The polymerization of actin: Extent of polymerization under pressure, volume change of polymerization, and relaxation after temperature jumps

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(Received 15 April 2005; accepted 24 June 2005; published online 22 August 2005)

The protein actin can polymerize from monomeric globular G-actin to polymeric filamentary F-actin, under the regulation of thermodynamic variables such as temperature, pressure, and compositions of G-actin and salts. We present here new measurements of the extent of polymerization (ϕ) of actin under pressure (P), for rabbit skeletal muscle actin in H_2O buffer in the presence of adenosine triposphate and calcium ions and at low (5–15 mM) KCl concentrations. We measured ϕ using pyrene-labeled actin, as a function of time (t) and temperature (T), for samples of fixed concentrations of initial G-actin and KCl and at fixed pressure. The $\phi(T,P)$ measurements at equilibrium have the same form as reported previously at 1 atm: low levels of polymerization at low temperatures, representing dimerization of the actin; an increase in ϕ at the polymerization temperature (T_p); a maximum in $\phi(T)$ above T_p with a decrease in $\phi(T)$ beyond the maximum, indicating a depolymerization at higher T. From $\phi(T,P)$ at temperatures below T_p, we estimate the change in volume for the dimerization of actin, ΔV_{dim}, to be -307 ± 10 ml/mol at 279 K. The change of T_p with pressure $dT_p/dP=(0.3015\pm0.0009)$ K/MPa$=(30.15\pm0.09)$ mK/atm. The $\phi(T,P)$ data at higher T indicate the change in volume on propagation, ΔV_{prop}, to be $+401\pm48$ ml/mol at 301 K. The $\phi(t)$ measurements yield initial relaxation times $r_p(T)$ that reflect the behavior of $\phi(T)$ and support the presence of a depolymerization temperature. We also measured the density of polymerizing actin with a vibrating tube density meter, the results of which confirm that the data from this instrument are affected by viscosity changes and can be erroneous. © *2005 American Institute of Physics*. [DOI: 10.1063/1.2001635]

I. INTRODUCTION

The aggregation or polymerization of globular G-actin to form filamentary F-actin is an important cellular process, serving major functions in cell structure and cell motility.[1] The mechanism for the aggregation of G-actin to F-actin can be viewed as having three main steps:[2–9]

(1) monomer activation or initiation, where A_1 is the monomeric G-actin,[10]

$$A_1 \rightleftarrows A_1^*, \text{ with an equilibrium constant } K_{\mathrm{act}}(T); \quad (1)$$

(2) dimerization of two monomers,[11]

$$A_1^* + A_1 \rightleftarrows A_2,$$

with an equilibrium constant $K_{\mathrm{dimer}}(T)$; and (2)

(3) trimer formation (nucleation) and propagation,[12]

$$A_2 + A_1 \rightleftarrows A_3, \text{ with equilibrium constant } K_{\mathrm{prop}}(T);$$

$$A_1 + A_i \rightleftarrows A_{i+1}, \text{ where } i \geqslant 3, \quad (3)$$

with equilibrium constant $K_{\mathrm{prop}}(T)$;

where the subscript denotes the number of G-actin monomers in a F-actin polymer. Step 3 assumes no dependence of $K_{\mathrm{prop}}(T)$ on the number of monomers in the polymer. The presence of a salt is necessary for aggregation to occur, and is thought to induce a configurational change that activates the monomer.[10]

In the presence of adenosine triphosphate (ATP), actin continuously hydrolyzes the ATP to adenosine diphosphate (ADP), and a steady state of this conversion is reached.[13] Under some conditions, the two ends of the F-actin have different affinities for G-actin, and a "treadmilling" of

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G-actin can result, in which case G-actin adds to one end of a F-actin molecule, moves down the molecule, and exits the other end of the molecule.[14] It has been said that "the hydrolysis of the actin-bound ATP is *not* tightly coupled to polymer formation."[15] Indeed, in the absence of ATP but in the presence of ADP, actin will polymerize to a true equilibrium with no treadmilling, but slowly and with a tendency toward denaturation.[15,16] On the other hand, a recent structural study[17] suggests that there may be coupling of hydrolysis and polymerization. Any such coupling is not explicit in the mechanism given above.

It is well known[3] that step 3 has a positive entropy change, ΔS_{prop}, and a positive enthalpy change, ΔH_{prop}, which means that, for fixed concentrations of initial G-actin and of salt, the propagation will occur only above a particular polymerization temperature or "floor" temperature.[18,19] Protein aggregations are more often studied at a fixed temperature and salt concentration, such that the propagation occurs only above a "critical concentration" of monomer (e.g., G-actin).[3] We focus here on the variation of temperature at fixed concentrations and pressures.

The sign and magnitude of the change in volume during actin polymerization have been controversial, as we will discuss below. We might expect that the aggregation of monomers into polymers will lead to a *decrease* in volume, as is observed for organic[20] and inorganic[21] polymerizations, which show volume changes in the range of -4 to -19 ml/mol.[19,22] However, the situation is more complex for proteins such as actin. (1) The volume changes for the steps in the reaction mechanism [Eqs. (1)–(3)] can be different in sign and magnitude; some experiments may measure the overall volume change and some may measure the volume change of one step of the mechanism. (2) The aggregation of the protein is thought to involve a release of water bound to the protein, which will cause an increase in the volume of the solution.[3,23,24] (3) The development of hydrophobic interactions among monomers to make a polymer may involve yet another volume change, the sign of which is hard to predict. (4) Changes in the binding of salts to the protein during the polymerization can increase or decrease the volume.[25] (5) The hydrolysis of ATP during the polymerization will involve a volume decrease of about -19 ml/mol.[26] (6) There may be a difference in packing efficiency as the polymer grows at the higher temperatures, since the polymeric filaments will be harder to pack as they get longer, and this may appear as an increase in volume.[27] The total change in volume during the polymerization will be the sum of these effects, and can depend on such variables as salt concentration, salt species, protein concentration, temperature, and pressure. Measurements on other aggregating proteins (e.g., tobacco mosaic virus[23] and flagellin[3]) show *increases* in volume upon polymerization.

Here we have extended our earlier work[28,29] on the dependence on temperature (T) of the extent of polymerization, ϕ, defined as the fraction of initial G-actin that has been incorporated into F-actin, by examining the effects of high pressure (P). At the same time we studied the relaxation of the actin system with time (t) after each temperature jump. The pressure studies on $\phi(T)$ and the relaxation data $\phi(t)$ corroborate our earlier work and support the presence of a reentrant depolymerization above the polymerization temperature. The pressure studies allow us to estimate the change of volume during actin dimerization and polymerization. We also attempted to measure the density of polymerizing actin using a vibrating tube density meter, in order to measure the volume change directly; we found that the vibrating tube density meter is prone to error for samples with high viscosities and thus does not give correct measurements of the volume change of actin.

