Osmoelastic Coupling in Biological Structures: Formation of Parallel Bundles of Actin Filaments in a Crystalline-like Structure Caused by Osmotic Stress†

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ABSTRACT: A high molecular weight inert molecule, poly(ethylene glycol) (PEG), or a soluble protein, ovalbumin, causes parallel bundles of actin filaments in a crystalline-like structure under physiological conditions of ionic compositions and pH. The bundle formation depends on the molecular weight of PEG, and a larger molecular weight of PEG can make the bundle at a lower concentration. Actin bundle formation has a discrete dependence on the concentration of PEG. The light scattering following PEG-induced bundle formation increased abruptly at 4.5% (w/w) PEG 6000, while at concentrations ≤4.0% (w/w) no increase was observed. Labeling actin filaments with heavy meromyosin indicated that the polarity of the filament in the bundle is random. The PEG-induced bundle formation depends on the ionic strength of the solutions and also the concentration of the filament, showing that a higher concentration of PEG was required at lower ionic strength or a lower concentration of the filament. The results described above cannot be explained on the basis of the postulation that the direct binding of PEG molecules to the actin filaments may cause bundle formation. Alternatively, the mechanism can be explained reasonably by the theory of osmoelastic coupling based on preferential exclusion of PEG molecules from the filament surface. High molecular weight molecules such as PEG should be preferentially excluded from the region adjacent to the actin filaments (exclusion layer) by steric hindrance, thereby making imbalance of osmolarity between the bulk and the exclusion layer. This imbalance puts an osmotic stress on the actin filament. To counterbalance it with the elastic pressure, the actin filament surrounded by the solvent is compressed elastically (osmoelastic coupling) and becomes progressively unstable with increased osmotic stress. Finally, above a critical intensity of the osmotic stress, the homogeneous distribution of the actin filaments separates into two phases; one is a diluted isotropic phase, and the other is a condensed anisotropic phase in which the filament is less surrounded with the solvent, i.e., a crystalline-like bundle structure.

Actin filaments are ubiquitous in the periphery of nonmuscle cells as well as in muscle cells, and are assembled in various structures (Stossel, 1984; Weber & Osborn, 1985). In particular, parallel bundles in a crystalline-like structure can be observed widely in the brush border microvilli of epithelial cells, in the cortical microvilli of fertilized eggs and oocytes, and in the progress extending from the surface of a variety of cells, including blood platelets and mammalian cells cultivated in vitro (Mooseker & Tilney, 1975; Matsudaira et al., 1983; Burgess & Schroeder, 1977; Small, 1981). Similar bundles can be formed in vitro by the aid of several kinds of actin binding proteins such as villin from epithelial cells, fimbrin from vertebrate cells, or fascin from marine eggs [reviewed by Stossel et al. (1985)].

Actin filaments can also align into a crystalline-like structure by themselves. Hanson (1973) and Yamamoto et al. (1975) observed bundle formation in a crystalline-like structure under a nonphysiological condition of high concentrations of magnesium ion (>50 mM) or low pH (<5.5). In addition, Ito et al. (1987) have suggested the possibility that the bundle formation of actin filaments may be induced under physiological ionic conditions and pH, if the filaments are subjected to an osmotic stress due to the imbalance of osmolarity caused by dialysis against a solution of high osmolarity. From thermodynamic analysis, they have indicated that the bundle formation should result from the coupling between the elasticity of the actin filaments and the osmotic stress (osmoelastic coupling).

In this study, we have demonstrated that addition of a high molecular weight poly(ethylene glycol) (PEG) or a protein, ovalbumin, can make a parallel bundle of actin filaments. The mechanism can be reasonably explained on the basis of osmoelastic coupling, in which the elasticity of the actin filaments is coupled with the osmotic stress due to the local imbalance of osmolarity that is caused by addition of a large additive such as PEG. The results in the present report suggest that, since cytoplasmic proteins should be able to have a similar effect, the actin filaments may form bundles in the cytoplasmic matrix more readily than expected. Such phenomena may also give the thermodynamic basis to analyze the molecular mechanism for specific actin binding proteins to assemble actin filaments in various structures.

Materials and Methods

Materials

Monomeric actin (G-actin) was prepared from rabbit skeletal muscles by the method of Spudich and Watt (1971).

