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Pressure Effects on Actin Self-Assembly: Interspecific Differences in the Equilibrium and Kinetics of the G to F Transformation†

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ABSTRACT: Purified skeletal muscle actins from species whose ambient pressures range from 1 to >500 atm were examined for the sensitivity to hydrostatic pressure of the globular (G) to filamentous (F) self-assembly reaction. Both the equilibrium position and the kinetics of self-assembly were affected by pressure. Increased pressure shifted the self-assembly equilibrium toward the monomer (G) state and reduced the rate of F-actin assembly. For most of the actins studied, the perturbation by pressure of F-actin formation decreased with increasing measurement of pressure, indicating that F-actin has a higher compressibility than G-actin. The increase in system volume and compressibility concomitant with the assembly of F-actin can be interpreted as reflections of the major role played by hydrophobic effects in stabilizing F-actin and of the existence of "hard" binding sites, in the terminology of Torgerson et al. [Torgerson, P. M., Drickamer, H. G., & Weber, G. (1979) *Biochemistry 18*, 3079-3083], in the actin subunits. For actin from the deepest occurring species studied, the teleost fish *Coryphaenoides armatus*, which occurs to depths of approximately 5000 m (equivalent to 501 atm of pressure), there was no difference in compressibility between G-actin and F-actin; that is, the effect of increasing pressure on self-assembly was linear over the entire pressure range examined, 600 atm. The self-assembly reaction of the actin from *C. armatus* also differed from that of the other actins examined in that the G to F equilibrium was relatively insensitive to increased pressure; i.e., the volume change (ΔV) of assembly was small. The changes in enthalpy (ΔH) and entropy (ΔS) accompanying self-assembly of *C. armatus* actin also were small, relative to the other actins examined [Swezey, R. R., & Somero, G. N. (1982) *Biochemistry 21*, 4496-4503]. Pressure effects on the kinetics of self-assembly were greater for this pressure-adapted actin than for actin from rabbit muscle, however. The actin from a shallower occurring congener of *C. armatus*, *Coryphaenoides acrolepis* (depth of occurrence of 250-2100 m), displayed responses to pressure similar to those of actins from shallow-living fishes, an observation which suggests that pressure effects on actin assembly may not become selectively important until pressures in excess of 200 atm are reached. On the basis of the calculated volume changes and the changes in protein compressibility upon self-assembly, we propose that the interspecific variations in the effects of pressure on the G to F transformation may result either from differences in the amount of hydrophobic effect contributing to the stabilization of F-actin or from varying degrees of "hardness" of the subunit contact sites in F-actin. In addition, for actin of *C. armatus*, we propose that concomitant with the assembly of F-actin there is an increase in exposure to solvent of polar or charged residues. Hydration of these residues during self-assembly would reduce the enthalpy, entropy, and volume changes accompanying self-assembly and would contribute to the negative free-energy change of self-assembly, thereby compensating for a lower contribution by the hydrophobic effect in this actin self-assembly reaction. The low ΔH, ΔS, and ΔV of self-assembly of *C. armatus* actin, and the similar ΔG of self-assembly relative to the other actins studied, support this model.

Despite the fact that more than three-fourths of the biosphere, when viewed in terms of volume (Childress, 1982), consists of environments where hydrostatic pressures lie in the range of several hundred atmospheres, the influences of pressure on biochemical systems, and the needs for adaptation to high pressure by deep-living organisms, remain poorly understood [cf. Jaenicke (1983), Somero et al. (1983), and Hochachka & Somero (1984)]. There are, in fact, two strong reasons for examining the influences of pressure on biochemical systems. First, such studies may lead to a closer understanding of the processes of molecular evolution, by which the bio-

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chemical systems of organisms are adapted to function under the different environmental conditions they encounter. There is evidence that adaptations of enzymes to pressure can play important roles in conferring tolerance to pressure and in establishing depth distribution patterns in the ocean (Siebenaller & Somero, 1978, 1979; Somero & Siebenaller, 1979; Somero et al., 1983; Siebenaller, 1984). Second, pressure, like temperature, can serve as a useful tool for analyzing the structural and functional attributes of macromolecular systems. For example, the study of proteins under high pressures has revealed the magnitudes of the volume and compressibility changes that accompany alterations in protein structure, and these data have been very important in the development of theories about the factors that stabilize the native structures of proteins (Brandts et al., 1970; Hawley, 1971; Zipp & Kauzmann, 1973) and contribute to the understanding of the catalytic efficiencies of enzymes (Low & Somero, 1975a,b; Greaney & Somero, 1979; Carter et al., 1978; Morild, 1981).

The present study, which examined the effects of elevated hydrostatic pressure on the globular (G) to filamentous (F) self-assembly of skeletal muscle actins purified from vertebrates adapted to widely different pressures and temperatures, was conducted with both of these objectives in mind. We sought to determine whether the self-assembly of actin, which earlier studies had suggested is strongly perturbed by increases in pressure (Ikkai & Ooi, 1966; Ikkai et al., 1966), was differently pressure sensitive for actins from animals adapted to 1-atm pressure and to pressures of up to approximately 500 atm. As in the case of other protein self-assembly reactions that have been studied under conditions of elevated hydrostatic pressure, e.g., tubulin (Salmon, 1975), myosin (Josephs & Harrington, 1968; Davis, 1981), fibrin (Collen et al., 1970), lactate dehydrogenase (Jaenicke, 1983), and enolase (Paladini & Weber, 1981), these earlier studies of pressure effects on actin involved only organisms adapted to 1-atm pressure. Thus, while the apparently high sensitivities of mammalian muscle actin to pressure suggest that adaptive modifications of actin assembly reactions in deep-living organisms are apt to be an important component of evolution in the deep sea, no data were available to test this conjecture. The second objective of our studies was to gain additional information about the types of bonding events and energy changes that are instrumental in stabilizing F-actin. Using the information we obtained on the pressure sensitivities of actin self-assembly, we wished to compute the changes in system (actin plus solvent) volume and actin compressibility during the G to F transformation and use these derived values to develop a hypothesis concerning the types of interactions that are important in stabilizing filamentous actin. This analysis was designed to provide additional insights into the stabilization forces common to all actin self-assembly reactions and to reveal how variations in these stabilizing forces, e.g., in the different types of noncovalent bonds used to form F-actin, adapt actins for self-assembly under widely different conditions of pressure and temperature.