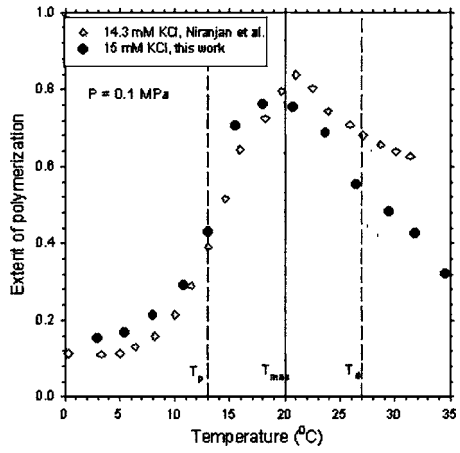

FIG. 1. The extent of polymerization as a function of temperature for the samples of rabbit muscle actin in H_2O buffer, at fixed initial concentration of G-actin and at various concentrations of KCl (see legend). The data from Niranjan *et al.* (Ref. 28) are at $[G]_0=2.93$ mg/ml; our new data are at 3.00 mg/ml. The polymerization is marked by a "floor" or polymerization temperature, T_p, a maximum temperature, $T_{\max}$, and a depolymerization temperature, T_d. These special temperatures are indicated by vertical lines.

A. Previous work on the extent of polymerization as a function of temperature

In previous work, we have studied $\phi(T)$ at atmospheric pressure in H_2O buffers[28] and in D_2O buffers.[29] Figure 1 (open symbols) shows the $\phi(T)$ of rabbit muscle actin as previously measured by labeled fluorescence spectroscopy, in H_2O buffer at 1-atm pressure.[28] The polymerization temperature, T_p, can be seen as the inflection point in $\phi(T)$ as the polymerization develops.[30–32] Such a T_p also exists for the assembly of the protein tubulin.[30–33] Above T_p, the extent of polymerization of a polymer would be expected to increase as the temperature increases and then reach a plateau.[19,30] However, Fig. 1 shows instead a maximum in $\phi(T)$. It has been proposed[28,29] that for actin, not only does the propagation step of the reaction mechanism [Eq. (3)] has a temperature of onset for polymerization, but the activation step [Eq. (1)] also has a positive entropy change, ΔS_{act}, and a positive enthalpy change, ΔH_{act}, which means that the activation step must also have a temperature of onset.[28,29] These two onset temperatures, one for activation and one for propagation, are thought to be only a few degrees apart.[29] The maximum in the extent (Fig. 1) at $T_{\max}$ is then a consequence of the competition between the two steps of the mechanism. The increase in the equilibrium constant for activation requires monomers, and those monomers are obtained by the depolymerization of existing polymers. The result is a depolymer-

TABLE I. Values of the change of molar volume for the polymerization of rabbit muscle actin (1–4 and 6) or chicken muscle actin (5), compared to new values from this work (7 and 8). ΔV is the total volume change of the polymerization and ΔV_{prop} is the volume change of the propagation step alone.

Reference	Method	Salt	ΔV (ml/mol)
(1) Ikkai and Ooi[a]	Dilatometer	$MgCl_2$, 1.0 mM	$\Delta V=+295$ at 25 °C
(2) Quirion and Gicquad[b]	Vibrating tube	$MgCl_2$, 0.1–7 mM	$\Delta V=-720\pm200$ at 25 °C
(3) Suzuki *et al.*[c]	Vibrating tube	$MgCl_2$, 0.5 mM +KCl, 100 mM	$\Delta V=(-4\pm1)\times10^3$ at 20 °C
(4) Ikkai and Ooi[d]	Pressure	KCl, 10 and 60 mM	$\Delta V_{\text{prop}}=+84$ at 25 °C
(5) Swezey and Somero[e]	Pressure	KCl, 100 mM	$\Delta V_{\text{prop}}=+63$ at 25 °C; +107 at 4 °C
(6) Garcia *et al.*[f]	Pressure	KCl, 100 mM	$\Delta V_{\text{prop}}=+328\pm63$ at 25 °C
(7) This work	Vibrating tube	KCl, 5–15 mM	$\Delta V_{\text{prop}}=(-9\pm2)\times10^3$ at 25 °C
(8) This work	Pressure	KCl, 15 mM	$\Delta V_{\text{prop}}=+401\pm48$ at 31 °C

[a]Reference 34.
[b]Reference 38.
[c]Reference 43.
[d]Reference 35.
[e]Reference 47.
[f]Reference 50.

ization temperature at T_d. The dimerization step of the mechanism [Eq. (2)] does not seem to have a strong influence on the extent of polymerization, other than producing the nonzero polymerization well below T_p.[29]

B. Previous experimental work on volume change and pressure effects

Here we use the symbol ΔV_{dimer} to indicate the change in volume of the dimerization step [Eq. (2)], ΔV_{prop} to indicate the change in volume of the propagation step [Eq. (3)], and ΔV to indicate the total change in volume on polymerization for all three steps [Eqs. (1)–(3)]. ΔV is obtained in experiments where initiating salt is added to actin in buffer under polymerizing conditions and then all three steps ensue. ΔV_{prop} is obtained in experiments when the salt is added to actin in buffer under nonpolymerizing conditions (e.g., at low temperature), and then conditions are changed (e.g., the temperature is increased) to cause polymerization. Under the latter conditions, it is possible to obtain ΔV_{dimer} from the effect of pressure on the low-level (dimerization) of polymerization at the lower temperatures (see below).

1. Direct measurements of the change of volume during the polymerization of actin

There have been three reports of direct measurements of the volume or density change for polymerizing actin, as listed in Table I. All these experiments were done in such a way as to yield ΔV.

First, in 1966, Ikkai and Ooi[34] used a dilatometer with a resolution of about 10 ppm to measure ΔV at (25.0000±0.0002) °C. The paper does not describe the actin preparation; we assume that it was rabbit muscle actin with Ca^{2+} as the divalent counterion, as in a related paper from the same group.[35] The technique required the mixing of two solutions: (1) 15 ml of 1.15-mg/ml G-actin in 2-mM tris-HCl buffer with 500-μM ATP at *p*H 8.1, and (2) 3 ml of 6-mM $MgCl_2$. Ikkai and Ooi corrected the observed volume change for the volumes of binding of the ATP and $MgCl_2$, and for the change of volume due to ATP hydrolysis; they claimed that the volumes of dilution of actin and $MgCl_2$ were negligible. They noted an equilibration time after mixing of about 12 min. They repeated the measurement at various actin concentrations, from 0.13 to 1.3 mg/ml. The final value obtained was $\Delta V=+391$ ml/mol (with no error estimate given), using a molecular weight for G-actin of 57 000. The value using a corrected molecular weight[36,37] of 43 000 is +295 ml/mol, where the value given is per mole or polymerized monomeric G-actin; this corresponds to a volume change of 21 ppm in their solutions.

