Mechanism of Heat-Induced Gelation of Pressurized Actomyosin: Pressure-Induced Changes in Actin and Myosin in Actomyosin

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The biochemical and physicochemical changes of myosin (myosin A), actomyosin (natural actomyosin), and actin after pressure treatment were investigated to elucidate the cause of the increased gel strength of pressurized actomyosin. Analysis of DNase I inhibition capacity of actomyosin demonstrated that in buffers containing 0.6 M KCl most of the actin in actomyosin was denatured by pressures of 150 MPa. However, at KCl concentrations greater than 0.6 M, a large part of actin in actomyosin was found to exist as the depolymerized form (native G-actin) after the release of pressure. Mg^{2+}-ATPase activity of actomyosin decreased with increasing pressure, whereas Ca^{2+}-ATPase activity was not affected at pressures below 200 MPa. Both sulfhydryl (SH) content and surface hydrophobicity of actomyosin increased with an increase in pressure. In conclusion, pressure effects on the heat-induced gelation of actomyosin are attributable to the denaturation of actin in actomyosin and the increased SH content and surface hydrophobicity.

INTRODUCTION

In the preceding paper (Ikeuchi et al., 1992), we reported that heat-induced gels of pressurized actomyosin were found to display storage modulus (G') higher than those of unpressurized actomyosin regardless of salt concentrations. Besides, the dynamic rheological measurements suggested the possibility that pressure-induced denaturation of actin in actomyosin was responsible for the increased storage modulus of pressurized actomyosin. In order to understand this pressure effect on the properties of heat-induced gelation of actomyosin, it is necessary to know how myofibrillar proteins form a gel on heating. The basic mechanism of the heat-induced gelation of myofibrillar protein system has been elucidated by Yasui and his co-workers (Yasui et al., 1980; Ishioroshi et al., 1983; Asghar et al., 1985). In summary, the following are some few important findings revealed by them: (1) A heat-induced gel of myosin at high ionic strength, where it exists as monomer, is formed by irreversible coagulation between the head portion molecules and subsequent cross-linking between tails. Myosin at low ionic strength, where it aggregates to assemble filaments, formed an ordered three-dimensional network structure termed strand-type. (2) A suitable amount of F-actin, which exists as an F-actomyosin complex in the system, remarkably enhances the heat-induced gelation of myosin at high ionic strength. On the contrary, at low ionic strength, F-actin exerts strong inhibitory effects on the heat-induced gelation of myosin filaments. (3) The SH groups of myosin molecules probably participate in the heat-induced gelation of myosin. Probably, the phenomena that occurred when actomyosin was pressurized and then heated could be explained by taking into account the contents of (1)-(3) described above. Therefore, studies on differences in the biochemical and physicochemical properties of myosin, actomyosin, and actin before and after pressure treatment are expected to facilitate understanding why pressurized actomyosin exhibits greater storage modulus than unpressurized actomyosin.

A primary objective of the present research was to correlate biochemical changes of myosin and actin in pressurized actomyosin detected by measurements of Mg^{2+}- and Ca^{2+}-ATPase activities and DNase I inhibition assay with the dynamic rheological behavior of pressurized actomyosin during heat gelation. The effect of pressure treatment on changes in the hydrophobicity and SH content of actomyosin was also studied with regard to variations in KCl concentration and pressure intensity because the hydrophobic and electrostatic interactions are important in the heat-induced protein network formation (Kinsella and Whitehead, 1989).

MATERIALS AND METHODS

Materials. All proteins were obtained from rabbit skeletal muscle. Myosin and actomyosin were purified by the procedure described in the preceding paper (Ikeuchi et al., 1992). Actin was prepared from acetone-dried powder according to the procedure of Spudich and Watt (1971). DNA and DNase I were purchased from Sigma Chemical Co. (St. Louis, MO). ATP was obtained from Oriental Yeast Co. Ltd. All other reagents used were of analytical grade.

Pressurization of Muscle Proteins. Pressure treatment for myosin, actomyosin, and actin was the same as in the preceding paper (Ikeuchi et al., 1992).

