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Partial Specific Volume and Adiabatic Compressibility of G-Actin Depend on the Bound Nucleotide

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We determined the partial specific volume and partial specific adiabatic compressibility of either ATP- or ADP-bound monomeric actin in the presence of Ca^{2+} by measuring the density of and sound velocity in a monomeric actin solution at 18°C. The partial specific volume of ATP-bound monomeric actin, equal to 0.744 cm^3/g, which is exceptionally high among globular proteins, was reduced to 0.727 cm^3/g when the tightly bound ATP was replaced with ADP. Associated with this, the adiabatic compressibility of ATP-bound monomeric actin, equal to 8.8×10^{-12} cm^2/dyne, decreased to 5.8×10^{-12} cm^2/dyne, which is a common value for globular proteins. These results suggested that an extraordinarily soft global conformation of ATP-bound monomeric actin is packed into a compact mass associated with the hydrolysis of bound ATP. When monomeric actin was limitedly proteolyzed at subdomain 2 with subtilisin, the nucleotide-dependent flexibility of the global conformation of monomeric actin was lost.

Key words: actin, adiabatic compressibility, nucleotide, partial specific volume, subtilisin.

Abbreviations: G-actin, monomeric actin; ATP-G-actin, ATP-bound monomeric actin; ADP-G-actin, ADP-bound monomeric actin; F-actin, filamentous actin.

It is well known that the native conformation of monomeric actin is stabilized primarily by the bound nucleotide (*1*). While the native conformation of monomeric actin is most stable in the ATP-bound form, the conformation of the ADP-bound actin monomer is stabilized if it is incorporated in polymeric actin. Physicochemical and biochemical studies of the actin monomer revealed significant structural differences between the ATP-bound and ADP-bound forms (*2–6*). Even though these studies indicated that the structure of monomeric actin differs with the nucleotide bound, it was not clear whether the difference was limited to local reaction sites or associated with its global conformation. In order to clarify this point, we determined the partial specific volume and partial specific adiabatic compressibility of both ATP-bound and ADP-bound monomeric actin. The results showed that the global conformation of monomeric actin changes significantly in association with the exchange of the bound nucleotide between ATP and ADP.

MATERIALS AND METHODS

Chemicals—ATP was purchased from Boehringer-Mannheim Biochemicals (Germany). ADP and subtilisin Carlsberg type VIII were obtained from Sigma Chemicals (USA). *N*-(1-pyrene)Iodoacetamide (P-29) was purchased from Molecular Probes Inc. (USA). All other chemicals were of reagent grade and purchased from Wako Pure Chemical Industries (Japan).

Miscellaneous—The concentration of G-actin was determined spectrophotometrically, using the absorption coefficient, $A^{1\%}_{1\,cm}$ = 6.3, at 290 nm. The absorption was corrected for light scattering by subtraction of the absorption at 320 nm (*7*). The molecular weight of actin, 43,000 Da, was used. Bradford's method with G-actin as the standard was used for the determination of pyrene-labeled and low actin concentration. We took the absorption coefficient and molecular weight of subtilisin-treated actin as being equal to those of intact actin. The preparation of pyrene-labeled actin and measurement of the critical concentration were performed according to Kouyama and Mihashi (1981) (*8*).

Preparation of G-Actins—G-Actin of rabbit skeletal muscle was prepared from acetone-dried powder as described previously (*9–11*). Further purification of ATP-G-actin was performed as follows: G-actin was polymerized in 0.1 M KCl and 2 mM $MgCl_2$ for 2 h. The F-actin solution was ultracentrifuged at 100,000 ×*g* 80 min and the F-actin pellet was dispersed in G-buffer (100 μM $CaCl_2$, 1 mM TrisHCl, pH 8.0, 1 mM NaN_3 and 1 mM 2-mercaptoethanol) containing 200 μM ATP. The dispersed actin solution was dialyzed overnight against 1 liter of G-buffer containing 200 μM ATP (ATP-G-buffer) at 4°C. Ultracentrifugation was carried out to remove undissolved actin, if any.

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ADP-G-actin was prepared by the method of Mihashi (1964) (*12*) with a slight modification, as described below. After centrifugation of F-actin derived from G-actin, the obtained F-actin pellet was dispersed in ADP-F-buffer (0.1 M KCl, 2 mM $MgCl_2$, and 5 mM TrisHCl, pH 8.0, in G-buffer plus 500 μM ADP). Dispersion was accelerated by ultrasonication on ice. After keeping on ice for 30 min, ultracentrifugation and pellet dispersal were carried out again. After ultracentrifugation, the F-actin pellet was dissolved in G-buffer containing 500 μM ADP (ADP-G-buffer). Depolymerization into ADP-G-actin was accelerated by gentle sonication on ice. The ADP-G-actin solution thus obtained was dialyzed against ADP-G-buffer overnight at 4°C for complete depolymerization. The ADP-G-actin was ultracentrifuged to remove any undissolved actin.

