum capacity of the solution in the bag was *ca.* 6 ml. Temperature was maintained at 25° by circulating water outside of the cell block from a temperature-controlled bath. Measure-

[1] Abbreviations used: ATP, adenosine triphosphate; ADP, adenosine diphosphate.

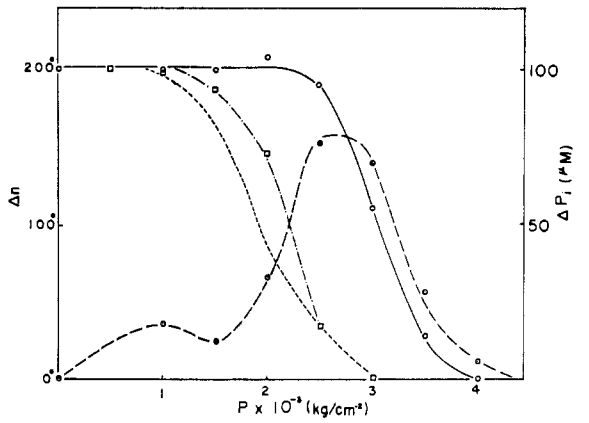

FIGURE 3: Curves of pressure denaturation for F-actin in the presence of ATP represented by flow birefringence, and of liberation of inorganic phosphate, ΔP_i, as a function of pressure. Each measurement was carried out after release of pressure which was applied for 10 min at 25°; protein concentration, 3.3 mg/ml; ATP, 500 μM; KCl, 0.06 M; Tris–HCl, 0.005 M, pH 8.1; ○, Δn in the absence of EDTA; □, Δn in the presence of 0.001 M EDTA; ⊙, ΔP_i in the absence of EDTA. A dashed line represents the denaturation curve for F-actin in the absence of ATP shown in Figure 1.

ments usually were carried out 10 min after release of pressure since time-dependent phenomena were observed in the presence of ATP.

The degree of flow birefringence (Δn) was measured by a Rao type instrument at a shear rate of *ca.* 33 sec^{-1}. On this instrument a value of $\Delta n = 64°$ corresponds to a concentration of 1 mg/ml of F-actin.

Difference spectrum was measured in a Zeiss PMQ II spectrophotometer with 0.2-cm quartz cells, taking the same solution without application of pressure as a reference.

Measurement of inorganic phosphate liberated by ATP split was carried out according to Martin and Doty (1949).

Exchange experiments by the use of ^{45}Ca were performed according to the usual method (Kasai *et al.*, 1965).

The amount of bound nucleotide was measured as follows: nucleotide irreversibly released from actin by applying pressure and free in the solvent was removed by treating the solution with Dowex 1 resin. The protein was precipitated by adding perchloric acid (to a final concentration of 5%), releasing bound nucleotide. Precipitated protein was filtered away and the ultraviolet absorption at 260 mμ of the filtrate was used as a measure of the residual bound nucleotide.

Sedimentation experiments were carried out in a Spinco Model E analytical ultracentrifuge at 59,780 rpm using Schlieren optics.

Viscosity was measured with an Ostwald type viscometer having a flow time of 68 sec for a buffer solution at 25°.

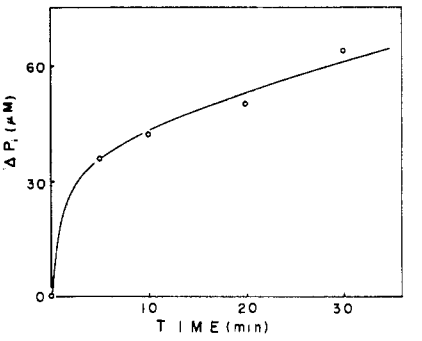

FIGURE 4: A time course of ATP splitting by F-actin under pressure of 2000 kg/cm² at 25°; protein concentration, 3.3 mg/ml; KCl, 0.06 M; Tris–HCl buffer, 0.005 M, pH 8.1; ATP, 500 μM.

Kerr effect was measured in the equipment reported elsewhere (Kobayashi *et al.*, 1964).

