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Review Article

My various thoughts on actin

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An enormous amount of research has been performed to characterize actin dynamics. Structural biology investigations have determined the localization of main chains and their changes coupled with G (Globular)-F (Filamentous) transformation of actin, whereas local thermal fluctuations that may be caused by free rotations of the tips of side chains are not yet fully investigated. This paper argues if the entropy change of actin accompanied by the G-F transformation is simply attributable to the changes in hydration. It took almost 10 years to understand that the actin filament is semi-flexible. This flexibility was visually confirmed as the development of optical microscope techniques, and the direct observation of actin severing events in the presence of actin binding proteins became possible. Finally, I expect the deep understanding of actin dynamics will lead to the elucidation of self-assembly mechanisms of the living creature.

Key words: actin thermodynamics, entropy changes in G-F transformation, hydration of actin, actin binding proteins, flexibility of actin filament

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Thermodynamic Parameters of Actin Polymerization

Actin is an extremely unique protein in the sense that it is possible to calculate the value of every thermodynamic quantity involved in the polymerization and depolymerization equilibrium. This calculation was performed based on the value of the critical concentration for polymerization (C_c) ([1–3] and Figs. 1 and 2), as published in the first part of Chapter V in Oosawa-Asakura's book entitled "Thermodynamics of the Polymerization of Protein" [1]. This book chapter also describes the required experiments for obtaining thermodynamic quantities. An example of the calculations obtained by Kikumoto is shown in Table 1 (see also [4]). The estimated thermodynamic quantities were not only different between Mg^{2+}-actin and Ca^{2+}-actin, but also between K^+ and Na^+ in the polymerization buffer.

Now, based on the answer to the calculation, we can obtain the value of entropy changes coupled to the monomer-polymer transition as shown in Table 1. The entropy change is quite big, and the value becomes considerably larger when actin takes a dimeric form as compared with the combined value of two independent monomers. Then the question arises: what causes such large entropy changes. Most people consider that, when actin molecules are dimerized, the amount of hydration water per molecule will become smaller than that for two separate actin monomers because the surface

◀ ***Significance*** ▶

Actin research is facing a paradigm shift as a result of recent rapid advances in scientific technologies. To share the current leading actin research, the international symposium "Now in actin study" was organized in December, 2016 at Nagoya University. This paper originates from my presentation there and is dedicated to the late Profs. Michiki Kasai (1937–2015) and Sho Asakura (1927–2016). This also introduces a letter from Prof. Donald L. D. Caspar, who collaborated with leading Japanese actin scientists such as Kasai and Asakura through his sabbatical stay. He has made outstanding contributions to our understanding of the functions and mechanisms of biological macromolecular assemblies such as TMV, flagella and actin filaments.

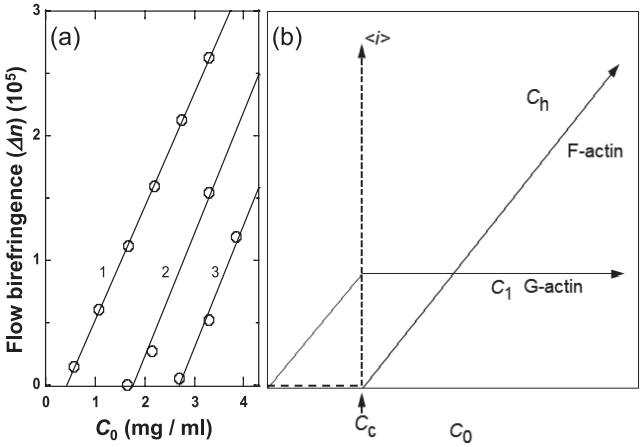

Figure 1 The specific features of the polymerization of actin as a condensation phenomenon. (a) The experimental relation between the degree of flow birefringence (Δn) (dependent on the concentration of F-actin) and the total actin concentration (C_0) at various temperatures, i.e., 20°C (1), 6°C (2) and 0°C (3). Actin concentration, 3.4 mg mL^{-1}; 0.4 mM $MgCl_2$; 7.5 mM veronal-HCl buffer of pH 8.3; 0.75 mM ATP. (b) The theoretical relation between the concentrations of G- (C_i) or F-actin (C_h) and the total actin concentration (C_0). The critical concentration of actin polymerization (C_c) is inversely proportional to the absolute temperature. The figure is reproduced from previously published figure [3].