Materials and Methods

Experimental Animals. Skeletal muscle actin was purified from the following species of vertebrates and used in the studies of F-actin assembly; body temperatures and, where relevant, depths of occurrence [values from Somero et al. (1983)] are given in parentheses: chicken (39 °C); desert iguana (*Dipsosaurus dorsalis*) (30–47 °C); and the teleost fishes (*Sebastolobus alascanus* (3–10 °C; 180–440 m), *Coryphaenoides acrolepis* (2–3 °C; 250–2100 m), *Coryphaenoides armatus* (2–3 °C; 1900–4800 m), and *Halosauropsis macrochir* (2–3 °C; 1500–5200 m). All specimens were killed and frozen shortly after capture and then held at −80 °C until used for actin purification.

Actin Purification. Acetone powders of the muscles dissected from the frozen specimens were prepared by the "low-salt EDTA" procedure of Strzelecka-Golaszewska et al. (1980). Actin was then purified from these powders by the method of Spudich & Watt (1981) to greater than 95% homogeneity, as judged by the sodium dodecyl sulfate–polyacrylamide (SDS–polyacrylamide)[1] gel electrophoresis method of Weber & Osborn (1969).

Preparation of Fluorescently Labeled Actins. Actins purified from chicken and *C. armatus* muscles were chemically modified by first reacting the proteins with *N*-ethylmaleimide (NEM)[1] to block available sulfhydryl groups and then reacting the NEM–actins with NBD-Cl to produce the fluorescent actin monomers (NBD-actin). All procedures were carried out as described by Detmers et al. (1981). These modifications do not affect the critical monomer concentration (C_c), the concentration of native G-actin that remains unpolymerized in the presence of F-actin, for actin polymerization, or the ability of F-actin to stimulate myosin ATPase activity (Detmers et al., 1981). The intensity of fluorescence at 530 nm when excited at 470 nm approximately doubles when the monomers become assembled into filaments (Detmers et al., 1981).

High-Pressure Studies. Two experimental approaches were taken to assess the effects of high pressure on the polymerization of actin. In one procedure, assembly was induced in the samples of unmodified G-actin [0.2 mg/mL in buffer G (5 mM imidazole hydrochloride, pH 7.0 at 20 °C, containing 0.1 mM ATP, 0.2 mM $MgCl_2$, 0.2 mM DTT, and 10 mg/mL NaN_3)] by the addition of KCl to 0.1 M. After the addition of KCl, the sample was sealed in a glass vial with a rubber septem. This vial was then placed into a stainless-steel pressure bomb, and the sample was quickly brought to the desired pressure by using a manually operated hydraulic pump. The pressurized sample was then incubated overnight at 4 °C and depressurized, and then the C_c at the elevated pressure was determined by assaying the amount of native monomeric actin in the sample, according to the DNase I inhibition assay of Blikstad et al. (1978), as modified by Swezey & Somero (1982a), at various times after release of pressure (see Results). We have previously shown that this assay measures only the actin in a solution that is competent to polymerize (Swezey & Somero, 1982a).

The second procedure for following actin polymerization under pressure involved use of the NBD-actin samples and a sensitive fluorometric procedure. The high-pressure cell used in the fluorescence spectrometer (Perkin-Elmer Model MPF-44A) was similar in design to the cell described by Mustafa et al. (1971), except that there was a third sapphire window added to the cell to allow measurement of light emitted at 90° to the incident beam. The pressure cell had a sample volume of 4.6 mL, it was maintained at the desired temperature by a circulating water bath, and its internal pressure was established by a manually operated hydraulic pump, using high-grade mineral oil as the pressurization fluid.

Measurement of actin self-assembly in the pressure fluorescence cell was carried out as follows. NBD-actin (4.14 mL, 0.04–0.40 mg/mL in buffer G) was placed into the cell, and the temperature of the sample was allowed to reach equilibrium. KCl (0.46 mL, 1 M in buffer G) was added to

[1] Abbreviations: DTT, dithiothreitol; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole; NEM, *N*-ethylmaleimide; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

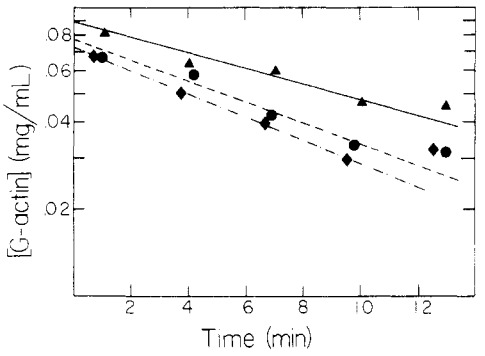


FIGURE 1: Determination of G-actin concentration (in milligrams per milliliter; the critical monomer concentration, C_c) as a function of time after depressurization. Solutions of chicken muscle actin (0.20 mg/mL) were incubated overnight at 4 °C at pressures of 200 (♦), 400 (●), and 600 atm (▲). The DNase I method (see Materials and Methods) was used to measure the concentration of G-actin ($=C_c$) in the solutions at different times after release of pressure. The decrease in C_c following release of pressure indicates reassembly of F-actin at 1-atm pressure. Assuming that the repolymerization is a monophasic function, extrapolation of the plot of G-actin concentration vs. time after pressure release to time = 0 yields the G-actin concentration in the solution under the particular incubation pressure.

the sample, and the solution was mixed. The pressure cell was quickly capped and brought to the desired pressure within 20 s of addition of the KCl. The assembly of actin was followed by recording on a strip chart recorder the increase in sample fluorescence accompanying polymerization. At the end of an experiment, an aliquot of the solution was removed from the cell and centrifuged at 179000*g* for 1 h to sediment the F-actin. Analysis of the protein concentration before and after centrifugation yielded the final value for the concentration of F-actin. In this manner, the changes in fluorescence with time could be quantitatively correlated with the amount of actin that had polymerized.