Second, in 1993, Quirion and Gicquaud[38] measured ΔV for actin polymerization using a vibrating tube density meter.[39] They used rabbit muscle actin in 2-mM tris-HCl, 0.2-mM ATP, 0.2-mM $CaCl_2$, 0.5-mM β-mercaptoethanol, and 0.02% NaN_3, at *p*H=8.0. Then 3.6 ml of 2.7-mg/ml actin in buffer was polymerized by the addition of $MgCl_2$ solution at various $MgCl_2$ concentrations (between 0.1 and 7 mM), allowed to equilibrate for 1 h (at a temperature not reported), and then introduced into a Sodev 03-D vibrating tube density meter (Sodev, Inc, Quebec, Canada) at (25.000±0.001) °C. Thus Quirion and Gicquaud varied the $MgCl_2$ concentration, while Ikkai and Ooi[34] had varied the actin concentration. Quirion and Gicquaud computed $\Delta V=(-720\pm200)$ ml/mol: a *negative* ΔV corresponding to a 51-ppm change for the solution.

Vibrating tube density meters are susceptible to errors from air bubbles, but Quirion and Gicquaud make no mention of degassing their samples. In addition, it has been established that early versions of the vibrating tube density meter were sensitive to viscosity changes as well as to density changes.[40] Since actin solutions show a significant increase in viscosity when the actin polymerizes,[41,42] measurements of the actin density made with vibrating tube density meters may be subject to error, as will be discussed further below.

The third direct measurement of ΔV came in 1996, when Suzuki *et al.*[43] measured the density of rabbit muscle actin solutions at various concentrations of actin in buffer. The densities of G-actin solutions and of F-actin solutions were measured and the differences taken. For G-actin, the buffer

contained 2-mM tris-HCl, 0.5-mM ATP, 0.1-mM $CaCl_2$, 1-mM β-mercaptoethanol, and 1-mM NaN_3, at pH=7.9. For F-actin, a buffer was used with 0.5-mM $MgCl_2$, 10-mM tris-HCl, 0.5-mM ATP, 0.1-mM $CaCl_2$, 1-mM β-mercaptoethanol, and 3-mM NaN_3, at pH=7.9; for a second F-actin buffer, 100-mM KCl was added. The instrument was again a vibrating tube density meter (Anton Paar DMA-02C, Anton Paar, Gratz, Austria). The samples were degassed and centrifuged to remove air bubbles. The temperature control was (20.000±0.003) °C. The densities of the F-actin solutions minus the densities of their buffers gave numbers larger than the corresponding differences between G-actin densities and their buffer densities, so the molar volumes of the F-actin would seem to be smaller and thus ΔV would appear to be negative. The densities were reported only as graphs of the differences from the buffers and as partial specific volumes. For example, from their Fig. 1, we read the data at 3-mg/ml actin as $(D-D_0)_{\text{F-actin}}=0.9$ mg/ml, where D is the density of the actin in low salt buffer and D_0 is the density of buffer alone, and $(D-D_0)_{\text{G-actin}}=0.7$ mg/ml. It is not possible to convert these densities to ΔV because these numbers are already small differences between larger numbers and are barely outside the error on those numbers, and because the inverse of a density difference is not a volume difference. However, the authors do report partial specific volumes for G-actin (+0.749 ml/g, no uncertainty given) and for F-actin (+0.63–0.66 ml/g), indicating a decrease in volume upon polymerization of (−0.09 to −0.12 ml/g). These values correspond to partial molar volumes (per mole of monomer) of 3.2×10^4 ml/mol for G-actin and 2.8×10^4 ml/mol for F-actin. The difference in the partial molar volumes gives $\Delta V=(-4\pm1)\times10^3$ ml/mol.

2. Previous work on the effect of pressure on actin polymerization

If we consider the polymerization temperature of actin as a phase-transition temperature,[44] then we can apply the equation given by Kennedy and Wheeler:[21]

$$\Delta V_{\text{prop}} = \Delta H_{\text{prop}}(dT_p/dP)/T_p, \tag{4}$$

which relates ΔV_{prop} (in m^3/mol) at the transition to the change in enthalpy (ΔH_{prop} in joules) at the transition, the dependence of T_p on pressure (P in pascal), and T_p. For the polymerization transition, ΔH_{prop} is positive when polymerization occurs above a transition ("floor") temperature and negative when polymerization occurs below a transition ("celling") temperature.[18] Thus for actin, ΔV_{prop} must have the same sign as (dT_p/dP).

Similarly, if we consider the polymerization of actin as a reversible chemical reaction, then for a reaction in a nonideal solution,[45,46]

$$(\partial \ln K_x/\partial P)_{T,c} = -\Delta V_{\text{prop}}/RT, \tag{5}$$

which relates the dependence on pressure of $K_x=[A_{n+1}]/\{[A_n][A_1]\}$, where the brackets indicate the concentrations of the species in Eq. (3), to ΔV_{prop} at a temperature T, where R is the gas constant. In the expression $(\partial \ln K_x/\partial P)_{T,c}$, the subscript requires constant temperature and constant compositions of all species (salts, ATP, pH, etc.), whereas these compositions are not necessarily held constant in experiments that change pressure.

The depolymerization of actin under hydrostatic pressure has been observed previously and indicates that ΔV_{prop} is positive by Eq. (5). Three studies of pressure effects on actin have given information on the behavior of ΔV_{prop}.

First, in 1966, Ikkai and Ooi[35] studied rabbit muscle actin (3.3 mg/ml) in a buffer with 2-mM tris-HCl and 0.5-mM ATP, at pH=8.0, at 25 °C and in the presence of 10- and 60-mM KCl. The extent of the polymerization under pressure was deduced from the measurements of flow birefringence. Increased pressure shifted the system toward the monomer, which implies that $(\partial \ln K_x/\partial P)_{T,c}$ in Eq. (5) is negative and therefore ΔV_{prop} must be positive. Equation (5) was used to calculate $\Delta V_t=+84$ ml/mol (no error estimate given). Compare the two values reported from the same group: the density measurement (discussed above[34]) yielded $\Delta V=+292$ ml/mol, whereas the pressure study yielded $\Delta V_{\text{prop}}=+84$ ml/mol. However, the shear stress necessary for the flow birefringence measurement in the pressure study could have broken the F-actin filaments and reduced the extent of polymerization.

The second indirect measurement of ΔV_{prop} was in 1985, when Swezey and Somero[47] reported a study of pressure effects on muscle actin from chicken, iguana, and several fishes, but not from rabbits. The extent of polymerization was measured by fluorescence labeling and/or DNase I inhibition assay, and Eq. (5) was used to calculate ΔV_{prop}. KCl at 100 mM was used to activate polymerization. Increased pressure shifted the system toward the monomer for every case; all values of ΔV_{prop} were positive, ranging from +2 to +139 ml/mol. The closest species to rabbit was chicken; rabbit muscle actin and chicken muscle actin differ by six amino acid replacements.[48,49] For chicken actin, ΔV_{prop} was found to be +63 ml/mol at 25 °C and +107 ml/mol at 4 °C (see Table I).