Actomyosin (15 mg/mL), myosin (10 mg/mL), and actin (2 mg/mL) were dissolved in solutions containing various KCl concentrations at pH 6.0 before pressure application. Sodium phosphate buffer (20 mM) was used as a buffer of pH 6.0 except that 20 mM MES (2-(*N*-morpholino)ethanesulfonic acid) was used for measurements of Mg^{2+},Ca^{2+}-ATPase activities or DNase I inhibition assay.

Measurements of Change in the Biochemical Properties. Ca^{2+}-activated ATPase activities of myosin and actomyosin treated with pressure were measured at 25 °C in a reaction mixture containing 10 mM $CaCl_2$, 225 mM KCl, 20 mM Tris-HCl (pH 7.5), 1 mM ATP, and 0.6 mg/mL protein. Similarly, Mg^{2+}-ATPase activities were measured in 2 mM $MgCl_2$, 75 mM KCl, 20 mM Tris-HCl (pH 7.5), 1 mM ATP, and 0.6 mg/mL protein. ATPase reaction was started by adding ATP and terminated by adding 20% trichloroacetic acid. The inorganic phosphate liberated was determined by the method of Fiske and SubbaRow (1925). Each ATPase activity was expressed as the relative remaining activity against the activity of unpressurized sample.

DNase I (EC 3.6.1.32) inhibition assay was used for estimating the denaturation of actin in actomyosin and actin under applied pressure. The detailed description of this assay is given in our previous paper (Ikeuchi et al., 1991). The denaturation of actin

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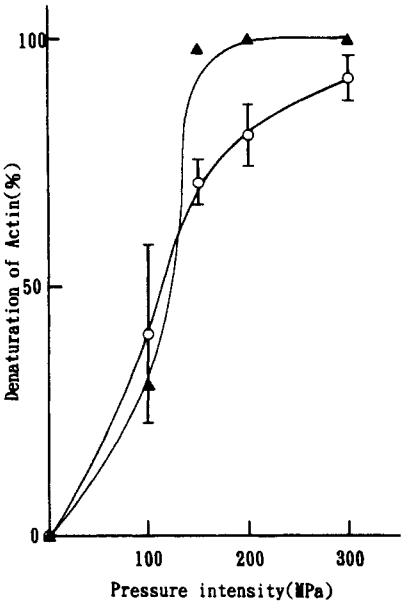

Figure 1. Changes in the percentage of denaturation of actin in actomyosin and F-actin at 0.6 M KCl and pH 6.0 (20 mM MES–NaOH buffer) as a function of pressure intensity: (○) actin in actomyosin, (▲) F-actin. The protein concentrations of actomyosin and F-actin were 15 and 2 mg/mL, respectively. Estimation of denaturation of actin was carried out by DNase I inhibition assay with KI treatment. Error bars indicate the SD derived from three different experiments. In the case of F-actin the displayed values are the means of duplicate measurements.

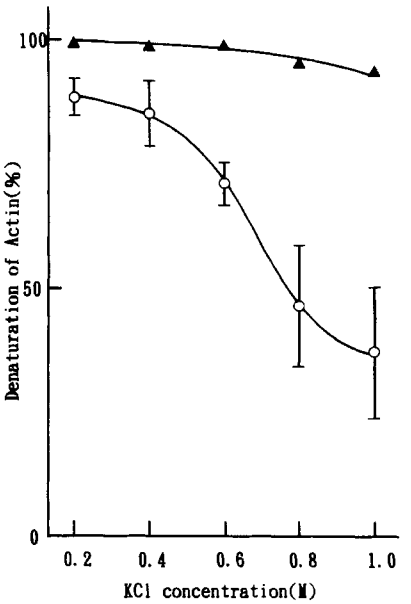

Figure 2. Effect of pressure treatment at various KCl concentrations and pH 6.0 (20 mM MES–NaOH buffer) on the denaturation of actin in actomyosin and F-actin: (○) actin in actomyosin, (▲) F-actin. Each sample was pressurized at 150 MPa for 5 min. Other conditions were the same as those in Figure 1. Error bars are as in Figure 1. In the case of F-actin the displayed values are the means of duplicate measurements.

was determined as the relative loss (%) against the DNase I inhibition capacity found in unpressurized sample. Native G-actin content in actomyosin was determined by measuring DNase I inhibition capacity of actomyosin sample solution without depolymerizing treatment with potassium iodide (KI).