Preparation of subtilisin-treated ATP-G-actin was performed according to Ooi and Mihashi (1996) (*13*) with a slight modification, as follows: G-actin at 2–4 mg/ml was digested at a weight ratio of subtilisin to actin of 1:1,000 to 1:2,000. The digestion was performed at 23°C for 50–60 min. Without the addition of phenylmethanesulfonyl fluoride (PMSF), to avoid its disturbance of sound velocity measurement, polymerization of actin was started by the addition of 0.1 M KCl, 2 mM $MgCl_2$, 5 mM TrisHCl, pH 8.0, and 2 mM ATP. After 30–60 minutes, the cycle of centrifugation and pellet dispersal in ATP-F-buffer (0.1 M KCl, 2 mM $MgCl_2$, and 5 mM TrisHCl, pH 8.0, in G-buffer containing 200 μM ATP) under gentle ultrasonic vibration was repeated twice. The dispersed F-actin solution was centrifuged and the pellet was dissolved with ATP-G-buffer. The solution of subtilisin-treated ATP-G-actin was dialyzed against 1 liter of ATP-G-buffer overnight at 4°C. Polymeric actin remaining was removed by ultracentrifugation. Subtilisin-cleavage of actin was always confirmed by Coomassie briliant blue-stained SDS-PAGE, with scanning with an image scanner (ES-2000; Epson) and analysis with NIHimage. The extent of digestion was over 93% for subtilisin-treated G-actin. The purity of the cleaved product was slightly decreased depending on the polymerization-depolymerization cycle.

For the preparation of subtilisin-treated ADP-G-actin, the process of nucleotide exchange was combined with subtilisin cleavage as follows. Firstly, ATP-G-actin was cleaved with subtilisin by the same method as mentioned above. Polymerization of subtilisin-treated actin was started by the addition of 0.1 M KCl, 2 mM $MgCl_2$, 5 mM TrisHCl, pH 8.0, and 2 mM ADP. The nucleotide exchange procedure was the same as for ADP-G-actin.

We modified the ordinary procedure for each G-actin preparation to avoid systematic errors in the measurement of density and sound velocity. Frequent polymerization-depolymerization cycles to replace the bound nucleotide or no use of a subtilisin inhibitor possibly causes degradation of actin. Therefore, we confirmed the polymerizability of the prepared actins by measuring their critical concentrations with pyrene-labeled actin. The results were in good agreement with the reported values (*4*, *14*, *15*). Furthermore, the critical concentrations of actins became almost zero in the presence of phalloidin, indicating that our actin preparations satisfactorily retained polymerizability.

The decreases of V_p and β_s were both not due to degradation of the protein, which was verified to evaluate the supernatant of F-actin derived through polymerization from G-actin. The concentration in the supernatant after ultracentrifugation for ADP-G-actin was higher than that for ATP-G-actin, while its polymerizability was changed on the addition of 200 μM ATP before polymerization to the same level as for ATP-G-actin.

Measurement of Density and Sound Velocity—To attain complete equilibrium of solutes, all G-actin solutions were further dialyzed for over 8 h against 1 liter of G-buffer containing either 200 μM ATP or 500 μM ADP at 4°C before the measurement of density and sound velocity. All G-actins were kept on ice after the last ultracentrifigation and used for the measurements within several hours. Each measurement sequence was completed within 30 min. Loss of polymerizability of actin samples kept at room temperature (22–23°C) for 5 h was checked after polymerization and ultracentrifugation. All G-actins retained the same polymerizability as the references at 0 h.