Poly(ethylene glycols) (PEGs) with various molecular weights (PEG 400, PEG 6000, and PEG 20 000) were purchased from Nakarai Chemical Ltd. and used without further purification. Nominal average molecular weights of PEG 400, PEG 6000, and PEG 20 000 are 400, 7500, and 15 000–25 000, respectively. Heavy meromyosin (HMM) was purchased from Nakarai Chemical Ltd. and ovalbumin from Sigma Chemical Co. HMM was dialyzed before use.

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Methods

Preparation of N-(1-Pyrenyl)iodoacetamide-Labeled (Pyrene-Labeled) G-Actin. Pyrene-labeled G-actin was prepared by a modified method of Kouyama and Mihashi (1981). *N*-(1-Pyrenyl)iodoacetamide dissolved in dimethylformamide was added to F-buffer containing 1.0 mg/mL F-actin (0.2 mM ATP, 100 mM KCl, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$, and 10 mM imidazole hydrochloride, pH 7.5). The molar ratio of the added fluorescent label to G-actin was 6.5 to 1, and the volume of dimethylformamide was kept less than 1% to avoid denaturation of the actin. After the mixture was allowed to stand for 24 h at 4 °C, it was centrifuged at 100000*g* for 2.5 h. The pellet was suspended in G-buffer (0.5 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM β-mercaptoethanol, and 2 mM Tris-HCl, pH 8.0) and dialyzed against the same buffer for at least overnight. The dialyzed solution was briefly sonicated and then passed through a gel filtration column of Sephacryl S-200 (2.5 cm × 10 cm). The molar ratio of fluorescent probe to G-actin in the eluted sample was 0.5–0.7.

Measurement of the Rate of Polymerization of Actin. A 0.5 mg/mL sample of the mixture of G-actin and pyrene-labeled G-actin (9:1 molar ratio) was polymerized in the F-buffer, and the time course of an increase in the fluorescent intensity at 407 nm which follows the polymerization was recorded at room temperature with a Hitachi 850-S spectrofluorometer.

Measurement of PEG-Induced Light Scattering. G-Actin was polymerized in a cuvette which contained 350 μL of F-buffer with an appropriate concentration of a PEG, and the time course of an increase in the 90° light scattering at 450 nm was recorded at room temperature with a Hitachi 850-S spectrofluorometer. For the measurement of the steady-state extent of PEG-induced light scattering, the solution of actin polymerized in the presence of PEG for 1 h was mildly agitated by a few gentle inversions of the cuvette before the light-scattering measurement.

Electron Microscopic Observation. The sample was adsorbed on a carbon-coated collodion grid for 1 min and stained with 2% uranyl acetate for 0.5–1 min. For the time course experiments, to adsorb the sample without agitation, the grid was put lightly onto a drop of the actin solution in appropriate time intervals after the start of the polymerization. For labeling actin filaments with HMM, the grid adsorbing the actin filaments was washed with ATP-depleted F-buffer, and then an aliquot of HMM solution (0.8 mg/mL in F-buffer) was put on the grid for 1 min. After the grid was washed, the sample was fixed with 0.04% tannic acid for 0.5 min and then stained with 2% uranyl acetate for 0.5 min. All of the samples were observed with a JEOL 100 B electron microscope.

RESULTS

PEG-Induced Increase in Light Scattering of Actin Filament Solutions. When G-actin was polymerized in F-buffer in the presence of various concentrations of PEG 6000, an increase in the light scattering of the solution was observed. The scattering increase depended on time and the concentration of PEG (Figure 1). At concentrations of PEG 6000 ≤4% (w/w), the increase in the light scattering was small and reached a plateau within 30 min. On the other hand, at concentrations ≥5% (w/w), the intensity of light scattering increased in a biphasic manner, i.e., a rapid increase within 30 min and then a gradual increase which continued for ~20 h.

A characteristic increase in the light scattering was observed when the sample solution was mildly agitated by a few gentle inversions of the cuvette. That is, at concentrations of PEG 6000 ≥4.5% (w/w), the light scattering increased very rapidly following agitation (arrow in Figure 1); the intensity became almost the same as that of a nonagitated sample standing for 20 h. On the other hand, at concentrations ≤4% (w/w), the intensity did not change significantly.