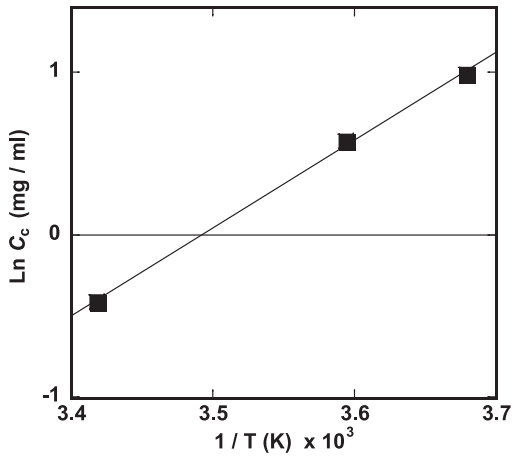

Figure 2 van't Hoff plot for the critical concentration (C_c) of actin polymerization originally obtained by Michiki Kasai. The figure is reproduced from previously published figure [1].

area of actin in contact with aqueous solution decreases, so that the entropy of the system will become larger due to the contribution to the entropy of the water molecules released from actin. Is it so simple and correct? Previously, an investigation of the hydration amount and the volume change caused from G-F transformations [5] suggested that about a quarter of hydration water of an actin monomer was released during the polymerization [6,7]. The hydration state of F-actin also depends on the bound cations [8]. Can the release of hydration water molecules completely explain the entropy change caused from the polymerization equilibrium? What should we do to make this point clearer? This is our next question.

On the Intramolecular Structure of Actin

In the above section, I wrote that the common approach may be to consider that the entropy changes of actin molecules occur due to the increase and decrease in hydration of the whole molecule. However, what happens if intramolecular hydration exists here and there in actin? My suggestion is to examine the intramolecular structure of actin in more detail without limiting the problem so narrowly. Three-dimensional structures of many protein molecules have been revealed by conventional X-ray crystallography. In the case of actin, its structure was revealed in 1990 and has not changed much since then [9]. It is perfect. However, it is only about the conformation of main chains. All amino acids have respective side chains. So my interests are on the states and conformations of side chains. The side chains of amino acids may be fluctuating and partly rotating, so that various cases are possible because their conformations and movements may be different under different conditions.

The current analysis of the molecular structure obtained, e.g., by X-ray crystallography, mainly reveals a static structure and hardly gives information of intramolecular movements. But, for example, if the tips of side chains freely rotate, the effect on the entropy changes would be large. There might be local thermal fluctuations that are explainable as local temperature. How deeply has such dynamic characteristics of the molecular structure been understood by the structural analysis so far? The papers on structural analyses of actin published since 1990 are listed in Table 2 with some comments [10–16].

Actually, in the case of actin molecule, since I imagine a local thermal ratchet in the structure, I have a deep interest in such fluctuations of internal atomic groups. In short, the dynamic analysis of the atomic structures of individual molecules is awaited in addition to the static analysis of the intramolecular structure. It also contains hydration problems.

Flexibility of Actin Filaments

In 1954, it was found that the thin filament aligned in parallel within the I-band of striated muscle was the actin filament (F-actin). The thin filaments were observed to "slide" against the myosin thick filaments by the interaction with myosin molecules without bending or changing the length [17,18]. Since then, researchers did not pay much attention to whether the actin filaments, i.e., the thin filaments, are flexible and pliant. However, it is natural to consider that the actin filament is not a rigid rod but is a semi-flexible polymer when the filament becomes longer and its tangent becomes measurable, because the actin filament is a helical polymer in which globular actin molecules are non-covalently linked together.

