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Random Copolymerization of ATP–Actin and ADP–Actin†

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ABSTRACT: The equilibrium of the copolymerization of ATP–actin and ADP–actin was investigated by an analysis of the critical concentrations of mixtures of ATP–actin and ADP–actin. The molar ratio of bound ATP to bound ADP was controlled by the ratio of free ATP and ADP. The experiments were performed under conditions (100 mM KCl, 1 mM $MgCl_2$, pH 7.5, 25 °C) where the ATP hydrolysis following binding of actin monomers to barbed filament ends was so slow that the distribution of ATP or ADP bound to the subunits near the ends of filaments was not affected by ATP hydrolysis. According to the analysis of the critical concentrations, the equilibrium constants for incorporation of ATP–actin or ADP–actin into filaments were independent of the type of nucleotide bound to contiguous subunits.

ATP hydrolysis, which occurs during polymerization of actin, has been shown to bring about treadmilling of actin due to different critical monomer concentrations of the two ends of actin filaments (Wegner, 1976; Pollard & Mooseker, 1981; Selve & Wegner, 1986a). Evidence has been provided that at polymerizing barbed ends ATP hydrolysis lags behind polymerization (Pardee & Spudich, 1982; Carlier et al., 1984; Pollard & Weeds, 1984; Grazi et al, 1984). Thus, polymerizing barbed ends carry terminal ATP subunits ("ATP-cap"). Polymerization at the pointed ends is so slow that the time between two association reactions is sufficient for ATP hydrolysis to occur. The pointed ends do not carry an ATP cap (Coué & Korn, 1986). Treadmilling of actin is brought about by the existence of ATP caps at the barbed ends and the terminal ADP subunits at the pointed ends. ATP-capped ends have a higher apparent affinity for actin monomers than ADP-bearing terminal subunits. Thus, the ATP-capped barbed ends tend to polymerize while the pointed ends depolymerize.

As ATP hydrolysis by actin is an important function which is a requirement for formation of copolymers of ATP–actin and ADP–actin and for treadmilling, many studies on the polymerization of ATP–actin, ADP–actin, and ATP hydrolysis have been reported (Straub & Feuer, 1950; Asakura & Oosawa, 1960; Hayashi & Rosenbluth, 1960). Structural differences between filaments polymerized from ATP–actin or from ADP–actin have been observed (Janmey et al., 1990). Filaments formed by polymerization of ATP–actin appear to be more stiff than ADP–actin filaments. Furthermore, ADP–actin filaments are more disordered. ATP–actin turned out to polymerize faster than ADP–actin. Filaments formed from ATP–actin are more stable than those formed from ADP–actin (Higashi & Oosawa, 1965; Cooke, 1975; Pantaloni et al., 1984; Lal et al., 1984).

Evidence has been provided which suggests that interactions between ATP subunits are rather weak as compared to heterologous interactions between ATP subunits and ADP subunits (Carlier et al., 1985; Pantaloni et al., 1985). However, this model had to be extended to accommodate for an intermediate of ATP hydrolysis which has been proposed to occur in filaments. Actin subunits with bound ADP and phosphate were thought to accumulate before the phosphate is released into solution (Carlier & Pantaloni, 1988; Korn et al., 1987).

In this paper, we investigate the question of cooperative interactions between ATP subunits and ADP subunits, thereby performing the experiments under conditions where there is no hint for the occurrence of subunits with bound ADP and phosphate. We measured critical concentrations of copolymers of ATP–actin and ADP–actin and analyzed the results in terms of cooperativity.

Materials and Methods

Preparation of Actin. Rabbit skeletal muscle actin was prepared according to Rees and Young (1967). Part of the

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protein was modified with *N*-ethylmaleimide at cysteine-374 and subsequently with 4-chloro-7-nitro-2,1,3-benzoxadiazole at lysine-373 to produce a fluorescently labeled actin (Detmers et al., 1981). The concentration of actin was determined photometrically at 290 nm by using an absorption coefficient of 24 900 M^{-1} cm^{-1} (Wegner, 1976).