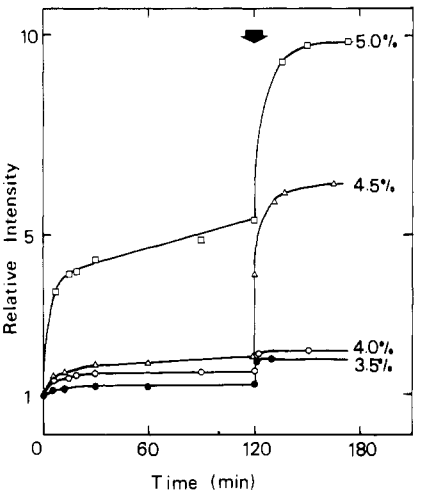

FIGURE 1: Time course of PEG-induced light scattering. G-Actin (0.5 mg/mL) was polymerized in F-buffer (100 mM KCl, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.2 mM ATP, and 10 mM imidazole hydrochloride, pH 7.5) containing an appropriate concentration of PEG 6000, and the light scattering of the solution was measured at room temperature. Two hours later after the polymerization was started, the cuvette containing the sample was gently inverted a few times (arrow in the figure), and then the light scattering was measured continuously. The numbers in the figure represent the concentrations of PEG in the individual sample solutions.

The PEG-induced increase in light scattering was reversible; the light scattering decreased to almost the same level as that of the PEG-free actin filament solution, when PEG 6000 in the filament solutions was diluted from 6% (w/w) to 3% (w/w). Further, the addition of PEG after completion of the polymerization had quite the same effect on the increase in light scattering.

The polymerization rate of actin was investigated at the same actin concentration as the light-scattering experiments were done, i.e., at 0.5 mg/mL, using the fluorescent method described by Kouyama and Mihashi (1981). In the absence of PEG, the polymerization was complete within 30 min. The addition of PEG increased the rate of polymerization, and also increased the extent of polymerization at the steady state slightly, as described by others (Tellam et al., 1983) (data not shown). Thus, the small increase in the light scattering within 30 min in Figure 1, which was also observed in the absence of PEG, should result from the polymerization of the filaments.

To investigate the cause of the PEG-induced increase in the light scattering, the ratio of the protein quantity in the pellet made by low-speed centrifugation (15000*g* for 5 min) to the supernatant was determined for various concentrations of PEG. The pellet ratio increased with increasing concentrations of PEG and correlated well to the intensity of PEG-induced light scattering, indicating that PEG-induced light scattering may be due to bundle formation of the actin filaments [(■) in Figure 2].

The PEG-induced light scattering depended on the molecular weight of PEG, and the largest molecular weight was the most effective (Figure 2). For example, 2% (w/w) was enough for PEG 20000 to induce the increase of light scattering, while even 20% (w/w) was not enough for PEG 400. A soluble globular protein, ovalbumin, also caused bundle formation at somewhat higher concentrations [(●) in Figure 2].

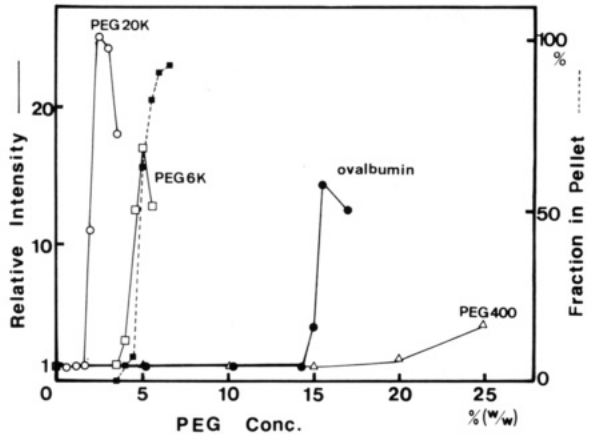

FIGURE 2: Molecular weight dependence of PEG-induced light scattering. G-Actin (0.5 mg/mL) was polymerized in F-buffer containing PEG of different molecular weight, and the stationary values of the light scattering (solid lines in the figure) or fractions of the pellet made by low-speed centrifugation (15000*g*, 5 min) (dashed line) were measured at various concentrations of the individual PEGs with different molecular weights: (○) PEG 20000; (■, □) PEG 6000; (△) PEG 400. The closed circles on the solid line represent the increase in the light scattering induced by ovalbumin.