RESULTS

Pressure Effects on Actin Assembly. (A) DNase I Method. The equilibrium established at high pressures for assembly of actin will be perturbed by the decompression of the samples which is necessary for the implementation of the DNase I assay of G-actin content in these samples. The relaxation of the equilibrium to its state at 1 atm was logarithmic in its dependence on time. Thus, the G-actin content of the pressurized actin samples was measured at four times following decompression, and semilogarithmic plots of these values vs. time were constructed. Extrapolation of these plots to the ordinate thus yields the G-actin concentration that was present, under equilibrium conditions, at the experimental pressure. Figure 1 shows a typical set of data collected by using actin purified from chicken muscle. For all of the actins examined, the monomer content decreased with time following decompression, indicating that high pressure shifted the equilibrium between G- and F-actin toward G-actin. The extent of this decrease in G-actin content was species dependent, however, as discussed below.

Quantitative evaluation of the G to F equilibrium was carried out as follows. The nucleated condensation model of actin polymerization (Oosawa & Kasai, 1971) assumes that the equilibrium constant for assembly, K, is equal to the reciprocal of the monomer concentration in equilibrium with the actin filaments, the critical actin concentration, C_c. Although this model for the actin self-assembly equilibrium is widely accepted and used, there has been some question as to whether actin assembly truly is at equilibrium or whether it is a steady-state process since the subunit which dissociates from the polymer, G-ADP–actin, is not the same as the subunit which adds to the growing polymer, G-ATP–actin. However, a number of studies indicate that the values of C_c are not affected by the nucleotide which is bound to the monomer (Kasai et al., 1965; Cooke, 1975; Pardee & Spudich, 1982). Furthermore, under the physiologically realistic solution conditions used in our studies, Pardee et al. (1982) demonstrated that there was negligible exchange of actin subunits between the monomer and polymer pools. Such an exchange would be expected if there were different values for the equilibrium constants at the two ends of the actin filaments (Wegner, 1976). Finally, the constancy of C_c in solutions containing different amounts of F-actin ends (generated by sonication), but identical total actin concentrations, is strong evidence favoring the nucleated condensation model for actin assembly (Oosawa & Kasai, 1971). Therefore, the application of equilibrium thermodynamics to the actin assembly process remains valid, and the association constant for assembly is defined by $K = 1/C_c$.

The equation describing the pressure dependence of K is $K(p)/K(1\text{ atm}) = \exp[-P\Delta V(p)/RT]$ where $\Delta V(p) = V_F(p) - V_G(p)$, i.e., the difference between the volumes of filamentous (V_F) and globular (V_G) actin at pressure p, where the volumes include both those of the proteins and those of their water of hydration. The volume of any species shows a dependence on pressure given by

$$V(p) = V(1\text{ atm})[1 - \beta(p - 1)]$$

where β, the coefficient of compressibility, is defined as

$$\beta = -1/V(dV/dP)$$

The value of ΔV is thus

$$\begin{aligned}\Delta V(p) &= V_F(p) - V_G(p)\\ &= V_F(1)[1 - \beta_F(p - 1)] - V_G(1)[1 - \beta_G(p - 1)]\\ &= V_F(1) - V_F(1)\beta_F(p - 1) - V_G(1) + V_G(1)\beta_G(p - 1)\\ &= V_F(1) - V_G(1) + (p - 1)[V_G(1)\beta_G - V_F(1)\beta_F]\\ &= V_F(1) - V_G(1) + (p - 1)[dV_F/dp - dV_G/dp]\\ &= \Delta V(1) + (p - 1)\Delta C\end{aligned}$$

where $\Delta V(1) = V_F(1) - V_G(1)$ is the volume change occurring at 1 atm, and $\Delta C = dV_F/dP - dV_G/dP$ represents the difference in compressibilities between the subunits when they are in their polymeric and monomeric forms.

Combining these results with the equation for the pressure dependence of K yields

$$K(p)/K(1) = \exp\{-p[\Delta V(1) + (p - 1)\Delta C]/RT\}$$

and

$$\ln [K(p)/K(1)] = (-\Delta C/RT)p^2 + \{[\Delta C - \Delta V(1)]/RT\}p$$

and, finally

$$\ln K(p) = (-\Delta C/RT)p^2 + \{[\Delta C - \Delta V(1)]/RT\}p + \ln K(1)$$

Thus, if the value of ΔC is negligible, a plot of ln K vs. pressure will be linear with a slope equal to $-\Delta V/RT$. However, if the value of ΔC is not negligible, then ln K will be a quadratic function of pressure, and the effect of pressure will be either magnified or attenuated at higher pressures, depending on whether ΔC is a positive or negative number, respectively.

Table I: Interspecific Variation in the Effects of Pressure on Self-Assembly of Muscle Actin

species (assay method)	$\Delta C \times 10^5$ [a]	$\Delta V(1)$ [b]	ln $K(1)_{calcd}$ [d]	ln $K(1)_{exptl}$ [e]	index of correlation, r^2 [f]
chicken (DNase I)	−12.80	107	13.74	13.85	0.9699
chicken (NBD-actin)	−4.15	63	13.84	13.85	0.9992
Dipsosarus dorsalis (DNase I)	−6.08	139	13.68	13.69	0.9970
Coryphaenoides acrolepis (DNase I)	−6.27	63	13.59	13.65	0.9900
Sebastolobus alascanus (DNase I)	−8.18	56	13.16	13.24	0.9356
Halosauropsis macrochir (DNase I)	−5.16	58	13.54	13.55	0.9910
Coryphaenoides armatus (DNase I)	−0.16	9	13.19	13.18	0.9369
		(8)[c]			(0.9333)[c]
Coryphaenoides armatus (NBD-actin)	1.22	2	13.18	13.18	0.9990
		(6)			(0.9330)

[a] Units of cubic centimeters per mole per atmosphere. [b] Units of cubic centimeters per mole. [c] Values in parentheses were computed by using a linear regression analysis; all other values were computed by using a nonlinear regression analysis (see Results). [d] Equilibrium constant for self-assembly (mol^{-1}) determined for 1 atm by nonlinear regression analysis (see Figure 2 and Results). [e] Equilibrium constant for self-assembly determined experimentally at 1 atm. [f] r^2 values for regressions of ln K vs. pressure (see Results).