Results

When pressures of various magnitudes were applied to an F-actin solution of *ca.* 3 mg/ml for 10 min in the absence of ATP, the degree of flow birefringence after the release of pressure began to decrease with increasing pressure at 1500 kg/cm², and became zero above a pressure of 3000 kg/cm², as shown in Figure 1. Since the degree of flow birefringence corresponds to the concentration of F-actin which is several microns in length, a decrease in the flow birefringence involves the destruction of F-actin structure. Once F-actin was destroyed by high pressure, the molecule had no further polymerizability. The difference spectra of F-actin solutions after pressure treatment compared with the same solution without treatment showed three peaks, one negative at 258 mμ, another positive at 284 mμ, and the other positive at 293 mμ (Figure 2). The shape of the difference spectrum resembles that obtained for the denaturation of actin in alcoholic solution (Mihashi and Ooi, 1965b). The peaks at 284 and 293 mμ would be attributed to a change in environment of tyrosine and tryptophan residues resulting from structural distortion by pressure. Also, the peak height at each wavelength increased with increasing pressure. The peak heights at 258 mμ were plotted *vs.* pressure as represented by a dashed line in Figure 1. Since this change is irreversible, the destruction of F-actin described above represents an irreversible denaturation of actin.

The same experiments were carried out on F-actin solutions in the presence of 500 μM ATP. The pressure at which denaturation began was 2500 kg/cm², *ca.* 1000 kg/cm² higher than without ATP, and the pressure where complete denaturation occurred was 4000 kg/cm², also 1000 kg/cm² higher than without ATP, as shown in Figure 3. That is, the addition of ATP protected the molecule from denaturation by pressure. The amounts of inorganic phosphate, ΔP_i, liberated by the application of pressure for 10 min were plotted

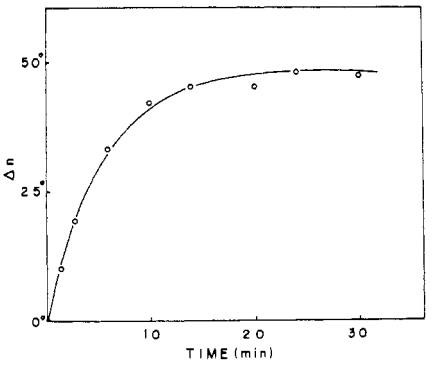

FIGURE 5: A time course of repolymerization of F-actin after release of pressure. The applied pressure was 3000 kg/cm² for 10 min at 25°; protein concentration, 2.1 mg/ml; KCl, 0.06 M; Tris–HCl buffer, 0.005 M, pH 8.1; ATP, 750 μM. The initial Δn was 134°.

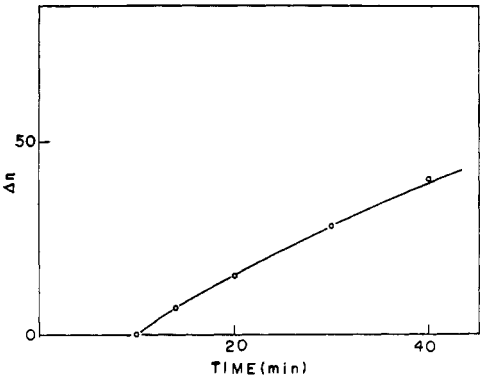

FIGURE 6: A time course of repolymerization of F-actin after release of pressure at low salt concentration. The applied pressure was 2000 kg/cm² for 10 min at 12.5°; protein concentration, 2.0 mg/ml; KCl, 0.01 M; Tris–HCl buffer, 0.005 M, pH 8.1; ATP, 500 μM. The initial Δn was 128°.

as a function of pressure in the same figure. The curve has a maximum at a pressure of 2800 kg/cm², where denaturation begins to occur. The maximum experimental value, 80 μM, is greater than the expected value, 50 μM, which will be liberated when F-actin of 3 mg/ml is depolymerized completely to G-actin, exchanges its ADP for ATP, and then polymerizes again. Therefore, the liberation of phosphate at the maximum is not accounted for by depolymerization of F-actin to G-actin by pressure, on which ADP is exchanged for free ATP in solution, but in addition, some cycle of exchange and splitting of ATP on the F-actin molecule, similar to ATP splitting by sonication or at high temperature, is thought to take place.

As a matter of fact, an increase in the amount of phosphate liberated with increasing time of application of pressure was found. This result is shown in Figure 4. Under a pressure of 2000 kg/cm², a linear increase in ΔP_i was observed after an initial sharp increase. Since ATP alone did not degrade to ADP under pressure within the time interval of this experiment, and the maximum amount of ΔP_i in this experiment is larger than the value expected from the complete depolymerization–polymerization process, 50 μM, the increase in ΔP_i within the time indicated in the figure is evidence that the above described cycle of ATP splitting occurs under pressure.