Table 1 Experimentally estimated thermodynamic quantities of actin polymerization equilibrium

[#]Conditions	temp. (°C)	T (K)	C_c (μM)	$\Delta G^{\$}$ (cal/mol)	$\Delta H^{\$}$ (cal/mol)	$\Delta S^{\$}$ (cal/molK)
K 30 mM Mg-actin	4	277.15	1.44	–7399	5334	45.9
〃	18	291.15	0.7	–8192	〃	46.5
〃	23.5	296.65	0.84	–8240	〃	45.8
Na 30 mM Mg-actin	4	277.15	0.43	–8069	2434	38.5
〃	18	291.15	0.26	–8752	〃	38.4
〃	23.5	296.65	0.35	–8751	〃	37.7
K 30 mM Ca-actin	4	277.15	3.01	–6993	3906	39.3
〃	18	291.15	2.41	–7475	〃	39.1
〃	23.5	296.65	1.81	–7786	〃	39.4
Na 30 mM Ca-actin	4	277.15	2.06	–7203	2652	35.6
〃	18	291.15	1.85	–7628	〃	35.3
〃	23.5	296.65	1.43	–7923	〃	35.6
*Low ionic condition (Kasai's [1,2])	temp. (°C)	T (K)	C_c* (μM)	ΔG* (cal/mol)	ΔH* (cal/mol)	ΔS* (cal/molK)
0.4 mM $MgCl_2$	0	273.15	61.9	–5254	14407	72
〃	6	279.15	40.8	–5600	〃	71.7
〃	20	293.15	10.4	–6674	〃	71.9

T; absolute temperature (K), C_c; critical concentration for polymerization, ΔG; Gibbs' free energy change of polymerization equilibrium, ΔH; enthalpy change of polymerization equilibrium, ΔS; entropy change of polymerization equilibrium. The 5% pyrene-labeled actin that bound Ca^{2+} or Mg^{2+} was polymerized in buffers including potassium or sodium ions overnight at each indicated temperature to measure C_c by monitoring pyrene fluorescence with excitation wavelength of 366 nm and emission wavelength of 407 nm [4]. Because the actin polymerization satisfies a two-phase thermodynamic equilibrium known as the *Clausius-Clapeyron* relation [1, 2], the thermodynamic quantities were determined from the slope and the 1/T axis-intercept of the linear regression line of the van't Hoff plot (ln $C_c=\Delta G/RT=\Delta H/RT-\Delta S/R$, where R is the gas constant [4]) (see Fig. 2).

[$] Other thermodynamic parameters calculated from C_c and T; $\Delta G=RT\ln C_c$, $\Delta H=R\cdot$(slope of $\ln C_c$-1/T plot), $\Delta S=(\Delta G-\Delta H)/T$

* Calculated and read data from the figures in refs [1] and [2].

[#] Conditions: K 30 mM Mg-actin and Na 30 mM Mg-actin indicate that Mg^{2+}-bound actin molecules were polymerized in 5 mM Tris-HCl, pH 8.0 and 30 mM KCl or NaCl, respectively. K 30 mM Ca-actin and Na 30 mM Ca-actin indicate that Ca^{2+}-bound actin molecules were polymerized in 5 mM Tris-HCl, pH 8.0 with 30 mM KCl or NaCl, respectively.

The very first experiment to examine whether an actin filament is practically a string-like bendable structure in aqueous solutions was done by Fujime [19]. He applied quasi-elastic scattering of laser light to observe the thermal bending fluctuation and quantitatively determined the bending stiffness of actin filaments [19]. This method detects small Doppler broadening of frequency of laser light due to scattering from the actin filaments showing Brownian translational, rotational and bending movements. From the degree of broadening, which depended on the scattering angle, Fujime attributed a part of the broadening to the bending movement of actin filaments and estimated their flexural rigidity. The results of this experiment were reported in 1970 [19].

Since then, it took nearly 10 years for the notion "the actin filament is semi-flexible" to be recognized. It was finally confirmed by direct visualization of the Brownian motions of individual actin filaments in solution by technical advances in dark-field optical microscopy by Nagashima and Asakura [20]. This technique allowed us to perform quantitative measurements of the average amplitude and temporal frequency of thermal bending motions of actin filaments, and the results were consistent with those obtained by quasi-elastic scattering of laser light [20]. The direct observation and analysis that demonstrated the semi-flexible structure of actin filament were reported in 1978 [20].

Although it may sound easy, it actually took 10 years to convince researchers about the flexibility of the filaments. Nowadays, no one sticks to the idea that actin filaments are "rigid rods".