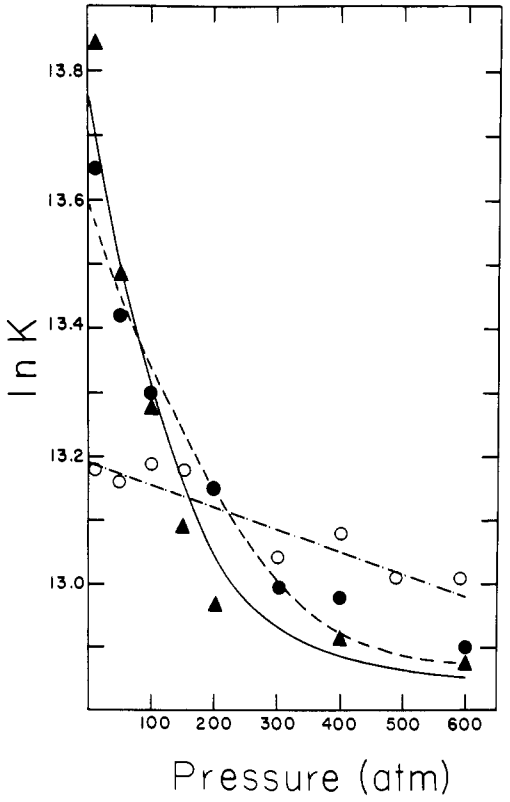
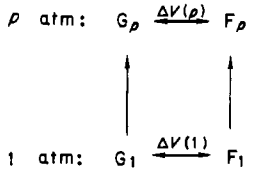


FIGURE 2: Interspecific differences in the effects of hydrostatic pressure on the assembly of actin as determined by using the DNase I assay for G-actin concentration (C_c). The equilibrium constant for the self-assembly reaction, K, was determined by using the relationship $K = 1/C_c$ (see Results). C_c values were measured by using the DNase I technique, as shown in Figure 1. Species shown in the figure are the chicken (▲), and the two fishes *Coryphaenoides acrolepis* (●) and *Coryphaenoides armatus* (○). The lines plotted on the figure were determined by using nonlinear regression analysis. From these plots, the values of $\Delta V(1)$ and ΔC given in Table I were calculated.

These relationships can be shown diagrammatically as follows:



If the reduction in volume upon going from 1 atm to p atm is not the same for both G-actin and F-actin, due to differential compressibilities for the two forms of the protein, then the values of ΔV at the two pressures will not be equal. Thus, if $\beta_G = \beta_F$, then $\Delta V(p) = \Delta V(1)$; if $\beta_G > \beta_F$, then $\Delta V(p) > \Delta V(1)$; if $\beta_G < \beta_F$, then $\Delta V(p) < \Delta V(1)$.

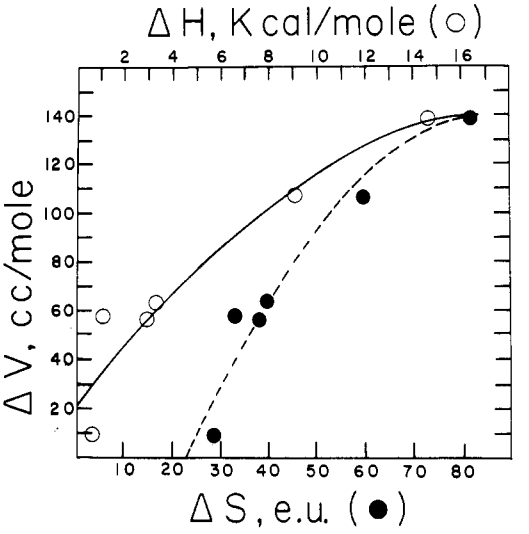


FIGURE 3: Interrelationships among the volume change (ΔV), enthalpy change (ΔH), and entropy change (ΔS) for self-assembly of actins from the six species used in this study. Values for ΔH and ΔS are from Swezey & Somero (1982a). The ΔV value is the change in volume during self-assembly at 1 atm (see Table I). Note the positive correlations between ΔV and ΔH and between ΔS and ΔV (see Discussion).

Figure 2 presents the results of the DNase I determinations of C_c and, therefore, of ln K as a function of pressure for actins of three species: the chicken; *Coryphaenoides acrolepsis*, a fish which occurs to depths of approximately 2100 m; and *Coryphaenoides armatus*, a fish which occurs to depths near 5000 m. For actins from all three species, high pressures disrupt the assembled state, and for actins from the chicken and *C. acrolepsis*, the depolymerizing effect is diminished at higher pressures. Therefore, the value of ΔC cannot be ignored in our analysis, and the data from these measurements of K vs. pressure were fitted by nonlinear regression analysis to equations of the form $\ln K = ap^2 + bp + c$. From knowledge of the coefficients a, b, and c, one can compute the values of ΔC and $\Delta V(1)$. The coefficient c is numerically equal to the value of ln $K(1)$. Table I shows the values of ΔC and $\Delta V(1)$ for all the actins tested, and it compares the experimentally determined values of ln $K(1)$ with the values calculated from the regression analysis. Also given in Table I are the values for the index of correlation of the fitted equations. The curves shown in Figure 2 are based on these regression lines.

For all species, $\Delta V(1)$ is a positive number, which means that the volume of the system is greater after the actin subunits have been assembled into filaments, and ΔC is, in all but one case (*C. armatus* NBD-actin), a negative number. The application of nonlinear regression analysis to the *C. armatus*

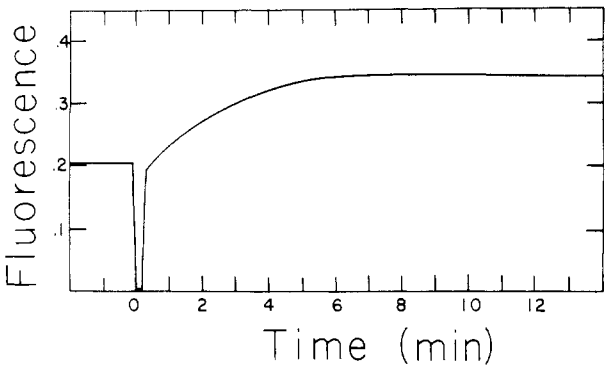


FIGURE 4: Time course of actin self-assembly at 1 atm as monitored by the increase in fluorescence intensity of NBD-actin. NBD-actin (chicken muscle; 0.086 mg/mL) was induced to polymerize at time = 0 by the addition of KCl (final [KCl] = 0.1 M). The intensity of fluorescence (λ^{ex} = 470 nm; λ^{em} = 530 nm) in arbitrary units was followed as a function of time at 25 °C.

data did not result in an improvement of the correlation coefficient over that obtained by linear regression analysis. Therefore, the quadratic term in the equation for the pressure dependence of ln K can be dropped, which means that ΔC for this species must be zero. The finding that ΔC is, for all but one actin, a negative number indicates that the compressibility of the subunits generally is increased upon their incorporation into filaments; that is, F-actin is more compressible than G-actin.