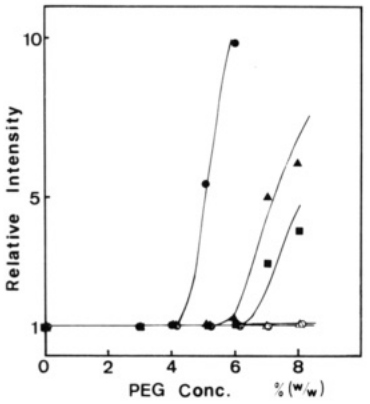

FIGURE 3: Ionic strength dependence of PEG-induced light scattering. G-Actin (0.5 mg/mL) was polymerized in the buffers with a variant concentration of KCl (*X* mM KCl, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.2 mM ATP, and 10 mM imidazole hydrochloride, pH 7.5) at an appropriate concentration of PEG 6000, and the stationary values of the light scattering of the individual solutions were measured. (●) 100 mM KCl; (▲) 75 mM KCl; (■) 50 mM KCl; (△) 25 mM KCl; (○) 0 mM KCl.

The ionic strength of the actin filament suspension pronouncedly affected the PEG-induced increase in the light scattering. Higher concentrations of PEG were required in lower ionic strengths (Figure 3). For example, the onset of the light-scattering increase was at 4.5% (w/w) PEG 6000 in F-buffer containing 100 mM KCl and at 6.5% (w/w) in 50 mM KCl, whereas in F-buffer depleted of KCl, the light-scattering increase was not observed at concentrations ≤8% (w/w), as observed by others (Tellam et al., 1983).

Electron Micrographs of PEG-Induced Bundle Formation of Actin Filaments. To observe the PEG-induced morphological changes of actin filament assembly, electron micrographs of the negatively stained actin filaments were taken under the same conditions as the light-scattering experiments (Figure 4). Actin filaments polymerized in PEG-free solutions were isotropically distributed at all times after the polymerization was complete (panel h in Figure 4). The actin filament assembly was changed dramatically in the presence of PEG 6000 at concentrations ≥5% (w/w). As a typical example, the time courses of the morphological change of the assembly structures of the actin filaments polymerized in the presence