Then, the recent advances in electron cryo-microscopy techniques have made it possible to examine the detailed intramolecular structure of actin filament. Each filament looks like a straight rod in electron cryo-micrographs of ice-embedded frozen-hydrated specimens. Not only the intramolecular structures but also intermolecular binding interactions between actin subunits within the filament have been visualized in detail [11–15]. To apply this technique, the solution of actin filaments has to be quickly frozen by plunging the electron microscopy grid into liquid ethane to avoid crystallization of ice. It is considered that the reason why each filament looks straight instead of curved seems to be attributable to this method of quick freezing without chemical fixation including staining.

By recent technical advances, it has become possible to custom-design the structure of actin filaments. For example,

Table 2 List of F-actin structures at near-atomic levels, associating with dynamics of actin polymerization and interactions with other actin binding proteins

1)	In 1990, Kabsch *et al.* and Holmes *et al.* showed that structures of G-actin [9] and F-actin [10] are not so extremely different, except the hydrophobic plug. This plug region was thought to stabilize the two strands of filament structure, but it was proved later by the structural analysis of F-actin by electron cryo-microscopy that the plug is actually a hydrophilic one by von der Ecken *et al.* [16].
2)	In 2009, Oda *et al.* showed the nature of G- to F-actin conformational transition by using X-ray fiber diffraction analysis together with cryo-electron microscope's data by Narita [11]. The transition is caused by a rotation of two major domains of actin, and actin molecules become flat by this rotation upon formation of the filament.
3)	In 2010, Fujii *et al.* used a novel electron cryomicroscopy technique and reported a three-dimensional density map of actin filaments [12]. The map showed all secondary structures of actin molecule in the filament structure. This map confirmed that actin is flat in the filament and that it forms one conformation. The map also visualized four residues at the N-terminus, which play important roles in the binding of other proteins such as myosin.
4)	In 2010, Murakami *et al.* showed the electron cryomicroscopic structure of actin filaments with intermolecular interactions mediated by Mg^{2+} or inorganic phosphate (Pi) [13]. They showed conformational changes orchestrated with actin polymerization and suggested four steps in actin ATPase cycle.
5)	In 2010, Galkin *et al.* found six different modes in the skeletal muscle F-actin structure [14].
6)	In 2015, Galkin *et al.* improved the resolution of electron cryomicrographs and reconstituted two classes of F-actin structure [15]. Both F-actin maps possess a significant opening of the ATP-binding cleft. These two actin structures are different from each other regarding the movement of subdomain 4 (SD4) toward SD2. One of these actin structures takes a form to easily release Pi after ATP hydrolysis.
7)	In 2015, von der Ecken *et al.* found that the binding of G-actin to the barbed-end of F-actin is initiated by the intra-strand binding of SD4 of G-actin to SD3 of actin molecule in F-actin [16]. Then the D-loop of G-actin is trapped in the hydrophobic cleft of SD3 of actin molecule in F-actin and pulls on SD2. However, the binding of G-actin at the pointed-end is more complicated. Including the processes described above, in total seven processes are required for G-actin to bind to the pointed-end of F-actin.

After 2010, the resolution of F-actin structural analyses is significantly improved. This allows us to discuss the dynamics of F-actin structure at a near-atomic level.

a circular bundle of actin filaments similar to the contractile ring, which appears on the occasion of cell division, has been reconstituted within a water-in-oil droplet covered with a mono-layered phospholipid membrane by Miyazaki *et al.* [21]. The rotational movement of actin rings was accidentally found by Higashi-Fujime when she squeezed out the cytoplasm of *Nitella* cells and observed the dynamics of filamentous structures under an optical microscope [22]. This ring showed a rapid rotational movement when ATP was present. I still do not know how such an ultra-high speed rotation can be achieved by myosin motors of *Nitella*.