Electron Microscopic Observation. After myosin and actomyosin containing 0.2 or 0.6 M KCl and 20 mM sodium phosphate buffer (pH 6.0) were pressurized at 150 MPa for 5 min, they were diluted with the same solutions to a protein concentration of 0.1 mg/mL. Each sample was put on carbon-coated 400-mesh grids and then negatively stained with 2% uranyl acetate. The specimens were observed in a Nippon Denshi JEM-100-B transmission electron microscope at an accelerating voltage of 80 kV.

Measurement of Hydrophobicity (S_0). Surface hydrophobicity was determined using hydrophobic fluorescent probe (1-anilinonaphthalene-8-sulfonic acid (ANS)) according to the method of Kato and Nakai (1980). The measurement was carried out in the absence of sodium dodecyl sulfate (SDS). Each sample was diluted with a solution containing 10 mM sodium phosphate buffer (pH 7.0) and 0.3 M KCl to obtain a final protein concentration ranging from 0.05 to 0.15 mg/mL. Then, 10 μL of 8 mM ANS in 0.1 M sodium phosphate buffer (pH 7.0) was added to the diluted sample. After the sample was permitted to stand for 2 h, the relative fluorescence intensity (FI) was measured with Hitachi fluorescence spectrometer F-3010. The wavelengths of excitation and emission were 380 and 490 nm, respectively. The net FI at each protein solution was estimated by subtracting the FI of each solution without probe from that with probe. The initial slope of the FI vs protein concentration (percent) was used as an index of the protein hydrophobicity (S_0).

Measurement of Sulfhydryl (SH) Content. SH content was determined by using Ellman's reagent (5′,5′-dithiobis(2-nitrobenzoic acid) (DTNB)) according to the method of Janatova et al. (1968). Each sample solution was diluted with a solution containing 74 mM sodium phosphate buffer (pH 8.0) and 0.6 M KCl to obtain a final protein concentration of 1–2 mg/mL, followed by centrifugation at 1500*g* for 10 min. To 1.5 mL of the resulting supernatant were added 0.1 mL of 25 mM ethylenediaminetetraacetic acid (EDTA) and 0.4 mL of 10 mM DTNB. After the solution was permitted to stand for 45 min at room temperature, the absorbance at 412 nm was measured with Hitachi spectrophotometer U-2000 (Ellman, 1959). SH content was calculated as mole/10^5 grams of proteins.

Determination of Protein Concentration. Protein concentration was determined by the biuret method using bovine serum albumin as a standard (Gornall et al., 1949).

Statistical Analysis. All analyses were performed in duplicate or triplicate, and average values were used in tables and figures. Statistical differences were estimated by a Duncan's multiple range test.

RESULTS AND DISCUSSION

Measurements of Biochemical Changes in Pressurized Myosin, Actomyosin, and Actin. Phosphate buffer is not suitable for the determination of inorganic phosphate in ATPase assay as indicated in Figures 3 and 4, and therefore we tried to use MES buffer (pH 6.0) instead for ATPase assay and DNase I inhibition assay.

DNase I inhibition assay gives information on how pressure affects the properties of actin in actomyosin (Ikeuchi et al., 1991). Figure 1 shows the percentage of denaturation of F-actin or actin in actomyosin pressurized at various pressure intensities for 5 min. A sigmoidal relationship was obtained between the percentage of denaturation of actin in actomyosin and the intensity of pressure applied. The degree of denaturation was accelerated by pressurization, and reached over 40, 75, 80, 90% at 100, 150, 200, and 300 MPa, respectively. In the case of F-actin without ATP, this change occurred more sharply between 100 and 150 MPa, which agreed with the report of Ikkai and Ooi (1966). Ko et al. (1990) reported that the repolymerization of the depolymerized actin (fish muscle actin in 0.1 M KCl and 1 mM $MgCl_2$ at pH 8.0), due to pressure treatment at 300 MPa for 60 min at 0 °C, occurred when incubated at 25 °C after the release of pressure, indicating that a large quantity of fish F-actin was still intact after a pressure treatment. This is evidently distinct from our result investigated by DNase I inhibition assay (Figure 1). But, this may be mainly due to the difference in pH, salt concentration, and actin species employed (Swezey and Somero, 1982). Figure 2 shows the effect of