Preparation and Purification of the Nucleotides. 1,N^6-Ethenoadenosine 5′-triphosphate (ε-ATP) was synthesized according to Secrist et al. (1972) with the modification that the crude product was applied to a DEAE-Sephadex A-25 column (2.5 × 40 cm). ε-ATP was eluted with a linear NH_4HCO_3 gradient (0.15–0.33 mM) (Wanger & Wegner, 1983). According to HPLC [Mono-Q-column, 5 mM triethanolamine hydrochloride, pH 7.5, KCl gradient (10–1000 mM)], no impurities were detected in the ε-ATP preparation.

ADP was separated from traces of ATP by chromatography on a DEAE-Sephadex A-25 column (2.5 × 40 cm). ADP was eluted with a linear NH_4HCO_3 gradient (0.15–0.33 mM). According to HPLC analysis (see above), the purified ADP was free of ATP. ATP was a commercially available product and used without further purification. Concentrations of the nucleotides were based on the following molar absorption coefficients: ε-ATP (275 nm), 5.6 × 10^3 M^{-1} cm^{-1} (Secrist et al., 1972); ATP, ADP (259 nm), 15.4 × 10^3 M^{-1} cm^{-1} (Dunn & Hall, 1970). ADP–actin was prepared by exchange of ATP for ADP on a Sephadex G-25 superfine column (2.5 × 70 cm) equilibrated with 0.5 mM ADP, 10 μM $MgCl_2$, 5 mM triethanolamine hydrochloride, pH 7.5, and 200 mg/L NaN_3. ε-ATP–actin was prepared by exchange of ATP for ε-ATP on a Sephadex G-25 superfine column (2.5 × 70 cm) equilibrated with 350 μM ε-ATP, 0.2 mM $CaCl_2$, and 5 mM triethanolamine hydrochloride, pH 7.5.

Fluorescence. Actin polymerization was followed by the 2.2–2.5-fold greater fluorescence intensity of polymeric actin compared to that of monomeric actin (Detmers et al., 1981). Five percent of fluorescently labeled actin was copolymerized with unmodified actin. This low proportion of labeled actin does not significantly alter the polymerization rate or extent of assembly of unmodified actin (Wegner, 1982). The excitation wavelength was 480 nm, and the fluorescence intensity was measured at 540 nm.

The exchange of actin-bound nucleotide was measured by the increase of the fluorescence intensity on binding of ε-ATP to monomeric actin (Miki et al., 1974). The excitation wavelength was 360 nm, and the emitted light was measured at 410 nm.

Critical Concentrations. The concentrations of monomeric ADP–actin and ATP–actin coexisting with polymeric actin (critical monomer concentration) were determined both after depolymerization of polymeric actin and after polymerization of monomeric actin onto actin filaments. Actin filaments (4 μM) were prepared by adjusting monomeric ATP–actin in buffer A (0.5 mM ATP, 0.2 mM $CaCl_2$, 5 mM triethanolamine hydrochloride, pH 7.5, and 200 mg/L NaN_3) and monomeric ADP–actin in buffer B (0.5 mM ADP, 10 μM $MgCl_2$, 5 mM triethanolamine hydrochloride, pH 7.5, and 200 mg/L NaN_3) to 100 mM KCl and 1 mM $MgCl_2$. Samples were prepared by combining 1 M KCl, 20 mM $MgCl_2$, buffer A, buffer B, monomeric ATP–actin, monomeric ADP–actin, and finally polymeric actin. The ratio between monomeric ADP–actin and ATP–actin was adjusted by the ratio between ADP (buffer B) and ATP (buffer A). The solutions were mixed in such a ratio that the final composition of the samples was 100 mM KCl, 1 mM $MgCl_2$, about 0.1 mM $CaCl_2$, 5 mM triethanolamine hydrochloride, pH 7.5, 200 mg/L NaN_3, and 0.5 mM nucleotides (ADP plus ATP). The initial concentration of polymeric actin was 1 μM; 0–2.4 μM monomeric actin was added to polymerized actin. The initial and the final fluorescence intensities were measured. The differences between the initial and the final fluorescence intensity were plotted versus the concentration of added monomers. The concentration of added actin monomers at which the fluorescence remains constant was taken as the critical concentration [see Figure 7 of Selve and Wegner (1986b)]. All experiments were performed at 25 °C because ADP–actin is known to be unstable at higher temperatures (Lal et al., 1984).