Formation and Severing of Actin Filaments

Actin filaments take various conformations from moment to moment, especially beneath the plasma membrane in living cells. Through elongation, branching, shortening by polymerization, severing, depolymerization and so on, actin filaments form a network structure over a wide area in a cell. This actin-based cell motility is orchestrated in cooperation with various regulatory proteins and other kinds of filaments formed by various cytoskeletal proteins. More than several tens of proteins are involved in the formation of such network structures. Here I will describe proteins having a severing effect on actin filaments, on which research has recently been advanced. This is the case of actin extracted from *Physarum polycephalum* by Hatano *et al.* [23]. In particular, in the presence of Ca^{2+}, this actin filament becomes short and flexible. Later, a novel protein that severs actin filaments was extracted from *P. polycephalum* and named fragmin, because *Physarum* actin filaments become more flexible and are easily severed when fragmin is added [24].

Upon adding fragmin and ATP to the solution of *Physarum* actin filaments in the presence of Ca^{2+}, the filaments are immediately broken to small fragments and scattered around. When I saw this phenomenon, I remembered the experiment done by Asakura a long time ago as described below. To visualize individual actin filaments under a dark-field microscope, he decorated actin filaments with heavy meromyosin (HMM: a proteolytic fragment of myosin molecule). This increased the light scattering intensity and enabled him to observe Brownian movements of actin filaments. It was not possible to visualize individual actin filaments in solution without HMM decoration when this experiment was done in 1978 [20]. This was an epoch-making experiment that made it possible to directly visualize individual actin filaments even though the filaments were thickened by decoration with HMM. This experiment proved that actin filaments are semi-flexible and show bending Brownian movements in solution.

During a series of these experiments, Asakura succeeded in visualizing individual actin filaments under a fluorescence microscope by labeling them with a fluorescent dye, i.e., fluorescein (FITC) [25]. Asakura investigated the effects of myosin subfragment-1 (S1: a proteolytic fragment of myosin molecule or a myosin motor domain, as a part of HMM having the ability to interact with actin and ATPase) on fluorescent actin filaments in the presence of ATP. Actin fila-

Table 3 Rate regulation of polymerization of actin*

Polymerization process (Bond formation)	Depolymerization process (Bond breaking)
Nucleotides	Mechanical agitation
Divalent cations	Thermal agitation
Myosin	Myosin+ATP
Polymer nuclei	
α-actinin, one of the actin accessory proteins, that was found more than 50 years ago [41, 42] has a regulatory function for polymerization of actin.	

* Adopted from Ciba Foundation Symposium at 1966 [27].

ments were severed rapidly into small fragments. I vividly remember that Asakura showed me this phenomenon several times. There was no need to use actin purified from *Physarum* or use fragmin. S1 (with ATP) possesses an intrinsic ability to sever actin filaments.

The reason why I focused on such properties would be because I had been carefully observing the "super precipitation phenomenon" that occurs in the mixed solution of actin filaments and myosin molecules with ATP. In this assay, we often observed the severing and fragmentation of actin filaments by using electron microscopy.

Note that, in 1969, Tawada and myself published a paper showing that the elongation of actin filaments stops when S1 and ATP are added at the same time [26] (This was the last paper that described my own experiments).

Many interesting experiments remain to be done to carefully investigate behaviors of actin filament and myosin in the presence of ATP at various levels of combinations and conditions. I wonder if there is a point overlapping between the properties summarized in the Proceedings of Ciba Foundation Symposium 50 years ago (Table 3, [27]) and those presented by Uyeda and his colleagues in the Actin Symposium held in 2016 in Nagoya [28,29]. It was a great pleasure to see many presentations on various severing proteins of actin filaments, such as fragmin [24], gelsolin (e.g., [30]), villin (e.g., [31]), ADF/cofilin (e.g., [32]) and Pf ADF1 (e.g., [33]), and electron microscopic observations on actin polymers at this Actin Symposium (see [4,29,34–40]).

At the end, it is my pleasure to share a letter from Prof. Donald L. D. Caspar at Florida State University, USA. He collaborated with Kasai and Asakura through his stay in Japan and has made outstanding contributions to our understanding of the functions and mechanisms of biological macromolecular assemblies such as TMV, flagella and actin filaments.

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Conflicts of Interest

The author declares that he has no conflict of interest.