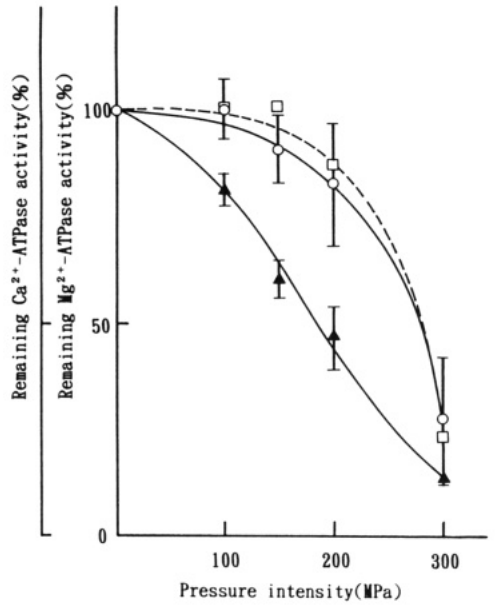

Figure 3. Changes in the remaining Ca^{2+}-activated and Mg^{2+}-activated ATPases of actomyosin and myosin at 0.6 M KCl and pH 6.0 (20 mM MES–NaOH buffer) as a function of pressure intensity: (○) Ca^{2+}-ATPase of actomyosin, (□) Ca^{2+}-ATPase of myosin, (▲) Mg^{2+}-ATPase of actomyosin. Error bars are as in Figure 1. In the case of Ca^{2+}-ATPase of myosin the displayed values are the means of duplicate measurements.

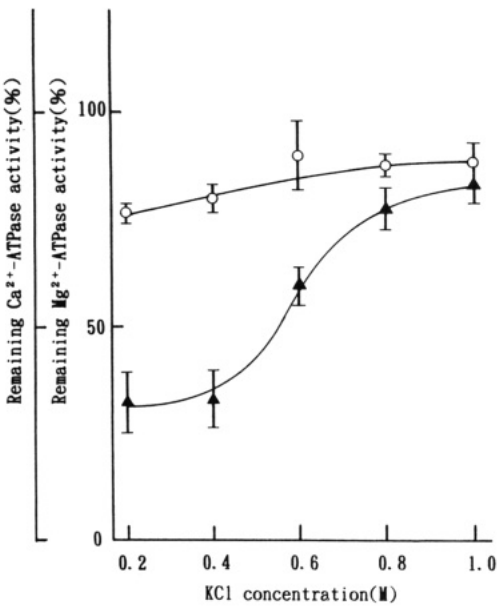

Figure 4. Effect of pressure treatment at various KCl concentrations and pH 6.0 (20 mM MES–NaOH buffer) on the Ca^{2+}-activated and Mg^{2+}-activated ATPases of actomyosin expressed as the relative remaining ATPase activity: (○) Ca^{2+}-ATPase of actomyosin, (▲) Mg^{2+}-ATPase of actomyosin. Error bars are as in Figure 1.

salt concentrations on the denaturation of F-actin or actin in actomyosin pressurized at 150 MPa. A considerable amount of actin in actomyosin remained undenatured with an increase in KCl concentration beyond 0.6 M. Then, to verify how much the remaining native actin in pressurized actomyosin existed as a depolymerized form (G-actin), DNase I inhibition assay was performed without KI treatment. As a result, more than 30–50% of the remaining native actin in actomyosin when treated with over 0.6 M KCl was found to exist as G-actin after the release of pressure (Table I). On the contrary, F-actin alone almost completely denatured in any KCl concentrations. The