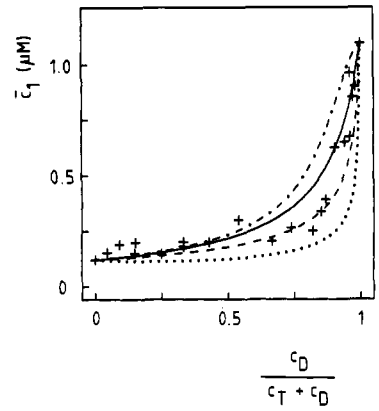

FIGURE 1: Plot of the critical monomer concentration ($\bar{c}_1$) versus the molar fraction of ATP–actin (c_T) and ADP–actin (c_D). (+) Measured critical concentrations. (—) Critical concentrations calculated for uncooperative random copolymerization (K_{TT} = 8.33 × 10^6 M^{-1}, K_{DD} = 0.91 × 10^6 M^{-1}, $K_{DT}K_{TD} = K_{TT}K_{DD}$). (- - -) Heterologous interactions between ATP subunits and ADP subunits are 4-fold stronger than homologous interactions ($K_{DT}K_{TD} = 4K_{TT}K_{DD}$). (···) Heterologous interactions are 16-fold stronger than homologous interactions ($K_{DT}K_{TD} = 16K_{TT}K_{DD}$). (-·-) Heterologous interactions are 4-fold weaker than homologous interactions ($K_{DT}K_{TD} = 1/4K_{TT}K_{DD}$).

Determination of ATP and ADP. ADP which is produced by actin filaments at the critical monomer concentrations was determined by HPLC analysis. The actin solution was obtained by dilution of polymeric actin (20 μM) to obtain a 3 μM actin sample dissolved in 100 mM KCl, 1 mM $MgCl_2$, about 0.1 mM $CaCl_2$, 0.1 mM ATP, 5 mM triethanolamine hydrochloride, pH 7.5, and 200 mg/L NaN_3. ATP hydrolysis was stopped by the addition of 0.2 mL of 2 M $HClO_4$ to 0.8-mL actin samples after various times. Following centrifugation, 2 M $KHCO_3$ was added to the supernatant to adjust the pH to a value of 7 and to precipitate ClO_4^- ions as $KClO_4$ crystals. The samples were applied to the HPLC [Mono Q column, 5 mM triethanolamine hydrochloride, pH 7.5, KCl gradient (10–1000 mM)]. The ADP peak was calibrated by solutions of known ADP concentrations.

Results and Discussion

Equilibrium of the Copolymerization of ATP–Actin and ADP–Actin. The critical concentration of actin was determined at various ratios of ATP–actin to ADP–actin. Figure 1 shows that, under the experimental conditions, the critical concentration of pure ADP–actin is about 10-fold higher (1.1 μM) than that of ATP–actin (0.12 μM). Relatively small amounts of ATP monomers decrease the critical concentration of ADP–actin appreciably. When 4% ATP monomers are present in ADP–actin, the critical concentration is reduced by 40% compared to that of ADP–actin.

The value of the critical concentration depends on the affinities of ATP monomers or ADP–actin for ATP ends or ADP-carrying subunits. For quantitative evaluation of the results depicted in Figure 1, it is neessary to derive a correlation between the critical concentration and the affinities of the two

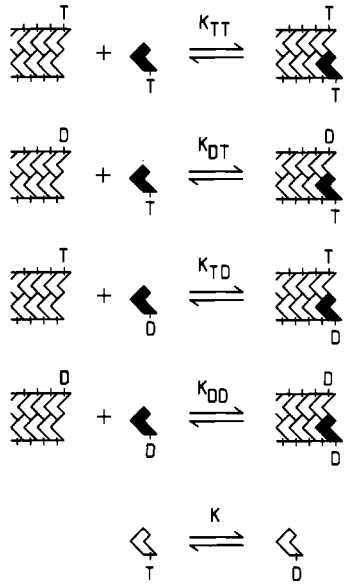

FIGURE 2: Reaction scheme of copolymerization of ATP-actin and ADP-actin and of the exchange of monomer-bound ATP and ADP.

types of actin monomers for the two types of filament ends.