Figure 3 shows the correlations between the entropy change (ΔS) of polymerization and ΔV, and between the enthalpy change of polymerization (ΔH) and ΔV, for the species used in this study. The values of ΔS and ΔH, determined at 1 atm, are from Swezey & Somero (1982a). The plots shown are based on nonlinear regression analysis of the data. The indices of correlation were 0.963 and 0.979 for ΔH vs. ΔV and for ΔS vs. ΔV. respectively. The significance of these correlations between the thermodynamic parameters of actin assembly is discussed later.

(*B*) *Fluorometric Analysis.* Figure 4 shows a typical time course for the increase in fluorescence due to the polymerization of actin at 1-atm pressure. Fluorescence intensity decreases at zero time due to the dilution of actin upon addition of KCl solution, which is added to induce polymerization. We found that the method of choice for assessing the effect of pressure with the fluorometric procedure was to initiate polymerization at 1 atm and then quickly bring the polymerizing sample to the highest pressure to be tested, 600 atm, and allow the system to come to equilibrium. Relieving the pressure in increments allows the system to relax to a new equilibrium, which we followed by the change in fluorescence intensity. A time course is shown in Figure 5 for such an experiment, using fluorescently labeled chicken actin. NBD-actin from *C. armatus* had a similar time course, except that the magnitudes of the increases in fluorescence upon release of pressure were much smaller (data not shown). This experimental strategy was taken because we found that the time required for the system to relax to a new equilibrium after the pressure was lowered, approximately 10–15 min, was much less than the relaxation time for a system when the pressure was increased, approximately 5.5 h (data not shown). In other words, assembly was much faster than disassembly. It should also be noted that the temperature used in the fluorescence experiments, 25 °C was higher than that used in the DNase I experiments, 4 °C, because low temperatures led to considerable fogging of the windows of the fluorescence cell, reducing this assay's reliability.

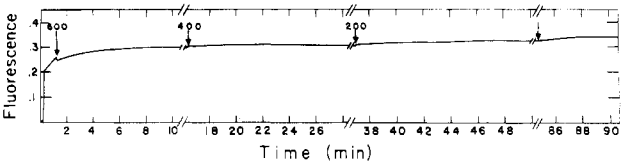


FIGURE 5: Effect of pressure on the assembly of NBD-actin as monitored by fluorescence intensity. Actin was induced to assemble by addition of KCl to a final concentration of 0.1 M (see Figure 4), and after 50 s of assembly at 1 atm, the pressure of the fluorometer cell was raised to 600 atm. The fluorescence intensity was then followed until it reached an equilibrium value at 600 atm. Then the pressure of the cell was sequentially lowered to the pressures (in atmospheres) indicated on the figure. Reassembly of depolymerized actin was followed as described under Results. The final F-actin concentration at 1 atm, determined by using the sedimentation assay described under Materials and Methods, was 0.066 mg/mL for this experiment and the one shown in Figure 4.

In the fluorescence assay system, the value of C_c at an elevated pressure was determined from the difference between the fluorescence intensity at that pressure and the fluorescence intensity at 1 atm, i.e., the final value in Figure 5. From a determination of the amount of actin which polymerizes at 1 atm, assayed as the concentration of protein which sediments at 179000g in 60 min, the differences in fluorescence intensity at higher pressures can be converted to increases in the monomer content, i.e., in the C_c, of the system. Control experiments showed that pressure had no effect on the fluorescence intensity of the buffer used and that high pressures (400–600 atm) caused an immediate 5% decrease in the fluorescence intensity of the G-actin present in the polymerized samples, along with a bathochromic shift in the emission spectrum of the fluorescent protein. These results are consistent with pressure-induced changes in fluorescence spectra of proteins found by other workers (Li et al., 1976). The time-dependent changes we observed thus can be ascribed to changes in the extent of actin self-assembly.

Figure 6 shows the effects of pressure on ln K for the actins from chicken and *C. armatus*, determined by using the fluorescence assay. Consistent with the results from the DNase I assay system (Figure 2), the effect of increasing pressure was to reduce K, and these effects differed between the two species. Again, the effects of pressure on K for actin from chicken muscle were nonlinear. The values calculated for ΔC and $\Delta V(1)$, using nonlinear regression analysis, are given in Table I. There is some discrepancy between the DNase I method and the fluorescence assay in the values for ΔV and ΔC, which we postulate arises from the higher experimental temperature used for the fluorescence measurements. In general, the values of ΔV for protein reactions show a temperature dependence that results from a difference in the expansibility (dV/dT) of the product vs. that of the reactant (Morild, 1981). For actin from chicken muscle, the ΔV is substantially lower under the conditions in the NBD-actin system; for actin of *C. armatus*, the difference in ΔV between the two methods is quite small in absolute terms, although large on a percentage basis (Table I). The difference in compressibility for G-actin and F-actin also differed between the two methods. We are unable to state the basis of these differences, albeit we conjecture that changes in the exposure of the NBD ligand to solvent during polymerization could contribute to volume and compressibility changes (see Discussion).