In the presence of ATP, an increase in flow birefringence, *i.e.*, repolymerization, proceeded after release of pressure under suitable conditions such as high pressure application or low temperature, whereas such a time-dependent phenomenon was not observed in the absence of ATP. In a lower pressure range, however, the recovery of G- to F-actin was too fast to measure, so that it was difficult to decide whether F-actin was depolymerized or not throughout the whole pressure range under the most favorable condition for polymerization, *e.g.*, 0.06 M KCl. At 3000 kg/cm², where *ca.* 50% inactivation occurs for 10-min application, the time course of repolymerization of F-actin after release of pressure could be measured, the result of which is illustrated in Figure 5. Since the extrapolated value of birefringence is 0, it is quite probable that F-actin under pressure exists in a depolymerized form, even though some of the protein is denatured.

In order to examine the state of the actin molecules under pressure, the following experiments were carried out. When the salt concentration is reduced to the minimum value required for polymerization, 0.01 M KCl or 0.5 mM Mg^{2+}, the recovery of F-actin depolymerized by pressure took longer with some latent period after release of pressure (Figure 6). We, therefore, could detect the molecular state by measuring the viscosity or sedimentation of depolymerized actin immediately after release of pressure. As listed in Table I depolymerized actin has the same specific viscosity as native G-actin, and almost the same sedimentation

TABLE I: Specific Viscosity and Sedimentation Constant of Depolymerized Actin and Native G-Actin.[a]

	η_{sp}/C	s_{20}
Control (G-actin)	0.05	2.50 S
Treatment under pressure of 3000 kg/cm² for 10 min	0.05	3.18 S

[a] Protein concentration, 2.4 mg/ml; ATP, 170 μM; Tris–HCl buffer, 0.002 M, pH 8.1; 0.5 mM of Mg^{2+} for viscosity measurement; 0.01 M KCl for sedimentation; 25°. The solution under the sedimentation run did not polymerize probably due to a pressure effect arising from centrifugal force. After taking the solution out, it polymerized again.

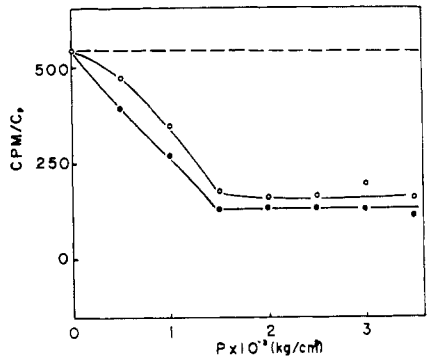

FIGURE 7: Exchange of Ca^{2+} on F-actin as a function of pressure. F-actin was prepared as follows: After free Ca^{2+} was removed by washing of G-actin with Dowex 50, 2 μmoles of ^{45}Ca was added, followed by complete polymerization with KCl; final salt concentration, KCl, 0.06 M; Tris-HCl buffer, 0.005 M, pH 8.1; Ca^{2+}, 0.001 M; ATP, 1 mM; protein concentration 2.7 mg/ml. The results of pressure application for 10 min (○) and for 20 min (●) are shown. Ordinate Scale: counts per minute (cpm) per protein concentration (C_p).

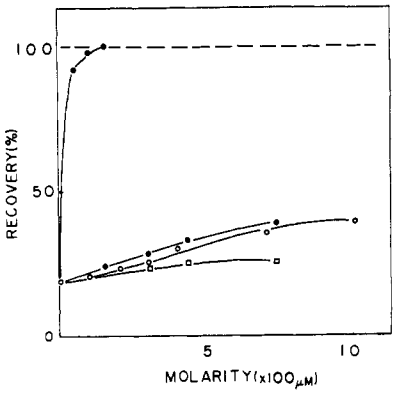

FIGURE 8: Protection effect of nucleotides and divalent cations from pressure denaturation for F-actin. A pressure of 2500 kg/cm² was applied for 10 min at 25°; protein concentration, 3.3 mg/ml; KCl, 0.06 M; Tris-HCl buffer, 0.005 M, pH 8.1; ○, ATP; ●, ADP; ○, Mg^{2+}; □, Ca^{2+}.

constant. After the measurements, the solutions were converted to F-actin. Furthermore, Kerr effects of the depolymerized solutions showed no negative pattern characteristic for F-actin, but a small positive one, suggesting that the protein is in its monomeric form (Kobayashi *et al.*, 1964).

When 0.001 M EDTA was added to F-actin solutions in the absence of ATP just prior to applying pressure, no appreciable difference was obtained for the denaturation curve without EDTA as shown in Figure 1. On the other hand, the addition of EDTA to F-actin solutions in the presence of ATP affected the pressure denaturation to a great extent. The denaturation curve in the presence of EDTA as a function of pressure is demonstrated in Figure 3. The curve is a little higher than, but almost the same as the one without ATP, the result suggesting that molecules protected from denaturation by ATP are quite likely to be in the G-form as inferred previously, since G-actin is easily denatured by the addition of EDTA, while F-actin is not (Tonomura and Yoshimura, 1961).