Critical Concentration of Copolymers of ATP-Actin and ADP-Actin. A scheme of the association and dissociation reactions of ATP-actin and ADP-actin at the barbed end of filaments is depicted in Figure 2. As assembly both of ATP-actin and of ADP-actin at the barbed end is considerably faster than at the pointed end (Pollard, 1986), the barbed ends have a great weight in determining the critical monomer concentration, and the contribution of the pointed ends to the critical monomer concentration is negligible. The equilibrium concentrations of actin monomers can be derived by using the equations of the law of mass action:

$$K_{TT}c_T = \frac{p_{TT}}{p_{TT} + p_{DT}} \quad (1)$$

$$K_{DT}c_T = \frac{p_{DT}}{p_{TD} + p_{DD}} \quad (2)$$

$$K_{TD}c_D = \frac{p_{DT}}{p_{TT} + p_{DT}} \quad (3)$$

$$K_{DD}c_D = \frac{p_{DD}}{p_{TD} + p_{DD}} \quad (4)$$

$$p_{TT} + p_{TD} + p_{DT} + p_{DD} = 1 \quad (5)$$

c_T and c_D are the concentrations of ATP monomers or ADP monomers, respectively. The association equilibrium constants are defined in Figure 2. p_{TT}, p_{TD}, p_{DT}, and p_{DD} are the probabilities that the next to the terminal subunit and the terminal subunit carry ATP (T) or ADP (D). The first subscript relates to the next to the terminal subunit, and the second subscript relates to the terminal subunit. The probabilities can be eliminated by an appropriate combination of eq 1-5:

$$K_{DT}K_{TD}c_Tc_D = (1 - K_{TT}c_T)(1 - K_{DD}c_D) \quad (6)$$

The concentrations of ATP monomers and ADP-actin are given by three independent equilibrium parameters, namely, K_{TT}, K_{DD}, and $K_{DT}K_{TD}$ (eq 6). Figure 3 shows the effect of cooperativity on the total critical concentration. If homologous interactions between ATP monomers and ATP ends or ADP monomers and ADP ends are stronger than heterologous interactions ($K_{TT}K_{DD} > K_{DT}K_{TD}$), the total critical concentration increases as compared to uncooperative copolymerization ($K_{TT}K_{DD} = K_{DT}K_{TD}$). In the case of strong heterologous

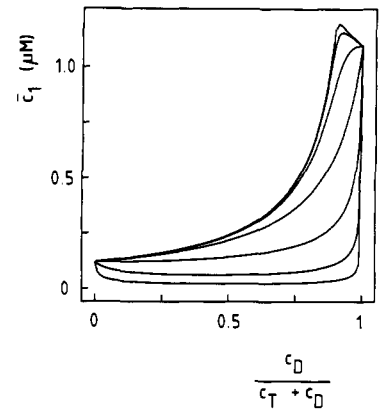

FIGURE 3: Calculated plots of the critical monomer concentration ($\bar{c}_1$) versus the molar fraction of ATP-actin (c_T) and ADP-actin (c_D). Upper three curves, heterologous interactions are weaker than homologous interactions ($K_{DT}K_{TD} = 10^{-3}K_{TT}K_{DD}$, $10^{-2}K_{TT}K_{DD}$, and $10^{-1}K_{TT}K_{DD}$). Middle curve, uncooperative random copolymerization ($K_{DT}K_{TD} = K_{TT}K_{DD}$). Lower three curves, heterologous interactions between ATP subunits and ADP subunits are stronger than homologous interactions ($K_{DT}K_{TD} = 10^{1}K_{TT}K_{DD}$, $10^{2}K_{TT}K_{DD}$, and $10^{3}K_{TT}K_{DD}$). For calculation of the curves, the values of K_{TT} and K_{DD} determined by measurements of the critical concentrations of pure ATP-actin and ADP-actin were applied ($K_{TT} = 8.33 \times 10^6$ M^{-1}, K_{DD} 0.9124×10^6 M^{-1}).

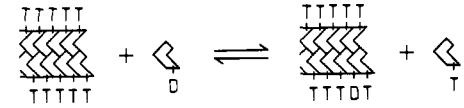

FIGURE 4: Exchange of an ATP subunit for an ADP subunit. The equilibrium of this exchange reaction is given by $K_{DT}K_{TD}/K_{TT}^2$.

interactions ($K_{DT}K_{TD} > K_{TT}K_{DD}$), the total critical concentration decreases below the value of uncooperative copolymerization (Figure 3).