Author Contribution

F. O. gave various thoughts on actin and wrote the manuscript.

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Appendix

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Dear Oosawa-sensei,

Congratulations on your 94th birthday and the celebration of your insightful analysis in collaboration with Kasai-san and Asakura-san of actin polymerization.
I first leaned about your and Kasai-san's classic 1962 Theory of Linear and Helical Aggregation of Macromolecules shortly after it was published in the Journal of Molecular Biology. At that time, with John Edsall's patient editorial encouragement, I was struggling to complete writing my theory of Assembly and Stability of the Tobacco Mosaic Virus Particle for the 1963 Volume of Advances in Protein Chemistry. Edsall recognized that the thermodynamic description that I was formulating for the equilibria in TMV helix assembly was fundamentally similar to the theory you had formulated for polymerization of the F-actin helix. In both systems, there is an equilibrium between monomers and polymers consisting on average of a very large number n of monomers. This equilibrium must depend on the nth power of monomer concentration and the equilibrium constant for growth. Since n is very large, the transition from dispersed molecules to long polymers takes place like a cooperative phase transition. In 1963, following Edsall's advice, I did note the homology between your analysis of the cooperative transition from the F-actin trimeric nucleus to polymer and my model for the transition from a hypothetical two-layer disk nucleus to helix; but at that time, I did not understand the nature of the physical forces that restrained nucleation.

The key control for these biological assembly processes is the energy barrier for starting off the process - that is nucleating the polymerization. You postulated a large energy barrier for forming a nucleating F-actin trimer from three linear G-actin monomers. By analogy with Asakura-san's demonstration that assembly of bacterial flagellin in vitro under physiological conditions requires a conformational change in the monomer, which can be catalyzed by preassembled polymer seeds, I postulated in 1980 that self-assembly of functional biological structures is self-controlled by an energetically unfavored switching from an unsociable to an associable conformational state, which can be stabilized by the energetically favorable bonds in the polymer.

Your $\sigma \sim 10^{-8}$ coefficient for forming the F-actin nucleus represents the low probability of switching three "kicking and screaming stochastic" G-actin monomers in solution into an orderly trimeric nucleus, stabilized by two sets of intermolecular bonds. Growth can then proceed by converting an unsociable monomer to the energetically unfavored associable conformation, which is stabilized by the set of energetically favored intermolecular bonds in the helical polymer. Since there are only two sets of bonds to stabilize the three associable monomers in the nucleating helix, the free energy change for its formation can be very

positive - thus an extremely low concentration of nucleating trimers. Since there is one set of stabilizing bonds for each monomer converted to the associable state in the growing polymer, the net free energy can favor growth. As you demonstrated, the critical concentration for polymer formation is the reciprocal of the equilibrium constant for monomer addition, which is defined by the free energy change for growth. Your formulation applies equally well to the linear polymerization of FtsZ, the bacterial tubulin, as it does to actin and eukaryotic tubulin and to any other polymer whose nucleation involves the energetically unfavorable conformational switching from an unsociable monomer to an associable polymer state.

Because G-actin in solution appears to be like one of Weber's "kicking and screaming stochastic" molecules, the nature of its conformational changes on assembly are ill defined. When a highly ordered F-actin molecule in a filament is confined to a thin layer of vitreous ice, it is a delight for a cryo electron microscopist who can determine its exact atomic coordinates. However, in solution, twisting and bending of F-actin filaments can remarkably modulate their functional properties. There is an attitude among many cell biologists that what actin can do on its own in solution is not of much interest since all its activities in cells are in concert with myriads actin related proteins. But what these related proteins can do to actin, depends on its intrinsic properties, which can be very adaptable depending on how they are coupled. Loose coupling, as conceived by you, implies more than one pathway between cause and effect and sometimes more than one possible outcome. Self-organization of cells in developing tissues or colony formation by social creatures may provide more appropriate analogies than mechanical models for the trial-and-error process by which actin molecules grow up and find their place in their societies. All actins from a given gene may be created equal, but the loose coupling involved in their folding and interactions allows for individual adaptability.

In conclusion, there is much to thank you for. Before I ever met you in the early 60s, your influence was evident. My brief sabbatical in Asakura-san's Nagoya lab that you organized for me in the spring of 1984 was a landmark. At the Nakatsugawa meeting that year, I learned how broad and creative your influence was on Japanese biophysical science and welcomed being included in your sphere of influence. Your legacy endures and will continue to grow.

With profound appreciation,

Donald L. D. Caspar