For the purpose of examining bound Ca^{2+}, exchangeability of Ca^{2+} on the molecule was measured by the use of ^{45}Ca in the presence of ATP. Figure 7 demonstrated the result of isotope exchange on F-actin as a function of pressure. As described before, F-actin does not undergo denaturation below a pressure of 3000 kg/cm² in the presence of ATP; nevertheless Ca^{2+} did exchange as a result of application of lower pressures for 10 min. The extent of exchange increases with increasing pressure up to 1500 kg/cm²; this increase is followed by a plateau region from 1500 kg/cm² to 3000 kg/cm², in which *ca.* 20% of the Ca^{2+} is not exchanged. When pressure is applied for twice as long, 20 min, the extent of the exchange became twice as much at 500 kg/cm², and the increment of the extent was lowered by increasing the applied pressure, probably due to a saturation effect. Usually Ca^{2+} on F-actin does not exchange with free Ca^{2+} in the solvent, so that the exchange result suggests that F-actin under pressure has a different structure from that at 1 atm, enabling it to exchange bound Ca^{2+} for free Ca^{2+} in the solvent, because EDTA cannot denature F-actin in the region below 1500 kg/cm² where exchange of Ca^{2+} takes place (Figures 1 and 3). The reason why 20% of the Ca^{2+} remains unexchanged even at high pressure is not clear.

As described before, the effect of ATP on protection from denaturation is significant. In Figure 8, the fraction of surviving F-actin after application of 2500 kg/cm² pressure for 10 min is shown as a function of ATP added. An amount of ATP equivalent to the calculated capacity of nucleotide binding estimated from the protein concentration was enough for almost full recovery of F-actin. Presumably ADP on each molecule would exchange for free ATP, forming a stable structure resisting pressure denaturation. ADP, on the other hand, does not have so significant an effect against pressure denaturation. This is the same effect exhibited by Mg^{2+} shown in Figure 8. Ca^{2+} has somewhat less of a protective effect *vs.* pressure denaturation than does Mg^{2+}. Furthermore, it is noteworthy that 1 mmole of Mg^{2+} does have a protective effect of 25%, whereas the effect of Ca^{2+} saturated at 6%, showing the difference between these cations.

As shown in Figure 3, *ca.* 50% of F-actin was denatured in the presence of 500 μM ATP under a pressure of 3000 kg/cm². However, further addition of 100 μmoles of Mg^{2+} produced no denaturation at all under the same condition, indicating that the protective effect of ATP and Mg^{2+} are not additive, but of a somewhat cooperative nature. The same effect was observed with Ca^{2+}. The result indicates that both nucleotide and

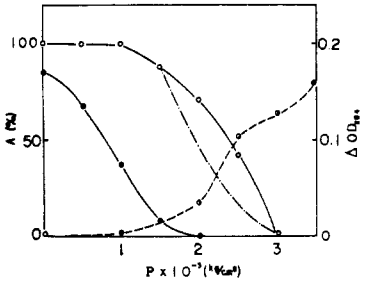

FIGURE 9: Curves of polymerizability, A, for G-actin as a function of pressure in the absence (O) and in the presence of EDTA (●), and of optical density of difference spectrum at 254 mμ, ΔOD_{254} (⊙). To each solution, KCl with buffer was added after release of pressure which was applied for 10 min at 25°; protein concentration, 1.9 mg/ml; Tris–HCl buffer, 5 mM, pH 8.1; ATP, 20 μM; EDTA, 0.001 M.

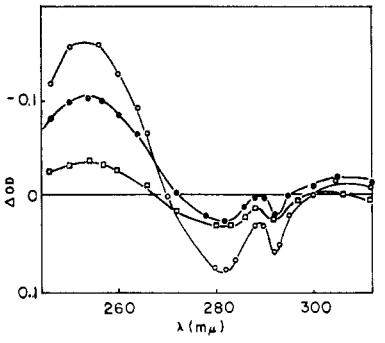

FIGURE 10: Difference spectra of pressure denaturation for G-actin. Measurements were performed by referring to the same solution without treatment of pressure; protein concentration, 1.9 mg/ml; Tris–HCl buffer, 5 mM, pH 8.1; ATP, 20 μM; □, 2000 kg/cm²; ●, 2500 kg/cm²; ○, 3500 kg/cm.²