Third, in 1992, Garcia *et al.*[50] reported on the pressure effects for rabbit muscle actin, varying the activating salt and using fluorescence spectroscopy to follow the depolymerization upon pressurization. The actin (0.2 mg/ml) was in a buffer with 2.4-mM tris-HCl and 0.2-mM ATP, at pH=7 at 25 °C. The temperature and its control are not given, but probably was 25 °C. Activating salts were of three kinds: 2-mM $MgCl_2$/100-mM KCl (giving "Mg-F-actin"), 100-mM KCl (giving "K-F-actin"), or 2-mM $CaCl_2$ (giving "Ca-F-actin"). Equation (5) gave $\Delta V_{\text{prop}}=(+74\pm14)$ ml/mol for Mg-F-actin, $(+79\pm12)$ ml/mol for Ca-F-actin, and $(+328\pm63)$ ml/mol for K-F-actin.

The conclusion from these pressure studies that ΔV_{prop} is positive depends on the assumption that the conditions of the derivative $(\partial \ln K_x/\partial P)_{T,c}$ of constant temperature and compositions are met. Constant temperature can be assumed. The concentration that is most likely to vary under pressure is that of hydrogen ions, but the pH of the buffer tris-HCl, used by Ikkai and Ooi and by Garcia *et al.*, has no significant dependence on pressure, and thus the criterion of constant composition can be satisfied.[51]

In summary, of the three published density measurements, all using $MgCl_2$ to activate polymerization, one gives

a positive value of ΔV and two give negative values; both negative values were obtained using vibrating tube density meters. Of the three studies of pressure effects, all using KCl as the initiating salt, all three give positive values of ΔV_{prop}.

II. EXPERIMENTAL METHODS

A. General

We first measured the density of polymerizing actin in solution as a function of temperature with a vibrating tube density meter. We then measured the extent of polymerization of actin solutions by labeled fluorescence spectroscopy, as a function of time and temperature at fixed pressures and concentrations. In all experiments, the activating salt (KCl) was added to the actin in buffer at 0 °C in H_2O buffer, and then the temperature was increased stepwise.

B. Sample preparation

Muscle acetone powder was prepared from the dorsal lateral skeletal and hind leg muscles of freshly sacrificed, fully exsanguinated rabbits by the standard method of Pardee and Spudich,[28,29,52–54] to produce acetone powder. G-actin was isolated from the acetone powder by cycles of polymerization and depolymerization. Polymerization of the G-actin in buffer A (4-mM tris-HCl, *p*H 8 at 25.0 °C; 0.2-mM Na_2ATP; 0.005% NaN_3; 0.2-mM $CaCl_2$; and 0.5-mM dithiothreitol) was accomplished by raising the concentrations to 50-mM KCl, 2-mM $MgCl_2$, and 1-mM Na_2ATP. The resulting F-actin was then depolymerized by dialysis for 12 h in a 12 000 molecular-weight cutoff colloidion bag (Spectra-Por, Rancho Dominguez, CA), against 1 l of buffer A at 4 °C with rapid stirring. The G-actin was removed as supernatant by ultracentrifugation at 4 °C for 1.5 h at 180 000 g. This recycled G-actin was further purified by size exclusion chromatography with Sephacryl S-200 (Amersham Biosciences, Piscataway, NJ), which had been previously equilibrated with buffer A.[55,56] The purified G-actin was either used within hours of purification, or repolymerized in the manner described above and stored on ice. Stored purified F-actin was recycled and column purified (as above) just prior to use. The actin was never frozen or lyophilized.

G-actin concentrations were determined by ultraviolet absorption spectroscopy at a wavelength of 290 nm using the extinction coefficient[55,57] $\epsilon_{290}=0.63\ \text{mg}^{-1}\,\text{cm}^{-1}$. Final actin purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).[58] The gel analyses indicated final G-actin purities of better than 99%; a typical gel analysis is shown in Fig. 2.

For the fluorescence experiments, 3% by weight of the initial G-actin was labeled with pyrene. The preparation of the *N*-(1-pyrenyl)iodoacetamide labeled actin has been described previously.[28,29,59]

C. Density measurement: Vibrating tube density meter

The density was measured with a commercial vibrating tube density meter, Paar Model DMA602 (Anton Paar USA, Ashland, VA), for which the frequency was measured with a Hewlett-Packard 3456A frequency counter. The instrument was calibrated using both H_2O and D_2O.[60] The accuracy of the calibration was 15 ppm. The precision of the measurements was about 3 ppm within a run. The reproducibility for independently prepared samples at (nominally) the same salt concentration was limited by the reproducibility (1%–4%) of the sample preparation (e.g., the precision of the pipets), and not by the precision of the density meter.

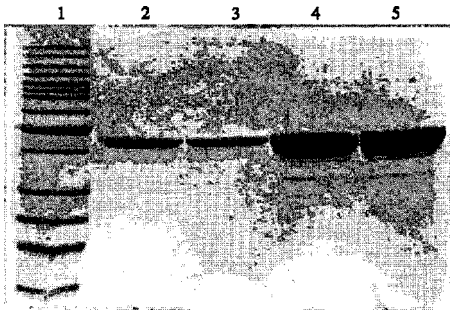

FIG. 2. SDS-PAGE gel analyses of the actin used for a sample of 3.0-mg/ml actin in buffer A with 15-mM KCl. The lanes are 1, markers, where the two bands bounding the actin bands are at 50 and 40 kD; 2, actin before density measurement, 5-g load; 3, actin after density measurement, 5-g load; 4, actin before density measurement, 40-g load; and 5, actin after density measurement, 40-g load. This gel compares well with actin gel analyses in the literature (see Ref. 54).

The temperature in the density meter was controlled by a circulating water/ethylene glycol bath with a range of −40–60 °C, and the temperature was measured by a calibrated thermistor in close proximity to the sample cell with an accuracy of 0.01 °C and a precision of 0.003 °C. The instrument was covered by insulating material to further reduce any thermal gradients and variations. For a temperature increase of 3 °C, the temperature bath took about 1 min to change, and the actual temperature near the cell required about 10 min to reach a steady value.

Attention was paid to air bubbles in the sample, to sample history, and to equilibration time. Once salt was added, the sample had to be kept below the floor temperature until the density measurements were begun. The samples were degassed just before injection into the density meter. The measurements were begun at low temperatures and advanced slowly to higher temperatures, because once polymers form, they are very slow to depolymerize.[52] Previous work in our laboratory[28,29] has shown that rabbit muscle actin, at these concentrations of salt and actin, requires about 30 min to reach the equilibrium extent of polymerization. We held the samples for 2 h at each temperature in order to be sure of full equilibration.

As discussed above, for the Paar DMA602 density meter, a change in viscosity can result in a systematic error in the density measurement, due to viscous damping of the vibrating tube. The viscosity of 1-mg/ml actin, in buffer at various concentrations of KCl, changes by about 100-fold during the polymerization.[28,29,42] Fitzgerald *et al.*[40] assessed the Paar model DMA55, which employs the same cells as the DMA602, and found that for a viscosity of 100 mPa s, the error in the density is +0.0006 g/ml; for a viscosity of 1000 mPa s; the error is about +0.0007 g/ml; between 800 and 4000 mPa s, the error levels off at +0.000 75 g/ml. We

measure (see below) a change in density during the polymerization of 3-mg/ml actin of +0.0005 g/ml, so the apparent increase in density due to viscous damping was of considerable concern. Correction equations are available[40] if the viscosity values are known, but there have been no quantitative studies of the viscosity of actin at these concentrations and temperatures.