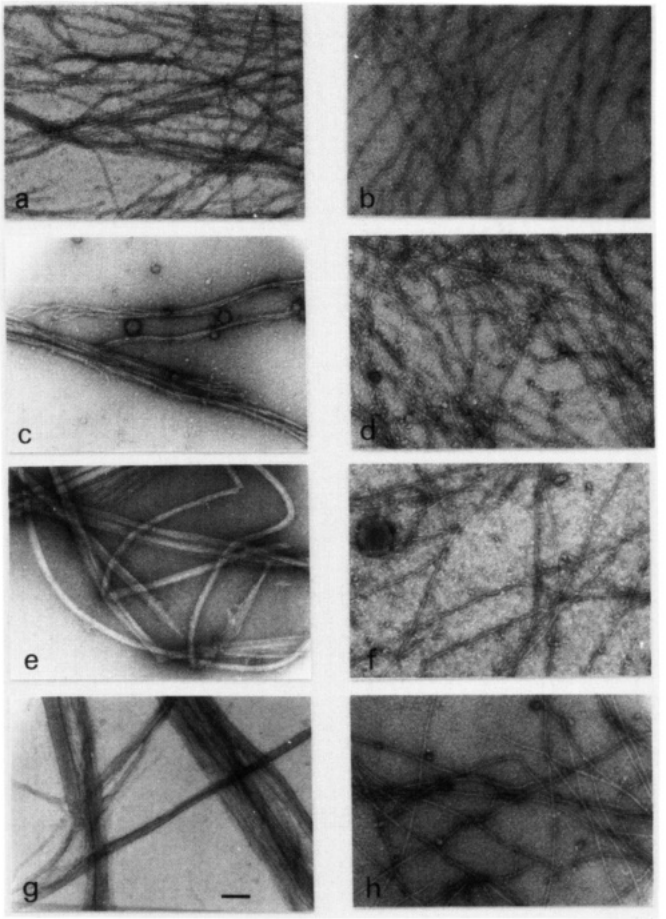

FIGURE 4: Electron micrographs of PEG-induced morphological change of actin filament assembly. G-Actin (0.5 mg/mL) was polymerized in F-buffer containing 7% (w/w) or 3.5% (w/w) PEG 6000. At an appropriate time after the polymerization was started, the sample was adsorbed to a carbon-coated collodion grid and negatively stained with 2% uranyl acetate as described under Materials and Methods. The photographs a–d and g represent the individual samples which were polymerized at 7% (w/w) for 30 min (a), at 3.5% (w/w) for 30 min (b), at 7% (w/w) for 120 min (c), at 3.5% (w/w) for 120 min (d), and at 7% (w/w) for 20 h (g), respectively. Photographs e and f represent the samples which were polymerized at 7% (w/w) (e) or at 3.5% (w/w) (f) for 60 min and then mildly pipetted before being adsorbed on the grid. Photograph h represents the control sample which was polymerized in the absence of PEG. Magnification, 13750×; bar, 0.28 μm.

of 7% (w/w) PEG 6000 are shown in Figure 4. The filaments gradually associated, progressively aligning their axes parallel to each other (panels a, c, and g in Figure 4). Two hours later, the filaments made an intermediate bundle in which the axes of filaments were almost parallel, though the packing of filaments was still loose (c). Gradually, the packing became closer, and 20 h later, most of the filaments took a crystalline-like structure (g). On the other hand, the filament assembly was not affected at all by the presence of PEG 6000 at concentrations ≤4% (w/w) (b, d).

Mild pipetting of the filament solutions accelerated significantly the formation of the closed packed bundles at concentrations of PEG 6000 ≥5% (w/w) (e), while it had no effect at lower concentrations (f). The acceleration of the bundle formation may correspond to the abrupt increase in the light scattering in Figure 1. The results suggest that subjecting the filaments to a mild shear force facilitates the formation of the closed-pack, crystalline-like bundles.

Addition of ovalbumin also induced bundle formation of the actin filaments at concentrations ≥ 15% (w/w), around which the observed light scattering of the actin filament solutions

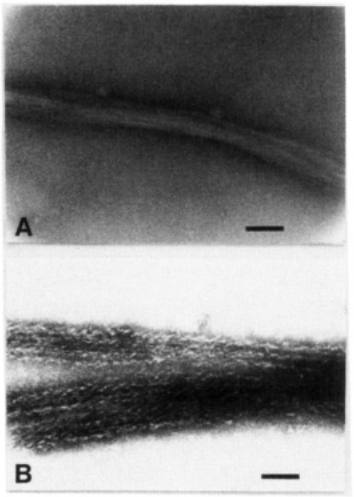

FIGURE 5: Electron micrographs of the actin filament bundle induced by ovalbumin (A) and HMM-labeled actin filaments in the PEG-induced bundles (B). (A) G-Actin (0.5 mg/mL) was polymerized in F-buffer containing 15% (w/w) ovalbumin. The electron micrographs was taken in the same way as described in Figure 4. Magnification, 40000×; bar, 0.125 μm. (B) After the formation of the bundle in the presence of 7% (w/w) PEG 6000, the actin filaments were labeled with HMM on the grid as described under Materials and Methods. Magnification, 40000×; bar, 0.125 μm.

abruptly increased (Figure 5A).

The micrograph of HMM-labeled actin filaments in Figure 5B showed that the polarity of filaments in the PEG-induced bundle was random.

Discussion

An inert synthetic polymer, PEG, can induce bundle formation of actin filaments in a crystalline-like structure. The abrupt increase in the light scattering indicates that the PEG-induced bundle formation may be a kind of critical phenomenon; the structure of the interfilament assembly of actin filaments changes discretely at a critical concentration of PEG 6000 [~4.5% (w/w)]; most filaments are distributed in bundle form, while below it they are distributed isotropically. The transformation of the isotopic distribution to the bundle form should be a diffusion-limited process. It takes about 1 day to form the closely packed bundle at 0.5 mg/mL concentration of actin. Subjecting the filaments to mild shear force quickens the bundle formation. Under the shear force, the filaments tend to align parallel to each other. Consequently, the diffusion-limited process should be much facilitated.

If PEG in the filament solution is diluted from a concentration above the critical one to below, the light-scattering increase due to the bundle formation disappears. Therefore, the transformation of the isotropic distribution to the bundle form should be a reversible process.