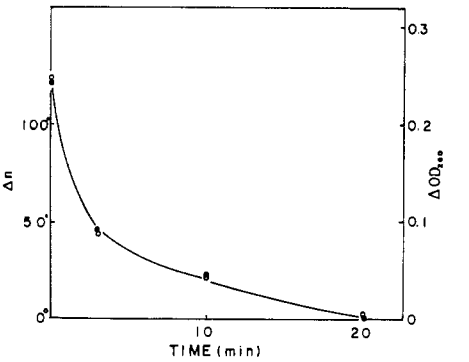

FIGURE 11: The polymerizability and the residual bound ATP of G-actin under an applied pressure of 3000 kg/cm² for various times at 25°; protein concentration, 1.9 mg/ml; Tris–HCl buffer, 0.008 M, pH 8.1; ATP, 30 μM; ●, ΔOD_{260}; ○, Δn.

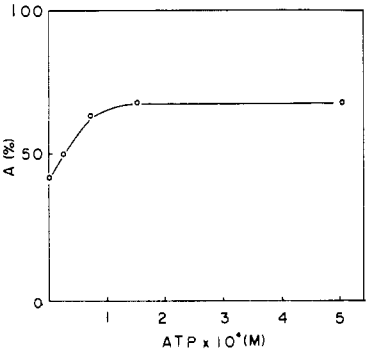

FIGURE 12: Protective effect of ATP from pressure denaturation of G-actin. Polymerizability, A, was measured after release of pressure. Pressure (2500 kg/cm²) was applied for 10 min at 25°; protein concentration, 1.9 mg/ml; Tris–HCl buffer, 0.005 M, pH 8.1.

Mg^{2+} or Ca^{2+} bound on G- or F-actin must play a role in stabilizing the native structure of these proteins in aqueous solutions, although polymerization without ATP can occur in sucrose solutions (Kasai *et al.*, 1965).

Experiments of the effect of pressure on the polymerizability and difference spectra of G-actin were carried out (Figures 9 and 10). Polymerizability of G-actin, after the application of various pressures for 10 min, began to decrease from a pressure of 1500 kg/cm² and was lost completely at 3000 kg/cm². With progressive denaturation, spectral peak heights at 254, 282, and 292 mμ became greater, giving a similar difference spectrum as that observed for F-actin so treated. Compared with the denaturation curve for F-actin in the absence of ATP, shown by a dotted–dashed line in the figure, G-actin seems to be a little more resistant to denaturation in the pressure range from 1500 kg/cm² to 3000 kg/cm². This could be due to a small amount of nucleotide in the dialysing solutions, which was necessary for maintaining the polymerizability of G-actin.

Next, the release of bound ATP from G-actin on applying pressure was compared with the pressure denaturation of the protein. Figure 11 demonstrates a time course for the decrease in polymerizability of G-actin at a pressure of 3000 kg/cm² together with its remaining bound ATP. The result shows that the extent of polymerizability loss was just parallel to the release of bound ATP, indicating that pressure denaturation resulted from the loss in nucleotide binding capacity associated with a structural change in the molecule which was suggested by the difference spectrum.

The same protective effect of ATP to pressure denaturation observed for F-actin was also found, although to a lesser extent, for G-actin. As shown in Figure 12, ATP beyond 100 μM gave no further increase

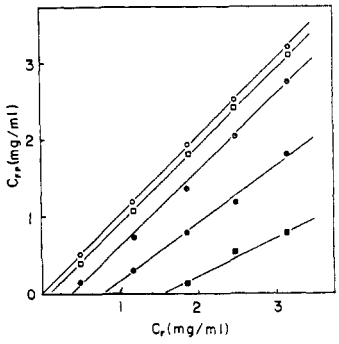

FIGURE 13: Concentration dependence of pressure denaturation for F-actin in the absence of ATP as a function of the applied pressure. The pressure was applied for 10 min at 25°. F-actin concentration before application of pressure, C_F, and that after pressure treatment, C_{FP}, was calculated from the value of flow birefringence, Δn, taking 64° for 1 mg/ml; ○, control; □, 1000 kg/cm²; ⊙, 1500 kg/cm²; ●, 2000 kg/cm²; ■, 2500 kg/cm.²

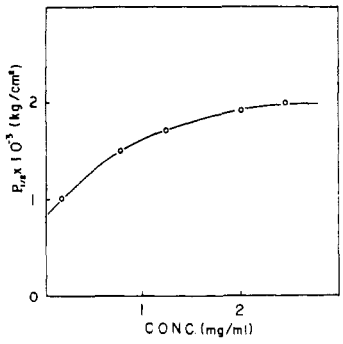

FIGURE 14; Plots of a pressure which gives half-denaturation ($P_{1/2}$) *vs.* protein concentration.