The cell of the vibrating tube density meter is made of Pyrex glass. Pyrex glass can cause the polymerization of salt-free actin samples because small amounts of sodium ions can leach from the glass.[61] We were careful to rinse the cell with the buffer several times. We do not consider this effect to be a problem for most of our samples because the added KCl was present in far larger amounts' than was any leached Na^+. The only samples where this was not the case are the samples of G-actin with no added salt, and there we see some small effects attributable to leached sodium (see Results).

For each sample, the density was measured as a function of temperature for that sample of actin in buffer with salt, and the density was also measured as a function of temperature for an aliquot of the very same buffer used for the actin sample, with the addition of salt to the same concentration as in the actin sample but without any actin.

D. Fluorescence spectrometry

Fluorescence measurements were made with the ISS PC1 Spectrofluorimeter, KOALA model (ISS, Champaign, IL). The lamp is a Perkin-Elmer Compact Xenon Arc Lamp and the slit width is 0.5 mm. There is a 365 (±10)-nm bandpass filter in front of the excitation monochromator and a 405 (±10)-nm bandpass filter between the cell and the photomultiplier tube. Measurements at atmospheric pressure (0.1 MPa) were taken in the ISS three-position sample compartment, with 600-μl quartz cuvettes.

The ISS HP200 pressure sample compartment operates at temperatures from −40 to +60 °C and at pressures from 1 to 3000 bars. A round quartz cuvette with a plastic cap is used as the sample cell for the high pressures.[62] The cuvette holds 0.80 ml, but the cap, which holds 0.4 ml, must also be filled to allow for transfer of pressure from the hydrostatic fluid to the sample though the cap. The total sample volume is therefore 1.2 ml. In the pressure cell, the sample cell is surrounded with spectroscopic-grade ethanol as the hydrostatic fluid. A manual pump compresses the fluid. The pressure is measured by an Astragage Bourdon gauge, with a range of 0–550 MPa and a resolution of ±1 MPa. If the pressure changes during a run due to thermal expansion of the ethanol, then the pressure must be manually reset to the desired value. It is known that ATP-actin does not denature under pressure until the pressure reaches 150 MPa.[35]

The temperature in the spectrometer was controlled by the same circulating water/ethylene glycol bath discussed above for the vibrating tube density meter, and the temperature was again measured by a calibrated thermistor in close proximity to the sample cell with an accuracy of 0.01 °C and a precision of 0.003 °C. The instrument was covered by insulating material to further reduce any thermal gradients and

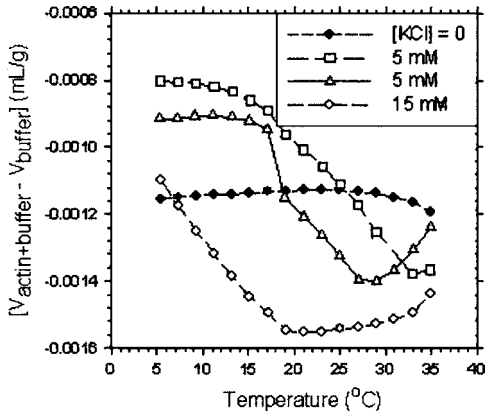

FIG. 3. Measurements made in a vibrating tube density meter of the difference between the specific volume (in ml/g) of $[G]_0$=3.0 mg/ml rabbit muscle actin in aqueous buffer, and the specific volume of the buffer alone, for various concentrations of KCl (see legend), as a function of temperature.

variations. For a 3° temperature increase, the temperature bath took about 1 min to change, and the actual temperature near the cell required about 10 min to reach a steady value.

After the final measurement of a run, $MgCl_2$ was added to the sample to a final concentration of 15 mM to completely polymerize the actin and the signal of maximum polymerization (I_{max}) was measured. The extent of polymerization $\Phi(t,T)$ is calculated from

$$\Phi(t,T) = I(t,T)/I_{max}, \tag{6}$$

where $I(t,T)$ is the intensity measured as a function of time and temperature. No correction was made for the intrinsic fluorescence of free G-actin because it is a small correction (4%) that is within the systematic errors due to sample inconsistencies.[28,29]

III. RESULTS

All experimental uncertainties are reported at the level of one standard deviation.

A. Density measurement: Vibrating tube density meter

For each sample and at each temperature, we measured (1) the density, $D_{actin+buffer}$, of the actin in aqueous buffer with KCl and (2) the density, D_{buffer}, of the buffer with KCl but without actin. The measurements of $D_{buffer}(T)$ were fitted by a polynomial in the temperature, and that equation was then used to calculate D_{buffer} at the temperature of each measurement of $D_{actin+buffer}$. We inverted the density data to obtain the respective specific volumes $V_{buffer}(T)$ and $V_{actin+buffer}(T)$ and then took the difference $[V_{actin+buffer}(T) - V_{buffer}(T)]$, which should equal ΔV_{prop}.

Figure 3 shows $[V_{actin+buffer}(T) - V_{buffer}(T)]$ as a function of temperature. Note that the total range of the left ordinate is only one part in a thousand of the volume of the buffer. One curve in Fig. 3 corresponds to the sample of G-actin in buffer with no salt: In the absence of salt, very little happens to the G-actin as the temperature is changed; the slight curvature of the data is probably due to a small amount of polymerization caused by the presence of a minute amount of Na^+, leached

from the Pyrex walls of the density meter cell.[61] The other data in Fig. 3 are for three samples of actin in buffer with salt. Two of the samples were independently prepared from different rabbit sources, but had the same nominal KCl concentration of 5 mM. The two 5-mM KCl curves are offset by about 0.0002 ml/mg, or about 200 ppm of the total solution volume, which is good reproducibility for protein samples.

Our earlier work[28,61] indicated that T_p for 3-mg/ml actin with 5-mM KCl is around 20 °C, consistent with a point of inflection in $[V_{\text{actin+buffer}}(T) - V_{\text{buffer}}(T)]$ in Fig. 3 at about 20 °C. Our earlier work also indicated that above T_p, at about 23 °C for 3 mg/ml with 5-mM KCl, $\Phi(T)$ decreases: This depolymerization is reflected in the $[V_{\text{actin+buffer}}(T) - V_{\text{buffer}}(T)]$ data for 5-mM KCl in Fig. 3 in that, after first decreasing, $[V_{\text{actin+buffer}}(T) - V_{\text{buffer}}(T)]$ then begins increasing at about 27–32 °C. For the sample with 15-mM KCl, the data in Fig. 3 show the same trends, consistent with the data at 15-mM KCl in Fig. 1, where T_p is at 10 °C and a reversal occurs at higher temperature. Thus the density data from the vibrating tube density meter have temperature profiles that reflect the measured behavior of $\Phi(T)$.