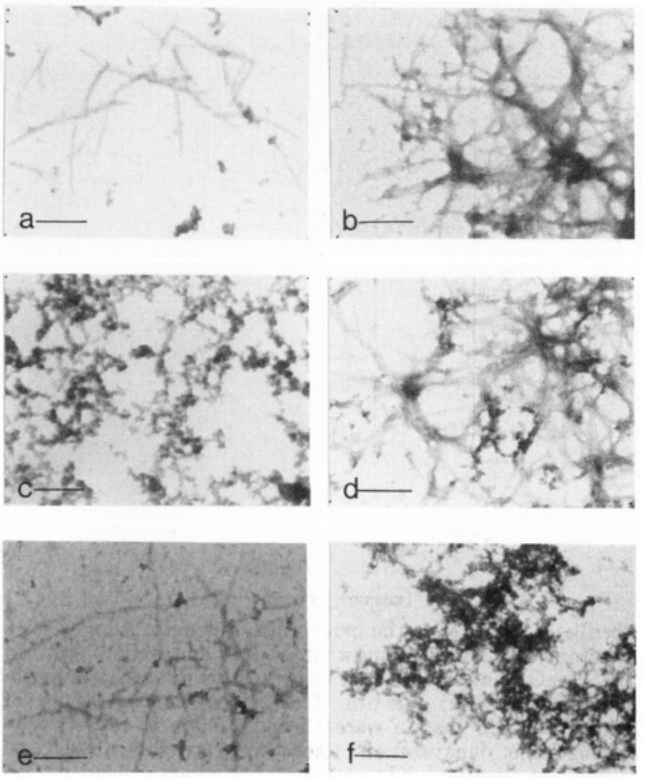

Figure 5. Electron micrographs of myosin and actomyosin at 0.2 or 0.6 M KCl and pH 6.0 (20 mM sodium phosphate buffer) before and after pressure application at 150 MPa for 5 min. All micrographs are of the same magnification (bar = 0.5 μm): (a) myosin at 0.2 M KCl; (b) pressurized myosin at 0.2 M KCl; (c) actomyosin at 0.2 M KCl; (d) pressurized actomyosin at 0.2 M KCl; (e) actomyosin at 0.6 M KCl; (f) pressurized actomyosin at 0.6 M KCl. It should be noted that the samples of myosin and actomyosin shown in (a)–(d) were obtained by directly diluting to final KCl concentration of 0.2 M without dialysis.

results in Figures 1 and 2 demonstrate that F-actin is subject to denaturation in suspensions which do not contain myosin (Ikkai and Ooi, 1969). Why the percentage of denaturation of actin in actomyosin declined as salt concentration increased is unknown. One interpretation is the following: the difference in the state of actomyosin at low salt concentration and at high salt concentration may be correlated with the difference in the resistivity of actin in actomyosin against pressurization (Figure 5). That is, myosin existing as a monomer at high salt concentration may protect actin from denaturation more effectively than myosin existing as a filament at low salt concentration does. There might be as yet unknown reason for pressure-induced denaturation of actin in actomyosin at varying salt concentrations. The answer will have to be addressed in future research.

Ca^{2+}-ATPase activity is an index of myosin denaturation in actomyosin and Mg^{2+}-ATPase activity reflects the properties of both actin and myosin in actomyosin. The Ca^{2+}-activated and Mg^{2+}-activated ATPase activities of actomyosin were measured as a function of pressure as shown in Figure 3. The increase in pressure from 100 to 200 MPa reduced the Ca^{2+}-ATPase activity of actomyosin slowly, and with a further rise in pressure to 300 MPa, the remaining activity rapidly diminished to about 25%. The dependence of the ATPase activity upon pressure was quite similar to that of myosin alone (dotted line). On the other hand, the remaining Mg^{2+}-activated ATPase activity of actomyosin decreased linearly with increasing pressure intensity, indicating that the denaturation of actin in actomyosin happened during pressure treatment as well as the result of Figure 1. Figure 4 illustrates the remaining

Table I. Changes in G-Actin Content in Actomyosin[a] at Various KCl Concentrations When Subjected to a Pressure of 150 MPa for 5 min

	total native actin content in actomyosin,[b] mg/mL	denaturation of actin, %	native G-actin content in actomyosin,[c] mg/mL	ratio of native G-actin to total native actin, %
0.6 M KCl nonpress	1.686	0	0.055	3.26
press	0.651	61.39	0.380	58.37
0.8 M KCl nonpress	1.725	0	0.147	8.55
press	1.019	40.94	0.372	36.56
1.0 M KCl nonpress	1.732	0	0.178	10.30
press	1.173	32.30	0.426	36.31

[a] Actomyosin (15 mg/mL) in 20 mM MES–NaOH (pH 6.0). [b] DNase I inhibition assay with KI treatment. [c] DNase I inhibition assay without KI treatment. Column values are means derived from two different experiments.