The kinetics of self-assembly can be evaluated from the time course of the increase in fluorescence in the following manner. Spudich & Cooke (1975), using a spectrophotometric assay of polymerization, found that actin self-assembly exhibited first-order kinetics with respect to the decrease in G-actin

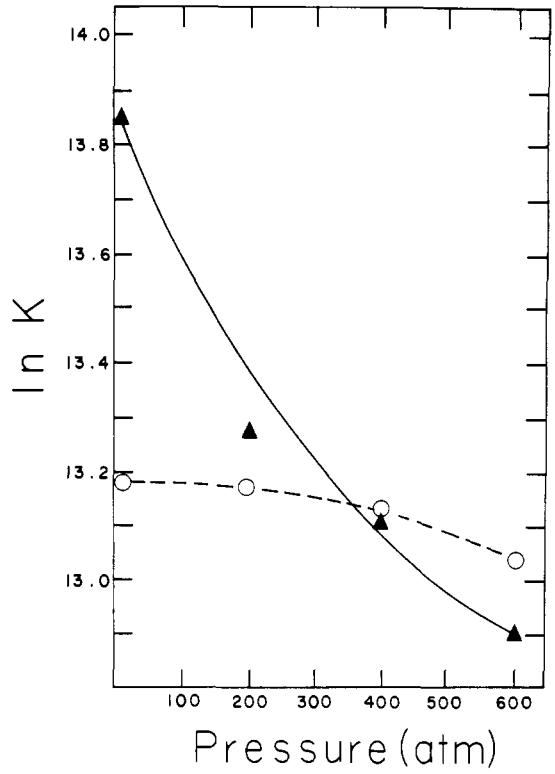


FIGURE 6: Interspecific differences in the effects of hydrostatic pressure on the assembly of actin as determined by using the fluorometric assay with NBD-actin. The values of K at elevated pressures were determined from the differences between the final value of the intensity of fluorescence at the given pressure and the value at 1-atm pressure (see Results). The species used were the chicken (▲) and *Coryphaenoides armatus* (○).

content. Likewise, we evaluated the amount of G-actin remaining at time t after polymerization was initiated, $C_{G(t)}$, as being proportional to $[F(0) - F(t)]/[F(f) - F(t)]$, where $F(0)$, $F(t)$, and $F(f)$ are the values of the fluorescence intensity at times zero, t, and infinity, respectively. The slope of a plot of ln C_G vs. time is equal to the first-order rate constant for assembly. There are two features of the kinetic analysis which support our assumption that first-order kinetics are valid for actin assembly. First, the regression coefficients for all of the time courses evaluated were greater than 0.99; six time points were used in each analysis. Second, the order of a reaction can be determined empirically from the slope of a plot of log C_G vs. log $-(dC_G/dt)$ (Castellan, 1971). All assays were subjected to this analysis by fitting the values of C_G vs. time to quadratic equations using a nonlinear analysis, and these equations were used to evaluate the term log $-(dC_G/dt)$. The order of the reaction as determined by this method was 1.02 ± 0.10 for chicken actin and 1.06 ± 0.11 for actin of *C. armatus*. There was no difference in the order of the reactions when they were measured at 1 atm or at 600 atm.

The values of the rate constants are given in Table II. It is evident that high pressure reduces the rate of assembly for both actins. For chicken actin, k_1 at 600 atm is about 67% of k_1 at 1 atm; for *C. armatus* actin, k_1 at 600 atm is only 46% of the k_1 at 1 atm. At either pressure, *C. armatus* actin assembles more rapidly than chicken actin.

Table II: Effects of Pressure on the Kinetics of Assembly of NBD-actin

species	pressure (atm)	k_1 (min^{-1})
chicken	1	0.446
chicken	600	0.299
Coryphaenoides armatus	1	0.977
Coryphaenoides armatus	600	0.458

Discussion

A common attribute of self-assembling protein systems is the sensitivity of the assembled state (quaternary structure) to hydrostatic pressure. Thus, for tubulin (Salmon, 1975), myosin (Josephs & Harrington, 1968; Davis, 1981), fibrin (Collen et al., 1970), lactate dehydrogenase (Schmidt et al., 1979; Schade et al., 1980; Jaenicke, 1983), sarcoplasmic reticulum ATPase (Champeil et al., 1981), and enolase (Paladini & Weber, 1981), increased hydrostatic pressure has been shown to shift the self-assembly equilibrium toward the monomeric state. For all of these multisubunit systems, then, the change in volume upon self-assembly is positive. Likewise, in the case of skeletal muscle actin, previous studies have suggested that self-assembly is characterized by substantial increases in system (protein plus solvent) volume. However, earlier analyses of actin self-assembly under pressure have suffered from critical experimental shortcomings, which have prevented quantitative estimation of volume and compressibility changes and have failed to contribute toward understanding the biological significance of these pressure effects. The early studies of Ikkai & Ooi (1966) with rabbit muscle actin involved measurement of the amount of actin that denatured after a 10-min treatment at high pressure. While the amount of actin which denatured in their study system was related to pressure-induced disassembly, their approach could not provide a quantitative measure of the equilibrium constant for self-assembly, and thus their data could not be used for computation of the thermodynamic parameters of assembly. Other work by Ikkai et al. (1966), in which the self-assembly of actin was studied by using dilatometry, did yield an estimate of the ΔV of self-assembly, +391 cm^3 mol^{-1}. However, these studies were conducted at unphysiologically low ionic strength, and since ionic strength and ion composition can both strongly influence the responses of protein systems to pressure (Neville et al., 1972; Low & Somero, 1975a,b; Greaney & Somero, 1979), it is unclear if the ΔV estimate obtained by Ikkai et al. (1966) is indicative of the in vivo ΔV of assembly. Nonetheless, the large size of the ΔV of assembly given by Ikkai & Ooi (1966) suggested to us that actin self-assembly might be an excellent study system for examining pressure effects in different species, and for investigating the bases of the pressure sensitivities of self-assembly reactions in protein systems. Thus, using their value of +391 cm^3 mol^{-1}, we calculated that the C_c for self-assembly of rabbit muscle actin would increase by approximately 3 orders of magnitude as the pressure was raised from 1 to 400 atm, a pressure near the average pressure of the marine water column (the average depth of the ocean is approximately 3800 m). This very large estimated effect on the self-assembly of actin suggests that evolutionary changes in the actins of deep-living marine species might be an important concomitant of adaptation to depth.