The apparent specific volume, V_{ap}, and apparent adiabatic compressibility, β_{ap}, of G-actins were calculated using the results for both sound velocity measurement ("sing-around pulse method") at 5 MHz, with an accuracy of 1 cm/s, and density measurement with a precision density meter, DMA-02C (Anton Paar, Gratz), with an accuracy of 1×10^{-6} g/cm^3. The temperature was controlled, with an accuracy of 0.003°C, with a thermobath (Neslab RTE-110) at 18°C. The details of the experimental procedures were reported previously (*16*, *17*). The adiabatic compressibility of a sample solution, β, was calculated with the Laplace equation:

$$\beta = 1/(\rho U^2) \tag{1}$$

where U is the sound velocity and ρ the density of the sample solution. The partial specific volume, V_p, and the adiabatic compressibility of the protein, β_s, at infinite dilution were calculated using the following equations (*16*, *18*):

$$V_p = \lim_{c \to 0} V_{ap} = \lim_{c \to 0} (1 - \gamma)/c \tag{2}$$

$$\begin{aligned} \beta_s &= -(1/V_p)(\partial V_p/\partial P)_s = \lim_{c \to 0} \beta_{ap} \\ &= \lim_{c \to 0} (\beta_0/V_{ap})(\beta/\beta_0 - \gamma)/c \end{aligned} \tag{3}$$

$$\gamma = (\rho - c)/\rho_0 \tag{4}$$

where P is the pressure, γ the apparent volume fraction of the solvent in solution, c the concentration of the protein in grams per milliliter of solution, and β_0 the adiabatic compressibility of the solvent. We chose the data set of each G-actin that exhibited least deviation along the linear regression line for both partial specific volume and adiabatic compressibility among 1–3 different preparations.

Molecular Weight Measurement by Sedimentation Equilibrium Measurement—Sedimentation equilibrium experiments were performed with a Beckman Optima XL-A analytical ultracentrifuge at a speed of 14,000 rpm at 20°C for 20 h using a 4-hole rotor and 12-mm double-sector centerpieces. Data analysis was performed with XL-A Data Analysis Software on an Origin (Microcal Software,

MA, USA). The apparent molecular weight, M_{app}, was determined using a single exponential model, "ideal 1." The solution densities and partial specific volumes of ATP-G-actin and subtilisin-treated ATP-G-actin were determined through the above described density measurements. The initial concentrations were 0.15, 0.22, and 0.30 mg/ml (3.4, 5.1, and 7.0 μM) for intact ATP-G-actin, and 0.14, 0.23, and 0.31 mg/ml (3.3, 5.3, and 7.2 μM) for subtilisin-treated ATP-G-actin, and the results were extrapolated to infinite dilution with linear regression.

RESULTS

The apparent specific volume and apparent adiabatic compressibility of ATP-G-actin determined from density and sound velocity measurements were expressed as a function of the actin concentration (Fig. 1). The partial specific volume, V_p, and partial specific adiabatic compressibility, β_s, were obtained by extrapolating the apparent values to infinite dilution with linear regression (Table 1). The partial specific volume, V_p, and the partial specific adiabatic compressibility, β_s, of ATP-G-actin are 0.744 cm^3/g and 8.8×10^{-12} cm^2/dyne, respectively. Both values are exceptionally high among globular proteins as was addressed firstly by Gekko and Noguchi (1979) (*18*).

In the case of ADP-G-actin, the partial specific volume, V_p, was 0.727 cm^3/g, and the adiabatic compressibility was 5.8×10^{-12} cm^2/dyne (Fig. 1). Both values were of the same order of magnitude as in the case of globular proteins in general (*18*), indicating that the anomalies in the partial specific volume and partial specific adiabatic compressibility of G-ATP-actin disappeared when the bound nucleotide was exchanged with ADP. The reduction in the partial specific adiabatic compressibility was particularly significant (34% loss). It should be noted for this determination, that extrapolation to infinite dilution was not thoroughly satisfactory since the protein concentration range examined was different for ATP-G-actin (from 3.3 to 5.2 mg/ml) and ADP-G-actin (from 2.4 to 7.3 mg/ml). The number averages of experimental values (namely the values obtained with the neglect of the concentration dependence) are shown in Table 1. Close inspection of individual values in Table 1 led us to conclude that the extraordinary flexible nature of ATP-G-actin disappears if the bound ATP is replaced with ADP. This suggested two possible scenarios: (i) the protein structure surrounding the bound nucleotide may change due to exchange of the bound nucleotide; (ii) the global structure including the configuration of subdomain 2, particularly of the long hydrophobic loop, the so called DNaseI-binding loop (residues 38–52), may change in association with the nucleotide exchange. The latter possibility was suggested by a previous study (*13*), where it was shown that limited proteolysis of the subdomain 2 is associated with a significant decrease in the rate of exchange the bound nucleotide. Then we performed measurements of the density of and sound velocity of a subtilisin-treated ATP-G-actin solution as well as a subtilisin-treated ADP-G-actin solution. Plots of the apparent values are presented in Fig. 2. The extrapolated values for subtilisin-treated ATP-G-actin were 0.727 cm^3/g for V_p and 6.6×10^{-12} cm^2/dyne for