The bundle formation might result from the direct interaction of PEG with the actin filament. Some of actin binding proteins such as α-actinin or ABP are known to cross-link actin filaments to cause gelation of the filaments. The above-mentioned discrete properties of bundle formation might correspond to the gelation caused by PEG. However, the results described below indicate that such a possibility should be implausible. First, assuming that the direct binding of PEG caused the gelation, the dissociation constant of PEG should be estimated to be larger than 10 mM. Compared with 0.5 μM ABP or 0.25 μM α-actinin (Hartwig & Stossel, 1981; Bennet et al., 1984), the dissociation constant may be too large to make a stable cross-linkage for the gelation. Second, the minimum concentration of PEG for the bundle formation was strongly dependent on the ionic strength, and a higher concentration was required in lower ionic strength (Figure 3). Such dependence indicates that direct binding of PEG with actin filaments should not be the cause of the bundle formation, since the binding of a neutral polymer such as PEG with actin filaments must be independent of the ionic strength. Third, the minimum concentration of PEG decreased with the increasing concentrations of actin filaments, e.g., 4.5% (w/w) at 0.5 mg/mL F-actin and 2.8% (w/w) at 5 mg/mL (Suzuki and Ito, unpublished results), which contradicts the gelation caused by the direct binding of PEG (Flory, 1953). Fourth, PEG molecules in actin filament solutions should be preferentially excluded from the filament surface rather than bind to it, as discussed below.

Alternatively, the PEG-induced bundle formation of actin filaments should be due to an indirect effect of PEG on the actin filament. As is well recognized in protein solutions containing a large cosolvent such as PEG (Kuntz & Kauzmann, 1974; Arakawa & Timasheff, 1985a,b), PEG molecules should be preferentially excluded from the region adjacent to the surface of the actin filament (exclusion layer). Phenomenologically, the effects of such preferential exclusion can be explained according to the analysis of Timasheff and his co-workers (Arakawa & Timasheff, 1985a,b). Let us introduce a parameter $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ at first, where m_i and μ_i represent molar concentration (moles per gram of water) and chemical potential, respectively, of the ith component. Components 1, 2, and 3 correspond to water, actin filament, and PEG, respectively. The value should be negative owing to the preferential exclusion. In fact, all values of the parameter hitherto obtained experimentally in protein in PEG/water mixtures are negative. From the standard cross-differentiation relation at equilibrium, the relation can be derived:

$$(\partial\mu_2/\partial m_3)_{T,m_2} = -(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}(\partial\mu_3/\partial m_3)_{T,m_2} > 0 \quad (1)$$

The result indicates that the preferential exclusion increases the chemical potential of the actin filament with increasing concentrations of PEG. Moreover, the values of $(\partial\mu_2/\partial m_3)_{T,m_2}$ should be larger for PEGs with larger molecular weights owing to more preferential exclusion. In fact, the values experimentally determined are 85 kcal (mol of protein)$^{-1}$ (mol of PEG)$^{-1}$ for PEG 4000 and 13 kcal (mol of protein)$^{-1}$ (mol of PEG)$^{-1}$ for PEG 1000 and 5 kcal (mol of protein)$^{-1}$ (mol of PEG)$^{-1}$ for PEG 400 in solutions of β-lactoglobulin in PEG/water mixtures (Arakawa & Timasheff, 1985a). Such an increase in chemical potential of the actin filament, represented by eq 1, should create a progressively more unstable situation for the actin filaments surrounded by the solvent, and above a critical concentration of PEG, the isotropic distribution of the filaments separates into a dilute isotropic phase and a concentrated anisotropic one in which the filaments are less surrounded by the solvent, a bundle formation in crystalline-like structure. PEG with larger molecular weight should be more effective, as demonstrated experimentally (Figure 2).

We can elucidate the molecular mechanism of the effects of the preferential exclusion as follows. As shown in the Appendix, the following equation can be derived from eq 1 by setting the partition coefficient between the exclusion layer and the bulk phase as K:

$$-(\partial\mu_2/\partial V_1')_{T,m_2} = RT(1 - K)C \quad (2)$$

where V_1' is the volume of water in the exclusion layer, C is the molar concentration of PEG, and R and T have their usual meanings. The right-hand term in eq 2 may correspond to the imbalance of osmolarity between the exclusion layer and

the bulk phase which results from the preferential exclusion of PEG. The left-hand term may correspond to the elastic pressure which should counterbalance the imbalance of osmolarity at equilibrium (Flory, 1953; Tanaka, 1981). The molecular events underlying the relation in eq 2 should be as follows. The addition of PEG should make the imbalance of osmolarity between the exclusion layer and the bulk phase, which exerts an osmotic stress on the filaments. Consequently, the filaments should be compressed elastically to counterbalance the osmotic stress by the elastic pressure (osmoelastic coupling).