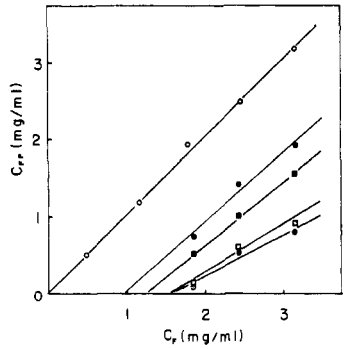

FIGURE 15: Concentration dependence of pressure denaturation for F-actin in the absence of ATP as a function of time. A pressure of 2500 kg/cm² was applied at 25°; KCl, 0.06 M; Tris–HCl, 0.005 M, pH 8.1; ○, control; ●, 2 min; ■, 3 min; □, 5 min; ⊙, 10 min.

in protective effect. When EDTA was added to G-actin, the molecule lost its polymerizability gradually as mentioned before. Application of pressure for 10 min on G-actin solutions containing EDTA accelerated the loss of polymerizability (Figure 9). At 2000 kg/cm², G-actin lost its polymerizability completely.

All the experiments described above were carried out at a fixed protein concentration of *ca.* 2–3 mg/ml. Even though denaturation was found to be dependent upon protein concentration, the effects described previously were the same at all concentrations studied. In Figure 13, the concentration dependence of F-actin for pressure denaturation is shown as a function of the pressure applied for 10 min. The result indicates that protein molecules are denatured more easily at a lower concentration. The pressure required for 50% denaturation, used as a measure of the stability of the molecule, decreases to *ca.* 800 kg/cm² with decreasing protein concentration (Figure 14). At higher protein concentrations the pressure seems to reach saturation around 2000 kg/cm². Although the absolute values obtained in separate experimental runs were a little different from sample to sample, because of small amounts of contaminants (nucleotide or divalent cations) which have a significant protective effect *vs.* pressure denaturation as mentioned before, similar results were obtained regardless of the preparation. The results indicate that no denaturation occurred below a pressure of 800 kg/cm² at low protein concentrations.

In the experiments described above, the pressure was applied for 10 min. The amount of surviving proteins, *i.e.*, protein still showing flow birefringence after various times of exposure to pressure, as a function of protein concentration is shown in Figure 15. For exposures of 3 min or less the curves are nearly parallel to the control line, indicating that the rate of production of denatured molecules is independent of protein concentration. After 3 min, the slopes of the lines begin to decrease, and finally seem to reach a limiting value. Therefore, there appear to be two phases to the denaturation on applying pressure: the first phase is independent of protein concentration and the second phase is concentration dependent. Furthermore, after application of pressure for *ca.* 10 min, a finite value for the amount of F-actin denatured, dependent upon the magnitude of the applied pressure, is attained.

When a small amount of ATP (10 μM) was added to the solutions, the curve for the protein concentration dependence of the pressure denaturation became parallel to the control, while the line without ATP was skewed. These curves are shown in Figure 16. This result indicates that the addition of ATP protects more molecules at higher protein concentrations.

Discussion

The results shown in Figures 1–7 may allow the following interpretation about the effect of pressure on F-actin. There are four states characterized by dif-

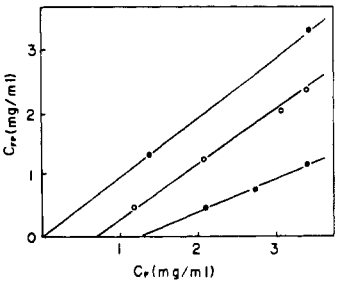

FIGURE 16: Concentration dependence of pressure denaturation for F-actin in the absence and presence of ATP. A pressure of 2500 kg/cm^2 was applied for 10 min at 25°; KCl, 0.06 M; Tris–HCl buffer, 0.005 M, pH 8.1; ●, control; O, ATP, 10 μM; ○, in the absence of ATP.