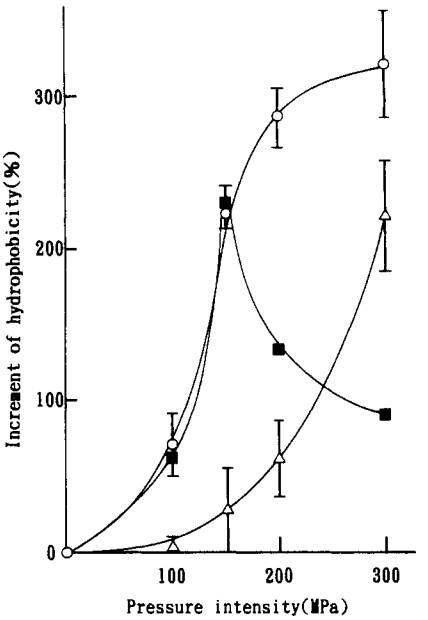

Figure 6. Changes in the surface hydrophobicity of myosin, actomyosin, and actin at 0.6 M KCl and pH 6.0 (20 mM sodium phosphate buffer) as a function of pressure intensity: (△) myosin, (○) actomyosin, (■) F-actin. The ordinate indicates the relative increment against the surface hydrophobicity of unpressurized sample. The protein concentrations of myosin, actomyosin and F-actin were 10, 15, and 2 mg/mL, respectively. Error bars are as in Figure 1. In the case of F-actin the displayed values are the means of duplicate measurements.

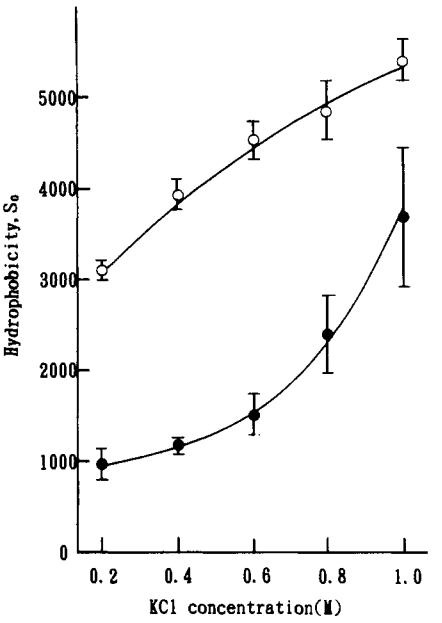

Figure 7. Effect of pressure treatment at various KCl concentrations and pH 6.0 (20 mM sodium phosphate buffer) on the surface hydrophobicity (S_0) of actomyosin: (●) unpressurized actomyosin, (○) actomyosin pressurized at 150 MPa for 5 min. The protein concentration was 15 mg/mL. Error bars are as in Figure 1.

Ca^{2+}-activated and Mg^{2+}-activated ATPase activities as influenced by KCl concentration at the constant pressure of 150 MPa for 5 min. At this pressure, the effect of KCl concentration on the extent of decrease in the Ca^{2+}-activated ATPase activity was not large. Ikkai and Ooi (1969) also described in their report that the state of heavy actomeromyosin modified by pressure was almost independent of the salt concentration. KCl concentration considerably affected the Mg^{2+}-ATPase activity, and the profile gave a sigmoidal curve, similar to denaturation of actin in actomyosin as shown in Figure 2.