Such elastic compression of actin filaments caused by osmotic stress had been predicted by Ito et al. (1987) from the analysis of the volume flow of actin filament solutions. In this case, the actin filaments are compressed elastically by the osmotic stress caused by dialysis of an actin filament solution against a solution of higher osmolarity. As quantitatively analyzed previously (Ito et al., 1987), the free energy increase (ΔF_d) due to the compression is proportional to the second power of osmotic stress (P_f), i.e.

$$\Delta F_d \propto P_f^2 \tag{3}$$

Equation 2 shows that the osmotic stress caused by addition of PEG in the present experiments can be represented as

$$P_f = RT(1 - K)C \tag{4}$$

Therefore, from eq 3 and 4, ΔF_d can be represented as

$$\Delta F_d \propto (1 - K)^2 C^2 \tag{5}$$

The free energy increase with increased concentrations of PEG, represented by eq 5, should create a progressively more unstable situation for the actin filaments surrounded by the solvent, and above a critical concentration of PEG, it would cause a bundle formation in crystalline-like structure, as mentioned previously.

The mechanism of bundle formation may correspond to that predicted theoretically by Flory (1956) in his analysis of the thermodynamic properties of rigid, solvent-impermeable rods. His analysis showed that a small increase in the rod–rod interaction energy, χ, which is defined as the energy change upon transferring a rod from pure rods to infinite dilution, can cause the isotropic distribution to separate into two phases, consisting of a nearly pure filament phase and a dilute isotropic one. The increase in the chemical potential of the actin filament represented by eq 1 or the increase in the free energy of the compression represented by eq 5 may be thermodynamically equivalent to an increase in χ in his analysis.

The strong dependence of PEG-induced bundle formation on ionic strength may be due to a contribution of the electrostatic repulsion between the filaments. The electrostatic repulsive force resulting from the negative surface charge of actin molecules acts effectively only on the filaments close enough to each other and hence increase the free energy of the filaments in the bundle. Therefore, higher concentrations of PEG should be required in lower ionic strength to compensate the increase in the electrostatic repulsive force with the osmotic stress (Figure 3).

Ovalbumin (molecular weight 45 000) also induces bundle formation of actin filaments, though the critical concentration is higher than that expected from the dependence on the molecular weights of PEGs. The high critical concentration should be attributed to much less effective exclusion of ovalbumin than PEG, owing to the much compacted structure of the molecule.

The results of the present analysis enable us to make the quantitative phase diagram of the actin filament solution which is represented by the filament–filament interaction energy as defined by Flory (1956) and the volume fraction of actin, and to study the effects of actin binding proteins on the phase diagram (Suzuki and Ito, unpublished experiments).

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APPENDIX

Analytical Derivation of the Equation Representing Osmoelastic Coupling. As shown by Timasheff and Kronman (1969), the following relation holds in a ternary system composed of component 1 = water, component 2 = protein, and component 3 = cosolvent:

$$M_1(\partial m_1/\partial m_2)_{T,\mu_1,\mu_3} = -(1/m_3)(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3} \tag{A-1}$$

where M_1 is the molecular weight of water and m_i the molar concentration of component i represented by units of moles per gram of water. Also, the following relation can be derived by using the standard cross-differentiation relation at equilibrium (Casassa & Eisenberg, 1964):

$$(\partial\mu_2/\partial m_3)_{T,m_2} = -(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}(\partial\mu_3/\partial m_3)_{T,m_2} \tag{A-2}$$

where μ_i is the chemical potential of the ith component. From eq A-1 and A-2:

$$(\partial\mu_2/\partial m_3)_{T,m_2} = RTM_1(\partial m_1/\partial m_2)_{T,\mu_1,\mu_3}$$

Therefore

$$\begin{aligned}(\partial\mu_2/\partial m_1)_{T,m_2} &= RTM_1(\partial m_1/\partial m_2)_{T,\mu_1,\mu_3}(\partial m_3/\partial m_1)_{T,m_2} \\ &= RTM_1(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}\end{aligned} \tag{A-3}$$