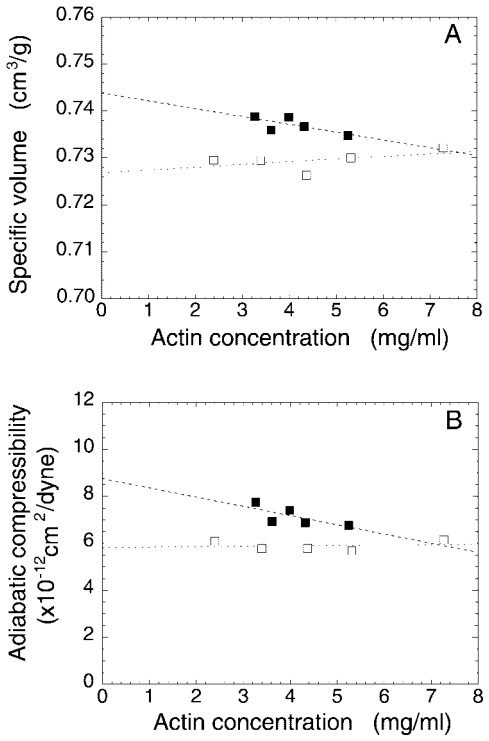

Fig. 1. **Plots of (A) the apparent specific volume against the monomeric actin concentration, c, and (B) the apparent adiabatic compressibility against the monomeric actin concentration.** The symbols are for ATP-bound monomeric actin (closed boxes) and ADP-bound monomeric actin (open boxes) in G-buffer (200 μM ATP or 500 μM ADP, 100 μM $CaCl_2$, 1 mM TrisHCl, pH 8.0, 1 mM NaN_3, and 1 mM 2-mercaptoethanol) at 18°C. The partial specific volume (V_p) and partial specific adiabatic compressibility (β_s) were obtained by extrapolation to infinite dilution using linear regression. The dotted lines are linear extrapolating functions with obtained the least square method.

Table 1. **The partial specific volume, V_p, and partial specific adiabatic compressibility, β_s, of ATP-G-actin, ADP-G-actin, subtilisin-treated ATP-G-actin, and subtilisin-treated ADP-G-actin in G-buffer (100 μM $CaCl_2$, 1 mM TrisHCl, pH 8.0, 1 mM NaN_3, and 1 mM 2-mercaptoethanol) containing 200 μM ATP or 500 μM ADP at 18°C.**

Type	$V_p \pm$ SE (Average of $V_{ap} \pm$ SD) (cm^3/g)	$\beta_s \pm$ SE (Average of $\beta_{ap} \pm$ SD) ($\times 10^{-12} cm^2$/dyne)
ATP-G-actin	0.744 ± 0.004 (0.737 ± 0.002)	8.8 ± 0.9 (7.2 ± 0.4)
ADP-G-actin	0.727 ± 0.003 (0.730 ± 0.002)	5.8 ± 0.3 (5.9 ± 0.2)
Subtilisin-treated ATP-G-actin	0.727 ± 0.002 (0.725 ± 0.001)	6.6 ± 0.4 (5.2 ± 0.5)
Subtilisin-treated ADP-G-actin	0.720 ± 0.005 (0.724 ± 0.004)	5.5 ± 0.7 (5.5 ± 0.5)

The error values are standard errors (SE) of linear extrapolation with the least square method and standard deviations (SD) of number averages. In the table, the values obtained by linear regression are compared with the simple number averaged values inside parentheses.