However, the decrease in $[V_{\text{actin+buffer}}(T) - V_{\text{buffer}}(T)]$ upon polymerization indicates a negative change in ΔV_{prop}. For example, the data at 5-mM KCl (open triangles in Fig. 3) give ΔV_{prop} to be about -9×10^3 ml/mol (see Table I). We believe that the values of these measurements cannot be trusted. Following the work of Fitzgerald *et al.*,[40] we tested the Paar DMA602 by measuring the densities of seven standard oils (Cannon Instrument Co., State College, PA) of known densities and viscosities. The densities varied from 0.866 to 0.879 g/ml and the viscosities varied from 14 to 585 mPa s. We found significant errors in the oil densities measured by the vibrating tube density meter when the viscosities were greater than 14 mPa s. The magnitude of the error increased with the viscosity: 600-ppm error at 200 mPa s, and 800-ppm error at 585 mPa s. The error in the density was such as to give a measured density higher than the true density. If the apparent density is too high, then the apparent volume is too low, which can explain the negative volumes of polymerization seen by every attempt to study the volume change of polymerization of actin using a vibrating tube density meter, including those listed in Table I and including our own data in Fig. 3. More recent models of vibrating tube density meters may have corrected this problem.

Figure 3 does follow Fig. 1 in showing changes with temperature, but the vibrating tube density meter is detecting the viscosity changes with polymerization and depolymerization, and is not measuring just the density changes. We conclude that we cannot measure the density change during actin polymerization by using the earlier models of the vibrating tube density meter. Our experiments indicate that the studies in Table I that used vibrating tube density meters (2, 3, and 7), all of which resulted negative values of the volume change, must be discounted.

TABLE II. Values of the polymerization temperature, T_p, the temperature of the maximum in $\Phi(T)$, $T_{\max}$, and the temperature of depolymerization, T_d.

Transition temperature (°C)	$P=0.1$ MPa	$P=10$ MPa	$P=20$ MPa
T_p	13±1	15±2	19±2
$T_{\max}$	19±1	22±2	28±2
T_d	26±2	35±2	34±2

B. Extent of polymerization measurements at three pressures, as functions of time and temperature

All the $\Phi(t,T)$ data discussed here are available for further analysis.[53]

1. P=1 atm (0.1 MPa), $[G]_0$=3.1 mg/ml, [KCl]=15 mM, H_2O *buffer*

Figure 1 (solid circles) shows the equilibrium $\Phi(T)$ data from this run as compared to the data from earlier work.[28] The agreement between the two data sets, taken about four years apart and by two different workers, is quite satisfactory. The new data extend to higher temperatures and give evidence of a second inflection point beyond the maximum in $\Phi(T)$, an inflection point that corresponds to a depolymerization temperature, T_d. See Table II for the values of T_p, $T_{\max}$, and T_d.

In this run and the runs described below, we also studied the response of the system to the reversal of the temperature step—that is, to a decrease in temperature. In all cases, we observed considerable hysteresis in the behavior of the actin system upon cooling. As reported before,[52] polymerized actin does not depolymerize on the same time scale (30–60 min) as it polymerizes—much longer times seem to be necessary for depolymerization. We do not show those measurements here, but they are available.[53]

Figure 4(a) shows Φ as a function of time after a positive temperature jump of 2 °C, at several temperatures near $T_{\max}$. Recall that the first 600 s are needed for the temperature to reach equilibrium. Then $\Phi(t)$ increases for temperatures below $T_{\max} \approx 19$ °C, remains constant near $T_{\max}$, and decreases above $T_{\max}$. The initial rates of polymerization were calculated by converting from $\Phi(t)$ to the amount of free monomer, $[G]$, remaining from the initial concentration of G-actin, $[G]_0$:

$$[G](t) = [G]_0\{1 - \phi(t)\}. \tag{7}$$

The overall plots of $[G](t)$ are not simple exponential functions and the interpretation of the complete relaxation times is not straightforward. As a first approach to the data, we consider just the initial rates of reaction. For the 10 min following the temperature equilibration period, $[G]$ is nearly linear in t and the slope $-d[G]/dt$ is taken as the initial rate of reaction, r_p. Figure 4(b) shows r_p as a function of temperature, as compared to $\Phi(T)$. It is interesting that $r_p(T)$ correlates with $\Phi(T)$: $r_p(T)$ shows a maximum at T_p, goes through zero at $T_{\max}$, and then shows a minimum at T_d.

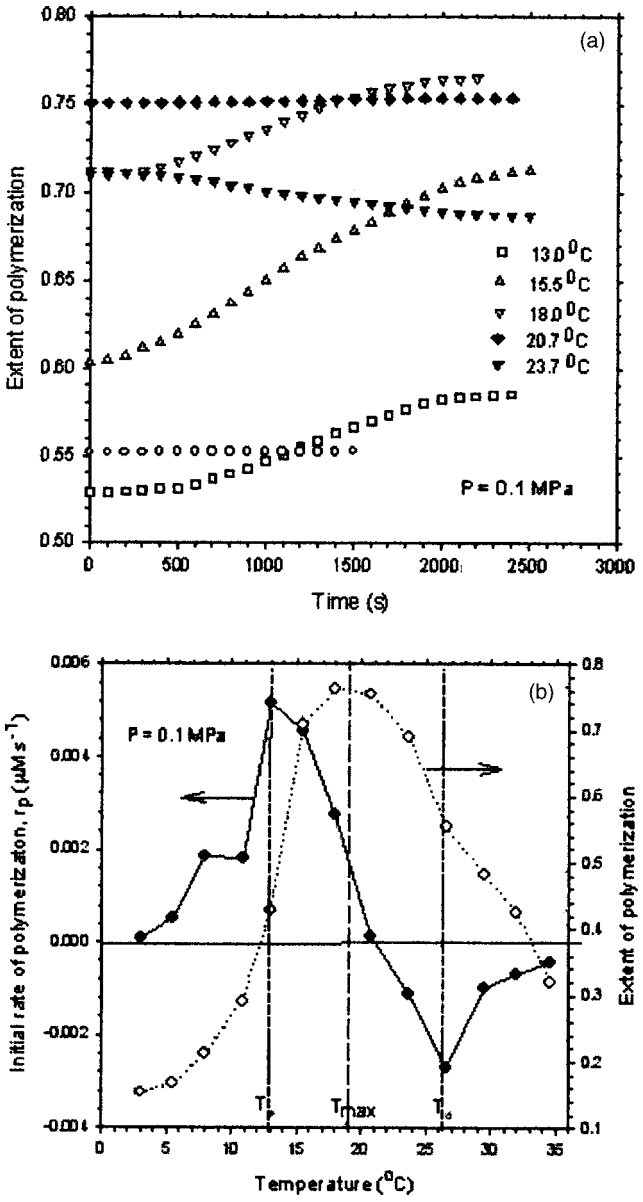

FIG. 4. Kinetics of actin polymerization at 0.1 MPa, [KCl]=15 mM, $[G]_0$ =3.1 mg/ml in H_2O buffer: (a) Relaxation of the extent of polymerization, $\Phi(T,t)$; (b) initial rate of polymerization of G-actin, r_p, as a function of temperature, as compared to the extent of polymerization, $\Phi(T)$. The error bars for r_p are smaller than the symbols.