Here let us assume there is the exclusion layer near the filament surface, where the cosolvent is preferentially excluded by steric hindrance. Setting the partition coefficient as K:

$$K = m_3'/m_3$$

$$(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3} = V_{excl}(m_3' - m_3) = V_{excl}(K - 1)m_3 \tag{A-4}$$

where m_3' and V_{excl} represent the molar concentration of PEG in the exclusion layer and the volume of the exclusion layer, respectively. From eq A-3 and A-4, we get

$$(\partial\mu_2/\partial n_1')_{T,m_2} = RTM_1(K - 1)m_3$$

or, using the concentration of the cosolvent (C) represented by units of moles per milliliter of water:

$$(\partial\mu_2/\partial V_1')_{T,m_2} = RT(K - 1)C \tag{A-5}$$

where $n_1' = V_{excl}m_1$ and $V_1' = (M_1/d)m_1'$ (d, density of water), and the former corresponds to the moles of water and the latter to the volume of water in the exclusion layer. Equation A-5 represents the osmoelastic coupling in equilibrium as discussed in the text.

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Tubulin Dimer Dissociation Detected by Fluorescence Anisotropy†

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ABSTRACT: We have demonstrated a concentration-dependent dissociation of bovine brain tubulin dimer covalently labeled with 5-[(4,6-dichlorotriazin-2-yl)amino]fluorescein (DTAF) or with fluorescein isothiocyanate (FITC) by fluorescence anisotropy and size-exclusion HPLC. The fluorescence anisotropy values decreased to a limiting value upon dilution of tubulin from 10^{-5} to 8×10^{-8} M. A dissociation constant in 0.1 M Pipes, pH 6.9, 1 mM EGTA, and 1 mM $MgSO_4$ at 20 °C was estimated to be $(8.4 \times 10^{-7}) \pm (0.4 \times 10^{-7})$ M. Control experiments using monomeric and other dimeric proteins, urea-denatured tubulin, and DTAF-tubulin diluted into solutions of bovine serum albumin or unlabeled tubulin were consistent with the finding that the changes in anisotropy upon dilution are due to protein dissociation. These results were supported by size-exclusion HPLC data where an increase in the elution volume of DTAF-tubulin and FITC-tubulin was observed with decreasing protein concentrations. Reversibility of the dissociation process and the lack of denaturation at high dilution were shown by the ability of reconcentrated protein to assemble into microtubules to about the same extent as undiluted protein. Fluorescent lifetimes and limiting anisotropy values were found to be approximately identical at different tubulin concentrations, indicating that the anisotropy changes reflect changes in size or rotational correlation time of the protein. Studies on the effects of tubulin ligands and promoters or inhibitors of assembly demonstrated that their effects on tubulin dimer–monomer equilibria are small but reproducible. Increasing the temperature to 36 °C resulted in about a 2-fold higher K_d value while lowering it to 10 °C caused a 2-fold decrease. Thermodynamic constants for the association reaction were calculated to be $\Delta H° = -9.5$ kcal/mol, $\Delta S° = -4.6$ eu, and $\Delta G°(20\ °C) = -8.1$ kcal/mol.

The major protein of microtubules, tubulin, is a heterodimer of two noncovalently linked subunits, α and β. The dimer binds two guanine nucleotides, divalent cations, colchicine, vinca alkaloids, and other mitotic inhibitors. To have a better understanding of the nature of the tubulin dimer under a variety of in vivo and in vitro conditions, it is important to understand the monomer association reaction. To date, only two physical studies of the reversible dissociation of bovine brain tubulin have been reported in the literature (Detrich & Williams, 1978; Detrich et al., 1982). In these studies, ultracentrifugal techniques and small zone gel filtration were used to calculate a dissociation constant of $(7-10) \times 10^{-7}$ M at 5 °C. Although there was a good fit of the centrifugation data with theoretical curves in these studies, the actual experimental points were somewhat limited at lower protein concentrations. We felt that it was important to examine the dissociation process by another physical technique which allowed the use of lower protein concentrations.

In this study, fluorescence anisotropy measurements were employed to investigate the tubulin monomer–dimer equilibrium. This method provides a highly sensitive and convenient tool for detection and analysis of oligomeric protein dissociation (Weber, 1953). The extent of dissociation of a protein covalently labeled with a fluorescent dye can be readily followed provided that the dimer and monomer exhibit characteristic anisotropy values depending on their rotational correlation times.

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