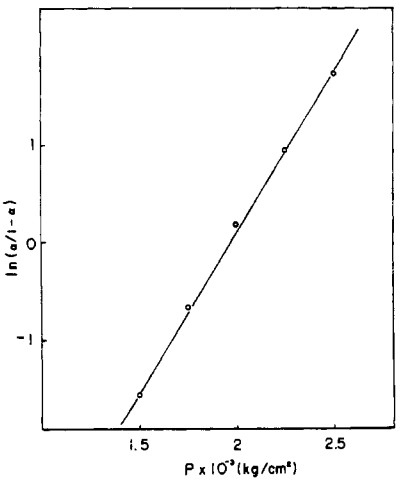

FIGURE 17: Plots of logarithms of ratio in G- (α) and F-actin ($1 - \alpha$) *vs.* pressure.

ferent properties in which F-actin can exist, depending upon the extent of applied pressure: first, the usual F-actin which is observed below 500 kg/cm^2, Ca^{2+} being not exchangeable in this state (Figures 1, 3, and 7); second, the F-actin structurally modified between 500 kg/cm^2 and 1500 kg/cm^2 (Ca^{2+} is now exchangeable in this state) (Figures 1, 3, and 7); third, the F-actin depolymerized between 1500 kg/cm^2 and 3000 kg/cm^2 which retains its polymerizability on releasing the pressure (Figures 5 and 6); and fourth, the actin which loses its polymerizability at pressure above 3500 kg/cm^2. The properties of the usual F-actin are still observed at low pressures, *i.e.*, denaturation does not occur even in the presence of EDTA, nor is Ca^{2+} exchanged, showing the evidence of the first state. The second state differs from the first one in its exchangeability of Ca^{2+} as indicated by Figure 7. Apparently, this is not the G-form of actin because the addition of EDTA did not denature the molecule but exchange of Ca^{2+} took place at pressures between 500 and 1500 kg/cm^2. It was difficult, with the present equipment, to detect whether or not F-actin was partially depolymerized within this range of pressure, but it seems likely that this form of F-actin is depolymerized to some extent, *i.e.*, an F-actin polymer perhaps not in a double helix (Oosawa *et al.*, 1965). The third state, pressure-depolymerized F-actin, was deduced from an extrapolation of the time course for recovery of the flow birefringence of the pressure treated actin to F-actin at both 0.06 M and 0.01 M KCl (Figures 5 and 6, and Table I). Therefore, the result that, between 1500 kg/cm^2 and 3000 kg/cm^2, the addition of ATP protected F-actin from pressure denaturation whereas EDTA could denature the molecule in the presence of ATP, can be explained by assuming a depolymerization of F-actin at the mentioned pressures. In addition, a part of the ATP split by application and release of pressure might be due to the proposed F–G transformation of actin under pressure. Thus we may infer that the effect of pressure would cause modification of F-actin structure, then depolymerization to G-actin (G–ADP actin, and its bound ADP would exchange for ATP in the solvent when present) and finally irreversible denaturation with increasing pressure. The only contradictory result with the foregoing presumption is the difference of the protective effect of ATP *vs.* pressure denaturation between F- and G-actin. The stronger resistance of F-actin to pressure in the presence of ATP than G-actin could be interpreted as arising either from an increase in the stability of the protein in the presence of salt or by some conformational change of the molecule which occurred on the addition of salt.

On the presumption described above the denaturation curve of F-actin in Figure 1 or that of F-actin in the presence of ATP and EDTA in Figure 3 is accounted for by the transformation of a structurally modified F-actin to G-actin caused by the application of pressure, *i.e.*, the curve represents the pressure dependence of the equilibrium between F- and G-actin. Since the change in equilibrium constant (K) at constant temperature (T) resulting from changes in the pressure (p) is related to the volume change per mole by the equation, $d \ln K/dp = -\Delta V/RT$, and approximate volume change arising from the transformation of F- and G-actin can be estimated from this curve. As shown in Figure 17, plots of logarithms of the ratio of G-actin to F-actin against pressure are linear. The volume change calculated from this slope is −84 ml/mole of polymerizing unit of actin, or monomer. That is, F-actin has a larger volume than that of G-actin, and the molar volume per actin decreases on depolymerization. This is consistent with Le Chatelier's principle according to which a change in state caused by pressure occurs in the direction where the molar volume is decreased. Dilatometric measurements of the volume change for the G–F transformation (T. Ikkai, H. Noguchi, and T. Ooi, to be published) gave a large positive volume change, a result which is consistent with the present calculation.