2. P=10 *and* 20 MPa, $[G]_0$=3.1 mg/ml, [KCl]=15 mM, H_2O *buffer*

Figure 5(a) shows $\Phi(T)$ at 10 and 20 MPa, as compared to the data discussed above at 0.1 MPa in H_2O buffer. We note the following from Figs. 5(a) and 5(b) and Table II.

(1) T_p increases as P increases, which means, from Eq. (4), that ΔV_{prop} must be positive. We obtain dT_p/dP $=(0.3015\pm0.0009)$ K/MPa$=(30.15\pm0.09)$ mK/atm.

(2) $T_{\max}$ increases as P increases.

(3) T_d increases between 0.1 and 10 MPa, but then is the same within error at 20 MPa. For the values of T_d at 10 and 20 MPa, we have drawn upon the initial rate measurements (discussed below) since $\Phi(T)$ did not clearly show points of inflection.

(4) The values of $\Phi(T)$ at all T are higher at 10 and 20 MPa than at 0.1 MPa. The values of $\Phi(T)$ are particularly high below T_p, where only dimerization is expected to take place. The values of Φ at the lower temperatures increase when the pressure is increased from 0.1 to 10 MPa, then decrease when the pressure is further increased to 20 MPa, but the difference at the higher pressure is close to the expected reproducibility.

The initial increase in the extent of dimerization with P suggests that the change of volume for dimerization is *negative* at 0.1–10 MPa. We can estimate the ΔV_{dimer} at 5.5 °C, a temperature at which there is no significant propagation even at 0.1 MPa, using Eq. (5) and the 0.1- and 10-MPa data; we obtain $\Delta V_{\text{dimer}}=-307\pm10$ ml/mol. It is possible that ΔV_{dimer} changes sign between 10 and 20 MPa, but we hesitate to interpret these data too closely. We are aware of no previous measurements of the change of volume for the dimerization of actin.

(5) We can also use Eq. (5) to estimate ΔV_{prop}, noting that the pH can be taken as constant because the tris-HCl buffer shows a negligible dependence of pH on pressure.[51] Below $T_{\max}$ there is no temperature at which all three runs in Fig. 5(a) show polymerization and only polymerization occurring. When the polymerization is occurring at 0.1 MPa, no polymerization is occurring at 10 or 20 MPa. When polymerization occurs at the higher pressures, depolymerization is happening at 0.1 MPa. Thus we estimate ΔV_{prop} from the data above T_p, at 301, 304, and 307 K, where depolymerization happens for all the samples; the change of volume obtained is that for depolymerization, which just has the opposite sign from the change of volume for polymerization. In Eq. (5), since $K_x=[A_{l+1}]/[A_1][A_l]\approx 1/[A_1]=1/[G]$, then $\ln K_x$ is taken as $-\ln[G]$. $[G]$ is obtained using Eq. (7). Figure 5(c) shows $K_x(P)$ for the data in Fig. 5(a). The resulting values of ΔV_{prop} are $+464\pm63$ ml/mol at 301 K, $+415\pm57$ ml/mol at 304 K, and $+324\pm63$ ml/mol at 307 K. Since the error bars represent one standard deviation, ΔV_{prop} does not change with temperature within a 99% confidence interval.

The initial rates of reaction at 10 and 20 MPa are shown in Figs. 5(d) and 5(e). As for the sample at 0.1 MPa [Fig. 4(b)], the behavior of the initial rate is correlated to the behavior of the extent of polymerization and supports the presence of T_d.

IV. DISCUSSION AND CONCLUSIONS

The response of polymerizing actin to changes in pressure as a thermodynamic variable has been used to measure changes in volume. The changes in volume have implications for the reaction mechanism, as discussed below. A knowledge of the effects of pressure differences on actin assembly may also be useful in understanding the effects of hydrostatic pressure on cell morphology,[63] the effects of intravascular pressure on cell structure,[64] the cytoskeletal structures of species that exist under high ambient pressures,[65] and the response of cells to changes in gravitational force.[66]

These new measurements of the extent of polymerization as a function of temperature at fixed pressures and con-