Our determinations of the effects of elevated hydrostatic pressure on C_c and, therefore, on ln K (Figures 2 and 4; Table I) present a more detailed picture of the effects of pressure on actin self-assembly than has previously been available. We found much smaller values for $\Delta V(1)$ than the value reported for rabbit muscle actin by Ikkai et al. (1966). Our value for the $\Delta V(1)$ of self-assembly for actin from chicken muscle, 63 cm^3 mol^{-1}, obtained at an experimental temperature close to the one used by Ikkai et al., can be used for direct comparison with their ΔV value for rabbit actin, since these two actins have identical primary structures (Vandekerckhove & Weber, 1978). The much smaller value for $\Delta V(1)$ found in our study

may be due to the differences in ionic strength between the two studies since, as mentioned above, ionic strength can strongly influence the responses of proteins to hydrostatic pressure [cf. Morild (1981)]. We also discovered that, with the exception of skeletal muscle actin from the deep-living fish *C. armatus*, the ΔV of self-assembly was not independent of pressure. Rather, the ΔV decreased with increasing pressure, as shown in Figures 2 and 6. We also found that increased pressure retarded the rate of assembly of F-actin (Figure 5, Table I), and these effects on assembly kinetics also differed between species. Our data on the effects of pressure on the equilibrium and kinetics of the self-assembly of actin thus provide a basis for conjectures about the types of intersubunit bonding interactions that contribute to the stabilization of F-actin, and about possible pressure-adaptive differences among the actins studied.

When the possible adaptive significance of the different responses to pressure shown by these actins is considered, i.e., differences between the actin from the very deep-living species, *C. armatus*, and the other actins studied, several points bear emphasis. First, as shown in Figures 2 and 5, the decrease in ln K with increasing pressure is much smaller for the actin from *C. armatus* than for those of the chicken and the shallower occurring congener *C. acrolepis*. The reduced response of K to pressure shown by the actin of *C. armatus* may be adaptive. For this species, which occurs over a wide range of depths (1900–4800 m, corresponding to pressures of 191–481 atm), the low sensitivity of K to pressure might facilitate a relatively constant ability for self-assembly at all depths. The differences noted between the actins of *C. armatus* and *C. acrolepis* are interesting in the context of threshold effects of pressure. For lactate dehydrogenase of marine fishes, it has been shown that apparently pressure-adaptive differences in kinetic properties of this enzyme are selected when pressure is increased to only 50–100 atm (Siebenaller & Somero, 1978, 1979; Somero & Siebenaller, 1979). Recent work of Siebenaller (1984) indicates that this same low-pressure threshold for adaptation also is characteristic of malate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase. In contrast, for the self-assembly of actin, much higher ambient pressures, possibly in excess of 200 atm, appear necessary to select for the types of pressure insensitivities shown by the actin from *C. armatus*.

From our results, it appears that the differences noted between the effects of pressure on self-assembly for actin of *C. armatus*, on the one hand, and shallow-occurring or terrestrial species, on the other, will not lead to extremely large differences in C_c among species at the highest pressures studied. Moreover, the observed pressure effects on C_c appear quite small when these effects on C_c are gauged against the total concentration of actin present in these muscles. We measured the actin concentrations of skeletal muscle in a wide range of terrestrial and aquatic vertebrates and found that, for all species, actin concentrations were near 20 mg of actin per g fresh weight of muscle (Swezey & Somero, 1982b). At the highest pressures studied, the differences in C_c among species were only 0.02–0.03 mg/mL, so it is apparent that actin assembly is possible, at high pressures, for all actins examined. Whether these slight differences in C_c are biologically significant is not clear. More important biologically than the actual value of C_c at high pressure may be the response of C_c to variations in pressure. In this view, the major adaptive difference among the actins is the reduced compressibility of the actin of *C. armatus*, which permits a stabilization of K in the face of varying pressures. Suffice to say that much further study of pressure effects on actin self-assembly, including studies of the effects of the other proteins that constitute the thin filaments of muscle, will be needed to develop an adequate understanding of pressure adaptations of actins.

Considering now the types of bonding interactions that are responsible for stabilizing F-actin, and for establishing the observed pressure sensitivities of the self-assembly equilibrium, we call attention first to the interspecific differences observed in ΔV (Table I), ΔH, and ΔS of self-assembly (Figure 3; Swezey & Somero, 1982a). The finding that self-assembly for all actins studied is characterized by positive changes in both enthalpy and entropy, i.e., self-assembly is an entropy-driven process, suggests a critical role for hydrophobic effects in actin polymerization. These are the only type of noncovalent bonds that form with positive changes in enthalpy and entropy. Key roles for hydrophobic effects in other self-assembling protein systems have been discussed by Lauffer (1975). The actins from muscles of high body temperature species (birds, mammals, desert reptiles) have the highest values for all three thermodynamic parameters, and the actins from the cold-adapted and deep-living fishes have the lowest values. The higher values for ΔH, ΔS, and ΔV of self-assembly for actins from high body temperature animals suggest that greater amounts of the hydrophobic effect are present in these systems than in those from low body temperature species; the additional hydrophobic bonding for the actins of high body temperature species may be responsible for the lower C_c values observed for these systems. The proposed predominance of hydrophobic effects between actin subunits is further supported by the covariation in the ΔV, ΔH, and ΔS of self-assembly shown in Figure 3. The formation of hydrophobic interactions is expected to involve an increase in system volume, due to alterations in water structure, as discussed below [cf. Suzuki & Taniguchi (1972)].

Another factor which has been proposed to establish the pressure sensitivity of the binding interactions of proteins is the compressibility of the binding sites. Torgerson et al. (1979) have interpreted the effects of pressure on protein interactions in terms of "hard" vs. "soft" binding sites on the protein surface. A soft site is defined as one capable of reducing its volume by rotation of its covalent bonds; if the lower volume conformation is the correct one for binding, then binding will be enhanced by increased pressure. This appears to be the case for the binding of substrate by lysozyme (Li et al., 1976). In terms of this model, the binding sites involved in the self-assembly of actin would be hard sites, i.e., sites for which rotation around covalent bonds, under the influence of pressure, cannot occur, and for which the sensitivity to pressure is established by the compressibility of the ligand in the site relative to the compressibility of the solvating water which it replaces. Torgerson et al. (1979) and Paladini & Weber (1981) have further shown that the disruption by pressure of binding at hard binding sites decreases at higher pressures. Thus, the introduction of a dead volume between the interacting subunits would result in a positive ΔV of assembly, which diminishes at higher pressure. This effect was observed in our studies.