The results obtained from these biochemical measurements indicate that actin is more apt to be denatured by pressure than myosin (Figures 1–4) and a large part of the remaining native actin exists as G-actin (Table I). Accordingly, it should be expected that pressure treatment at 100–150 MPa would effectively diminish the negative effect of an excess amount of F-actin in actomyosin on the gel strength of myosin by denaturing or depolymerizing actin (Yasui et al., 1980). As a result, pressure treatment increases the heat-induced gel strength of actomyosin but not myosin. The dynamic rheological behavior of actomyosin shown in the preceding paper certainly reflects the change in the property of actin in actomyosin under pressure.

Electron Microscopy. The structure of myosin or actomyosin in the solution (i.e. aggregated, filamentous, and solubilized forms) influences its heat-induced gelation (Asghar et al., 1985). Transmission electron microscopic observation was carried out to investigate the state of actomyosin in 0.2 or 0.6 M KCl and myosin in 0.2 M KCl immediately after being pressurized at 150 MPa for 5 min (Figure 5). In Figure 5a, the dispersed myosin filaments and no interfilamentous association were visible in the electron microscopic fields before pressure application. Also the filaments obtained by diluting to 0.2 M KCl were shorter than those formed by prolonged dialysis against 0.1 M KCl at pH 7.0 or 6.0 (Yamamoto et al., 1987, 1990; Tumminia et al., 1989). With pressure application and release, the myosin filaments gathering into aggregates were observed (Figure 5b). The detailed study concerning pressure-induced gelation of myosin filament at low pH has recently been reported by Yamamoto et al. (1990). In the case of actomyosin in 0.2 M KCl, the aggregated form (Figure 5c) was transformed into a structure which was quite similar to that of pressurized myosin (Figure 5, parts b and d). This suggested that the disintegration of actin filaments in actomyosin at 0.2 M KCl took place under

Table II. Changes in SH Contents of Myosin,[a] Actomyosin,[b] and F-Actin[c] under Various Pressure Intensities

	pressure intensity				
	0 MPa	100 MPa	150 MPa	200 MPa	300 MPa
myosin	3.69 ± 0.16[d]	3.75 ± 0.20	3.81 ± 0.20	3.79 ± 0.18	3.90 ± 0.16
actomyosin	2.70 ± 0.09[d]	2.95 ± 0.11	3.54 ± 0.27	3.77 ± 0.12	3.83 ± 0.19
F-actin	3.19[e]	4.49	6.84	3.91	4.03

[a] SH content: SH mol/10^5 g of protein. Myosin (10 mg/mL) in 0.6 M KCl and 20 mM sodium phosphate (pH 6.0). [b] Actomyosin (15 mg/mL) in 0.6 M KCl and 20 mM sodium phosphate (pH 6.0). [c] F-actin (2 mg/mL) in 0.6 M KCl and 20 mM sodium phosphate (pH 6.0). [d] Data are means of three replicates ± SD. [e] Data are means of two replicates.

Table III. Changes in SH Contents of Myosin,[a] Actomyosin,[b] and F-Actin[c] at Various KCl Concentrations When Subjected to a Pressure of 150 MPa for 5 min

	KCl concentration				
	0.2 M	0.4 M	0.6 M	0.8 M	1.0 M
myosin nonpress	3.74[d]	3.90	3.99	4.01	3.42
press	3.75[d]	3.91	3.98	3.99	3.61
actomyosin nonpress	2.26 ± 0.35[e]	2.26 ± 0.18	2.31 ± 0.01	2.93 ± 0.02	3.05 ± 0.01
press	2.78 ± 0.02[e]	3.20 ± 0.31	3.30 ± 0.04	3.69 ± 0.06	3.44 ± 0.06
F-actin nonpress	2.20[d]	2.77	3.54	3.12	5.44
press	3.76[d]	6.02	6.56	6.57	6.31

[a] SH contents: SH mol/10^5 g of protein. Myosin (10 mg/mL) in 20 mM sodium phosphate (pH 6.0). [b] Actomyosin (15 mg/mL) in 20 mM sodium phosphate (pH 6.0). [c] F-actin (2 mg/mL) in 20 mM sodium phosphate (pH 6.0). [d] Data are means of two replicates. [e] Data are means of three replicates ± SD.

pressure. On the contrary, the structure of actomyosin at 0.6 M KCl which probably consists of a large number of myosin monomers attached to the surface of actin filaments, although not detected in the photomicrograph, was transformed into the aggregated form by pressure treatment (Figure 5, parts e and f).