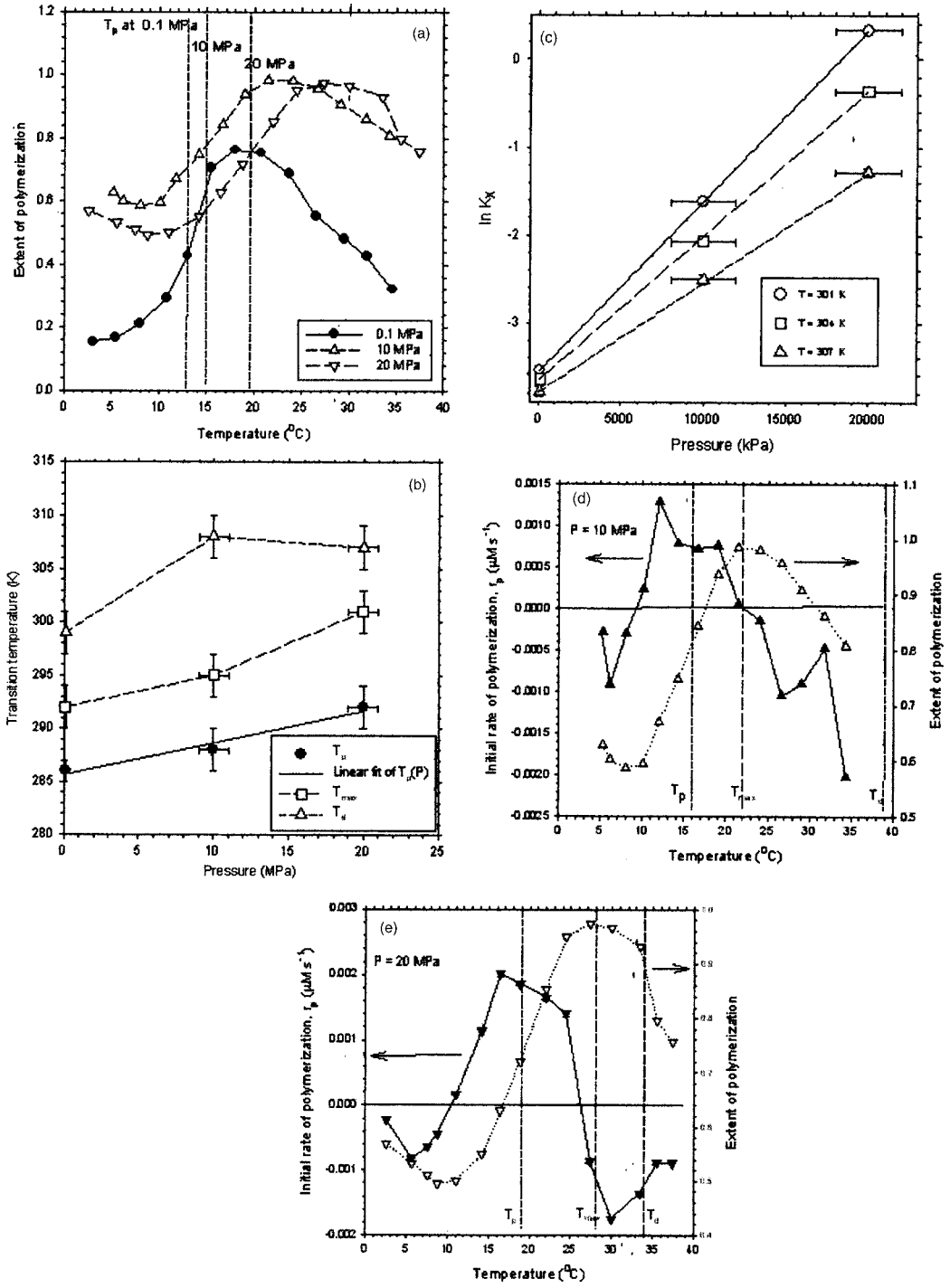

FIG. 5. (a) Extent of polymerization, $\Phi(T)$, at 0.1, 10, and 20 MPa, [KCl]=15 mM, $[G]_0$=3.1 mg/ml in H_2O buffer; (b) dependence of T_p, T_{max}, and T_d on pressure, where the solid line is a linear fit of $T_p(P)$; (c) $\ln K_{prop}$ as a function of pressure at three temperatures; (d) initial rate of polymerization, r_p, as a function of temperature, as compared to $\Phi(T)$, at 10 MPa; (e) initial rate of polymerization, r_p, as a function of temperature, as compared to $\Phi(T)$, at 20 MPa. For (d) and (e), the error bars for r_p are smaller than the symbols.

centrations, near the polymerization line of rabbit muscle actin at low KCl concentrations, agree with our previous measurements at 1 atm (Refs. 28 and 29) in that all experiments indicate the onset of polymerization at a polymerization or "floor" temperature, and all experiments indicate a reversal of the polymerization at a temperature of about 15 °C above the polymerization temperature. The measurements of the initial rates of polymerization show behavior that is correlated to the extents of polymerization and that support the presence of reentrant depolymerization at the higher temperatures. The cause of this high-temperature reversal of the polymerization has been suggested to be a competition between the floor temperature for the propagation step of the polymerization and a floor temperature for the activation step of the polymerization.[28,29]

We measure the dependence of the polymerization temperature on pressure to be $dT_p/dP=(0.3015\pm0.0009)$ K/MPa$=(30.15\pm0.09)$ mK/atm. From the pressure depen-

dence of the extent of polymerization, we find that the sign of the ΔV_{prop} for rabbit muscle actin in positive, consistent with what has been reported for other entropically driven processes.[23] The average of three measurements is $\Delta V_{\text{prop}} = +401 \pm 48$ ml/mol at 31 °C, where the uncertainty is one standard deviation. For comparison, ΔV, the change in volume for all the steps of the polymerization in tobacco mosaic virus is about +413 ml/mol,[23] and in flagellin is $+157 \pm 4$ ml/mol.[67] From data below T_p, we determine that for the dimerization of actin, $\Delta V_{\text{dimer}} = -307$ ml/mol at 5.5 °C.

We have reviewed the literature on the change of volume for actin propagation. In Table I, measurements 2, 3, and 7 have to be dismissed because vibrating tube density meters are sensitive to viscosity changes and thus yield biased measurements of the density of polymerizing actin. Measurement 4 used flow birefringence to detect polymerization, a method that applies shear to the sample and could affect the extent of polymerization. Measurement 5 is on chicken muscle actin, not rabbit muscle actin. Thus the best measurements in Table I on rabbit muscle actin are 1 (Ref. 34) and 6 (Ref. 50), which agree rather well with our new measurement, 8. Measurement 1 is for the total volume change ΔV; while 6 and 8 are for the volume change for propagation, ΔV_{prop}. Measurement 1 used $MgCl_2$ as the initiating salt, whereas measurements 6 and 8 used KCl as the initiating salt; Garcia *et al.*[50] found (see discussion above under Sec. I B) that $MgCl_2$ and $CaCl_2$ give smaller values of ΔV_{prop} than does KCl. Note also that measurements 6 and 8 are at rather different [KCl].

We can use the value $\Delta V_{\text{prop}} = +401 \pm 48$ ml/mol and $dT_p/dP = 0.3015$ K/MPa = 30.15 mK/atm, in Eq. (4) to estimate ΔH_{prop} at [KCl] = 15 mM and $[G]_0 = 3.1$ mg/ml. We obtain $+380 \pm 46$ kJ/mol. A fit of a lattice model to the $\Phi(T)$ data for such samples has given $\Delta H_{\text{prop}} = +180$ kJ/mol,[28] differing significantly, but the model did have six correlated free parameters.

At the microscopic level, what in the mechanism of the polymerization of actin can cause ΔV_{prop} to be positive?

(1) Release of water of hydration. The current explanation of entropically driven processes, including the polymerization of actin, is that the increase in entropy comes from the release of bound water during the polymerization.[3,23,24] A hydration layer of water is bound to the actin. It is assumed that the bound water is denser than the released water, and thus that the volume will increase due to the released water.

(2) ATP hydrolysis. When ATP is present in the actin buffer, the ATP is hydrolyzed to ADP during the polymerization. The volume change associated with this reaction is about −19 ml/(mol of ATP),[26] and 1 mol of ATP is hydrolyzed when 1 mol of actin is polymerized, so this is a negative change in the volume during polymerization.

(4) Hydrophobic interactions. The G-actin monomers aggregate to form F-actin polymers by noncovalent hydrophobic interactions. These interactions may themselves cause a volume change. The volume change for the coming together of two hydrophobic entities is thought to be negative.[68]

(5) Packing efficiency. Actin, tubulin, and tobacco mosaic virus form semiflexible polymers. As the polymer chains grow longer, their packing can be expected to be less efficient, leading to a volume increase.[27–29]

We note the comment of Israelachvili and Wennerstrom[69] that "macromolecular associations in water [depend] on a competition between solute-solute, solute-solvent, and solvent-solvent bonds (including hydrogen bonds), the outcome of which is generally not obvious or simple to analyze."

ACKNOWLEDGMENTS

This work was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases, a part of the National Institutes of Health (AR45191), and by the Chemistry Division of the National Science Foundation. We thank Xin Wen and Michael L. Alessi for assistance at various stages of this project, and Jack F. Douglas for helpful discussions. One of the authors (J.F.) thanks Kuan Wang, Chief of LMB, NIAMS, and NIH, for encouragement and support in this project. Another author (S.G.) acknowledges the support of the University of Maryland College Park, Division of Research and Graduate Studies.

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