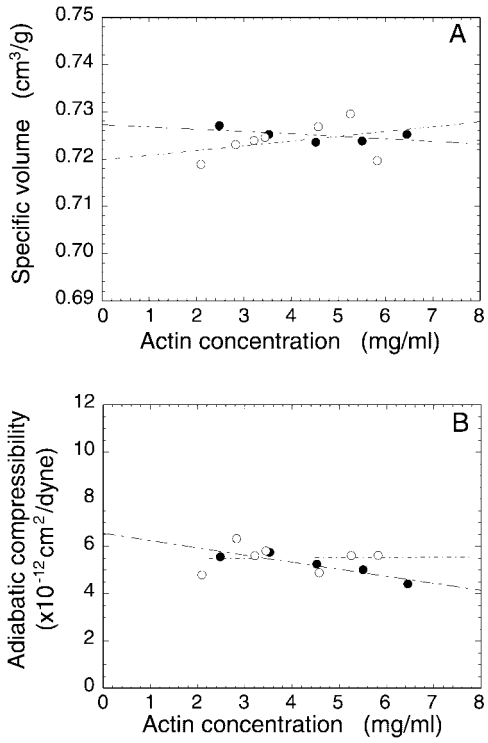

Fig. 2. **Plots of (A) the apparent specific volume against the monomeric actin concentration, c, and (B) the apparent adiabatic compressibility against the monomeric actin concentration of subtilisin-treated monomeric actin.** The symbols are for subtilisin-treated ATP-bound monomeric actin (closed circles) and subtilisin-treated ADP-bound monomeric actin (open circle) under the same conditions as in Fig. 1. The partial specific volume (V_p) and partial specific adiabatic compressibility (β_s) were obtained by the same methods as in Fig. 1. The broken lines show linear extrapolating functions.

β_s. These are very close to those of intact ADP-G-actin (Table 1). Moreover, both V_p and β_s became insensitive to the exchange of ATP with ADP. The data for subtilisin-treated ADP-G-actin were widely spread compared to in the case of other G-actins, because it was very unstable, and it was thus difficult to measure both sound velocity and density for subtilisin-treated ADP-G-actin.

The molecular weight measurement of subtilisin-treated ATP-G-actin showed that the apparent molecular weight plotted as a function of the initial protein concentration gave extrapolated values equal to 42,000 Da for subtilisin-treated G-ATP-actin and 44,000 Da for G-ATP-actin, which were not significantly different from each other. This indicated that the loss of sensitivity to nucleotide-exchange of subtilisin-treated actin is not due to aggregation of the protein after limited proteolysis in solution but due to some intra-molecular change. Thus, we concluded that the extraordinarily flexible conformation of ATP-bound monomeric actin becomes a compact form associated with the exchange of ATP with ADP (or hydrolysis of the bound ATP).

DISCUSSION

The partial specific volume of ATP-G-actin, equal to 0.744 cm^3/g, and partial specific compressibility, equal to 8.8×10^{-12} cm^2/dyne, obtained in this study were both compatible with the previously reported values (*17*). We should mention that reliability of the experimentally obtained values was increased in the present work, because we measured both the density of and sound velocity in solution using the same solution. In the case of ADP-bound monomeric actin bound to Mg^{2+} instead of Ca^{2+} , the partial specific volume, equal to 0.732 cm^3/g, was previously obtained (*12*). This value is very close to 0.727 cm^3/g obtained in the present study for V_p of ADP-G-actin bound to Ca^{2+}. This means that the effect of the divalent cation bound to ADP-G-actin on its V_p is not large like that of nucleotide exchange, as supported by the results of a polymerization kinetics experiment (*15*), although several previous studies involving antigenic assaying and fluorescence spectroscopy were in accord, suggesting a difference in the conformation of the protein depending on the bound divalent cation (*19–21*). Bound divalent cations may only affect a very local structure of the protein.

Among globular proteins, ATP-G-actin was so far known to have an exceptionally large partial specific volume and adiabatic compressibility (*18*, *17*). The present study not only confirmed previous studies but also provided very strong evidence that the extraordinarily compressible nature of ATP-G-actin disappears when the bound ATP is exchanged with ADP, or after hydrolysis of the bound nucleotide. This finding may provide a reasonable explanation as to the different reactivities of G-actin with many actin binding proteins, which showed nucleotide-dependent regulation of a versatile biological function of actin (*22–25*).

The results obtained for subtilisin-treated G-actins in the present study suggested that subdomain 2 (or the DNaseI-binding loop) is responsible, at least partly, for the extraordinarily compressible structure of ATP-G-actin. The great thermodynamic flexibility of ATP-G-actin may indicate significant thermal fluctuation of protein volume in solution, because both the partial specific volume and compressibility are linked to volume fluctuation (*26–28*). The great thermal volume fluctuation of ATP-G-actin may be responsible for the high sensitivity of ATP-G-actin to various environmental changes. The atomic models of G-actin proposed in a study of crystals of the actin:DNaseI complex suggested that the structure of ATP-bound monomeric actin is very similar to that of the ADP-bound form (*29*). Very recently, however, an ADP-actin model deduced from actin crystals without other proteins bound (*30*) indicated that ADP-bound monomeric actin exhibits a small but distinct difference from that in the model of Kabsch *et al*. (*29*). These differences in actin structure that depend on the species of bound nucleotide may explain the difference in the global conformation of G-actin found in the present study.

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