Thus, the result of Figure 5d, together with the result that actin in actomyosin pressurized at 150 MPa was mostly denatured at 0.2 M KCl (Figure 2), gave one of the strongest pieces of evidence in support of the speculation that pressurized actomyosin at 0.2 M KCl behaves like myosin in regard to the dynamic rheological behavior (Figure 2B,D in the preceding paper).

Measurements of SH Content and Surface Hydrophobicity (S_0). Many hydrophobic residues and the SH amino group buried in the interior of native muscle proteins would be exposed at the molecular surface under pressure, altering the properties of heat-induced gels (Kato and Nakai, 1980; Ishioroshi et al., 1980). The hydrophobicity and SH content of myosin, actomyosin, and actin were determined before and after pressure treatment. The increments of surface hydrophobicity (initial slope, S_0) of such proteins are plotted as a function of pressure in Figure 6. The S_0 values of actomyosin and myosin were increased with increasing pressure. Actin alone showed an abnormal profile with regard to the increment of S_0; it attained a maximum at 150 MPa and decreased sharply with other pressure intensities. A decline of the increment of S_0 in actomyosin observed at above 200 MPa may be connected with this change in the S_0 of actin. The S_0 value of the pressurized actomyosin increased to 2 or 3 times that of control sample at all KCl concentrations (Figure 7). Tables II and III show the change in SH content of actomyosin, myosin, and actin under various conditions of pressure. As shown in these tables, the marked increase in SH content for myosin was not observed, unlike in the case of hydrophobicity. The SH content of actomyosin increased with increasing pressure intensity and KCl concentration. On the other hand, the SH content of F-actin pressurized at 150 MPa was more than 2-fold higher than that of unpressurized F-actin and was maximum in analogy with the case of hydrophobicity. The decreases in SH content and hydrophobicity over 200 MPa were thought to be due to the aggregation of actin. Also, the change in the SH content of actomyosin seems to reflect, on the whole, the change in that of actin in actomyosin because the change in myosin alone was small regardless of pressure intensity and KCl concentration.

The increases of hydrophobicity and SH content in pressurized actomyosin would be seemingly one of the reasons for the increase of the heat-induced gel-forming capacity of actomyosin because hydrophobic and SH amino acid groups are believed to play a role in protein–protein interaction to form a protein gel on heating (Kinsella and Whitehead, 1989). However, the results of Figures 3 and 4 cannot always be explained by merely considering the increase of hydrophobicity or SH content, because the change in these values do not correspond to the change in the gel strength (G' and G'') of actomyosin. In the case of pressurized myosin, the increase of hydrophobicity may, however, compensate for the decrease in the heat-induced gel strength of myosin which was denatured by pressure at 300 MPa (Figure 3, and Figure 3 in the preceding paper). If the difference in the heat-induced gel strength between unpressurized actomyosin and pressurized actomyosin does not arise from the difference in the extents of hydrophobicity and SH content, then how does pressurized actomyosin at low or high salt concentration form a gel firmer than unpressurized actomyosin? Presumably, it is more realistic to consider the denaturation or depolymerization of actin in actomyosin due to pressure as a fundamental cause of the increased gel strength of pressurized actomyosin (Figures 1–5 and Table I). This is because a large amount of F-actin exhibits a negative effect on the heat-induced gelation of myosin at low and high KCl concentrations according to the Yasui's theory (see Introduction). Of course, there is no doubt that increases of hydrophobicity and SH content in actomyosin by pressure are partially responsible for the increased gel strength of pressurized actomyosin.

ACKNOWLEDGMENT

We express our thanks to Nippon Kokan Co., Ltd., of Niigata who made the cold isostatic press available to us. We are grateful to Dr. K. Katsuta for her valuable suggestions and Mr. N. Sallah for reading the manuscript.

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Received for review November 15, 1991. Revised manuscript received March 16, 1992. Accepted July 13, 1992.

Registry No. ATPase, 9000-83-3; KCl, 7447-40-7.