The volume change calculated from the pressure denaturation curve represents the volume change per monomeric unit associated with an F–G transformation

in the presence of salt, so that we may deduce the interaction force between monomers in F-actin. A large volume decrease on F–G transformation means the same volume increase on G–F transformation which is considered to be endothermic (Asakura *et al.*, 1960). Therefore, on polymerization of G-actin to F-actin in the presence of salt, the molar volume increases by 84 ml and also the molar entropy increases by forming intermolecular bridges. These changes can be attributed to some melting of the protein's bound waters either by neutralizing charges in forming salt bridges, or by hydrophobic bonding (Johnson *et al.*, 1954; Kauzman, 1959). On the reverse transformation, both the volume and the entropy decrease by breaking bonds, *i.e.*, when pressure is applied to F-actin solutions, an F-actin molecule tends to have a smaller volume, resulting in destruction of the bonds between monomeric units and dissociation into G-actin molecules which presumably have more bound waters. Since this change occurs in the presence of salt, the assumption of salt bridges between monomers does not seem to be reasonable, unless some special interaction occurs between specified ions. The possible such interaction is between ATP^{4-} and Mg^{2+} (or Ca^{2+}), the volume decrease on dissociation of these ions having been reported as -22 ml/mole of reactants (Noguchi *et al.*, 1964; Rainford *et al.*, 1965). This value is one-fourth of the calculated value mentioned above. Therefore, it is probable that the volume change mainly results from the formation or cleavage of hydrophobic bonds. If three-quarters of the volume change is assumed to be due to interactions between hydrophobic side chains, several residues are involved in the polymerization of G-actin to F-actin. Presumably similar experiments on pressure denaturation at various temperature would be helpful for getting further information on the nature of the bonding between the monomeric units in F-actin.

The significant protective effect of nucleotides and divalent cations, especially ATP and Mg^{2+}, suggests that these molecules are tightly bound to a depolymerized molecule so as to maintain the native conformation, thus playing the role as supporters of the structure. Also the cooperative property of protection by ATP and divalent cations suggests that both binding sites are close to one another.

Denaturation by pressure is considered to be due to the destruction of a native structure of actin by hydrostatic compression with an accompanying volume decrease. Therefore, in the absence of ATP, the depolymerized molecule might be compressed at the binding site of the nucleotide, giving rise to irreversible denaturation with an accompanying change in conformation as suggested by the spectral changes of the denatured molecule. Consequently, some interaction between pressure-inactivated actin and native actin is expected to exist. In fact, addition of the denatured actin to the native actin solution protected molecules to some extent from denaturation.

The lack of proportionality in the concentration dependence of pressure denaturation shown in Figures 13 and 15 could be interpreted in several ways. One interpretation is the following: the G–F transformation can be explained as a kind of condensation phenomenon (Oosawa *et al.*, 1959). Pressure displaces the equilibrium state of the system, so that a shift occurs in the critical concentration for polymerization, above which F-actin can be formed. Therefore, the concentration of G-actin produced under pressure according to a phase shift is expected to be constant at every protein concentration, resulting in a constant rate of production of denatured molecules. Another explanation is that the denaturation occurs more easily at lower concentrations in general and that the parallel line in the figure is accidental. Last, there might exist an as yet unknown reason for pressure denaturation. We cannot decide which is the case in this study until further information is obtained.

In pressure studies, especially on protein, there are several problems concerning the solvent (Johnson *et al.*, 1954). Among them, the pH change caused by pressure is thought to have a dominant effect. Preliminary experiments using two kinds of buffer, Tris–HCl (pH 8.1) and borate–NaOH (pH 8.1), were performed to distinguish the effect of the pH change on pressure denaturation, since the former under pressure is expected to change toward a basic pH and the latter toward an acidic pH. The amount of denatured protein produced under a pressure of 2000 kg/cm^2 applied for 10 min in the absence of ATP was 62% in Tris buffer and 45% in borate buffer. Since the F–G transformation is favored by higher pH, the above results suggests that pH had some effect on the F–G transformation under pressure. However, the pH change is not the only reason for the F–G transformation, because a similar denaturation curve was obtained in the borate buffer as in the Tris buffer. It is presumed that teh change in pH under pressure might shift the denaturation curve. In order to obtain further information on the pH dependence, control experiments to determine the direction and extent of pH changes of the buffer with increasing magnitude of pressure are required. Another problem is the change in water structure under pressure. In the pressure range in this study, there is not enough knowledge to speculate the effect of the change in water structure itself on F–G transformation so far.

All the measurements in this study were made after the release of pressure. Of course, it is desirable to make measurements while pressure is being applied; and results of such experiments can be analyzed by thermodynamical relation. However, such experiments require the construction of an apparatus capable of measuring a number of properties under pressures up to 5000 kg/cm^2. In our laboratory, we have constructed a cell with optical windows. Experiments using this cell are now in progress, though only a limited number of properties are measurable with this apparatus.

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