The exponential decrease in the effect of pressure on ln K at higher pressures is evidence that F-actin is more compressible than G-actin, and it provides indirect evidence for the nature of the bonding and structural changes entailed in the assembly of F-actin. The compressibility of a protein is influenced by several factors, among which are the existence of dead or void spaces within the protein, which increase compressibility, and the extent of protein hydration, which tends to reduce the compressibility of the system (Gekko &

Noguchi, 1979). For actin self-assembly, both of these effects could contribute to the compressibility differences we found between F-actin and G-actin. Water organized around the nonpolar groups involved in the intersubunit bonding is removed and enters the bulk solution, thereby decreasing the hydration state of the protein. As discussed above, the formation of dead spaces, e.g., at subunit contact sites, during polymerization could contribute to a higher compressibility of the system, as well as to an increased volume upon self-assembly. All of these interpretations, then, are consistent with the hypothesis that the assembly of F-actin is stabilized largely by hydrophobic interactions, which occur at subunit contact sites which, in the terminology of Torgerson et al. (1979), are hard sites, where dead volumes are established during self-assembly.

The one actin which deviated sharply in pressure response from the other actin homologues studied is from the deep-living teleost fish *C. armatus*. For this actin, the effect of pressure on ln K was relatively small, and it was linear over the range of pressures examined (Figure 2). In addition to suggesting putative adaptive significance (see above), these two differences from the other actins examined are intriguing in terms of what they imply for subunit assembly processes in the actin of *C. armatus* and, by implication, in other protein assembly processes from high pressure adapted organisms. The apparent lack of compressibility noted in the actin self-assembly reaction of *C. armatus* suggests that different types of subunit–subunit interactions are present from those just discussed for the other actins. We hypothesize, first, that if dead spaces between the subunits form when actin of *C. armatus* assembles, these dead spaces are very small and do not establish a significant increase in compressibility for F-actin. Second, the lower values of ΔH, ΔS, and ΔV, coupled with the lack of elevation in compressibility of F-actin for this species, suggest that hydrophobic effects play a reduced role in stabilizing F-actin relative to the other actin homologues studied. Although the overall self-assembly process for actin from *C. armatus* is entropy driven, as in the case of all actins examined, the positive ΔS is much smaller than for the other actins, even though the strength of subunit–subunit interactions is similar to those of F-actins of other species, as shown by the similar values of K. To account for the conservation of K in the face of decreased amounts of the hydrophobic effect, we conjecture that during self-assembly of actin from *C. armatus*, one or more charged or polar groups increase their exposure to solvent. This increase in exposure of charged or polar groups would have several important effects on the energy and volume changes of self-assembly and on the inherent compressibility of the system; all of these expected effects are fully consistent with our data. First, this hydration would be strongly exergonic and thus would favor self-assembly. This contribution to the negative ΔG of assembly would tend to compensate for the proposed reduction in entropy-driven assembly due to a lowered reliance on hydrophobic effects. Therefore, these exergonic hydration reactions would favor a conservation of K. Second, the hydration of polar or charged groups would tend to reduce the compressibility of the system. Again, these hydrations of polar or charged groups would offset the influences of hydrophobic effects. As stated earlier, the formation of hydrophobic interactions will favor protein dehydration and an increase in compressibility. The lack of significant compressibility of actin from *C. armatus* may, therefore, reflect the occurrence of a "titration" of compressibility-increasing effects (hydrophobic interactions) by compressibility-decreasing effects (hydration of polar or charged groups). "Titrations" of this sort were earlier predicted on theoretical grounds (Somero & Low, 1977). Lastly, the hydration of polar or charged grups would lead to a reduction in system volume, i.e, to a lower ΔV of assembly. Our data again are consistent with this prediction.

In conclusion, we propose that the actin of *C. armatus* is different from the other actins we examined in that the types of bonding interactions stabilizing the polymerized state, and the changes in water organization around the protein during self-assembly, facilitate both a low value of ΔV per se and a reduction, if not a full elimination, of the difference between the compressibilities of the monomeric and polymeric forms of the protein. Both of these effects may be important for a species that occurs in very deep waters (up to approximately 5000 m) and which experiences large changes in pressure during its life history. We find it interesting that actin from the other deeply occurring fish we studied, *Halosauropsis macrochir*, does not exhibit the putative pressure adaptations discussed for actin from *C. armatus*. Does this apparent lack of adaptation to pressure suggest a "failure" to adapt, or has the contractile apparatus of *H. macrochir* adapted to pressure via different means from those suggested to occur for actin from *C. armatus*?

Turning now to the effects of pressure on the kinetics of self-assembly, our data indicate that high pressure reduced the rate of assembly to a greater extent for actin from *C. armatus* than for chicken actin. From the relationship $K = k_1/k_{-1}$, we can also assess the effect of pressure on the depolymerization rates for these two actin homologues. For chicken actin, the observed decrease in k_1 of 33% at 600 atm is accompanied by an increase in k_{-1} of 72%. For actin of *C. armatus*, the value of k_1 and k_{-1} decreased by 54% and 46%, respectively, as pressure was increased to 600 atm. Because it is unclear what the rate-limiting step is in actin polymerization or depolymerization, and because the nature of transition states in these structural changes is not known, we have no basis for making mechanistic interpretations of these effects of pressure on the kinetics of assembly and disassembly. We point out, however, that the more rapid polymerization of actin of *C. armatus* (Table II) at both pressures studied does suggest that the rate-limiting step in assembly is not the alteration in G-actin conformation that occurs concomitantly with self-assembly (Pardee & Spudich, 1982). We argue this because the actin of *C. armatus* has a more stable structure, as judged by resistance to thermal denaturation (Swezey & Somero, 1982a), than actin from chicken. If thermal stability can serve as an index of the energy costs entailed in conformational changes, then one would predict that self-assembly of *C. armatus* actin would proceed more slowly than that for the apparently more flexible actin of chicken muscle.

In summary, these comparative analyses of actin self-assembly have provided additional evidence that the self-assembly of actin is strongly dependent on hydrophobic effects, at least for the majority of species studied, and the subunit contact sites resemble hard binding sites, in the terminology of Torgerson et al. (1979). Despite a widespread qualitative similarity among all actins studied, e.g., all actin self-assembly reactions are entropy-driven, the interspecific differences we observed, e.g., in the ΔH, ΔS, and ΔV of assembly and in differences in compressibility between F-actin and G-actin, show that significant evolutionary change has occurred in muscle actins, despite a high degree of conservation in actin primary structure.

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