The equilibrium distribution of ATP subunits and ADP subunits in the filament depends on the three equilibrium parameters K_{TT}, K_{DD}, and $K_{DT}K_{TD}$. In Figure 4, an example for a change of the distribution of ATP subunits and ADP subunits within a filament is depicted. The equilibrium of this exchange of an ATP subunit for an ADP subunit is given by two of the three equilibrium parameters ($K_{DT}K_{TD}/K_{TT}^2$). In a similar manner, it can be demonstrated that the equilibrium between different distributions of ATP subunits and ADP subunits in filaments depends only on the three equilibrium parameters. Thus, on the basis of measurements of critical concentrations and their evaluation, one can make predictions about the equilibrium distribution of the two types of subunits in filaments. Strong interactions between homologous subunits bring about formation of long clusters of ATP subunits and of ADP subunits with a few interfaces between the clusters. Strong interactions between heterologous subunits lead to a distribution in which ATP subunits and ADP subunits occur preferentially alternately in a filament (Zimm & Bragg, 1958). However, it should be mentioned that, according to the definition given in this paper, in the case of uncooperative copolymerization an ATP monomer binds to an ATP end not necessarily with the same affinity as to an ADP end although the distribution of ATP subunits and ADP subunits within a filament is random. For instance, it is possible that the affinity of ATP monomers for an ATP end is greater than that for an ADP end by a factor of n ($K_{TT}/K_{DT} = n$). This difference could be compensated by a n-fold lower affinity of ADP monomers for ADP ends as compared to that for an ATP end ($K_{DD}/K_{TD} = 1/n$; $K_{DT}K_{TD}/K_{TT}K_{DD} = n/n = 1$).

Exchange of Monomer-Bound ATP and ADP. The ratio between the concentrations of ATP monomers and ADP

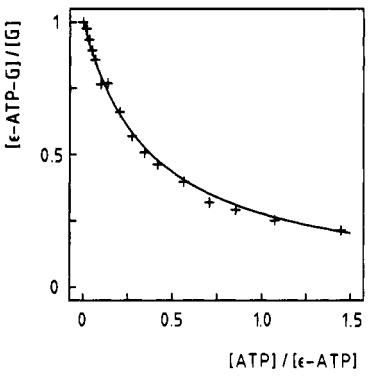

FIGURE 5: Titration of ε-ATP–actin with ATP. [ε-ATP], concentration of ε-ATP; [ATP], concentration of ATP; [ε-ATP–G], concentration of actin monomers with bound ε-ATP; [G], total concentration of actin monomers. (—) Fit of the equilibrium constant of exchange of actin-bound ε-ATP for ATP, K_T (K_T = 2.6).

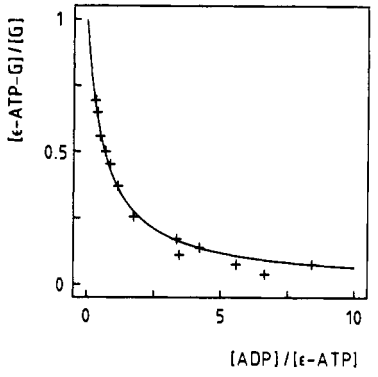

FIGURE 6: Titration of ADP–actin with ε-ATP. [ε-ATP], concentration of ε-ATP; [ADP], concentration of ADP; [ε-ATP–G], concentration of actin monomers with bound ε-ATP; [G], total concentration of actin monomers. (—) Fit of the equilibrium constant of exchange of actin-bound ε-ATP for ADP, K_D (K_D = 1.5).

monomers depends on the exchange of these nucleotides. If this ratio is known, the total actin monomer concentration, $\bar{c}_1$, which comprises ATP monomers and ADP monomers ($\bar{c}_1 = c_T + c_D$), can be evaluated with the aid of eq 6.

The exchange of monomer-bound ATP for ADP was measured by an assay which has been described by Miki (1974), Waechter and Engel (1975), Neidl and Engel (1979), and Wanger and Wegner (1983). This assay takes advantage of the 40-fold increase of fluorescence on binding of the ATP analogue ε-ATP to actin monomers. ε-ATP–actin monomers were titrated with ATP or ADP. Displacement of ε-ATP by ATP or ADP was measured by a decrease of the fluorescence intensity. The results are depicted in Figures 5 and 6. The equilibrium constants for exchange of ε-ATP for ATP (K_T) or ADP (K_D) were fitted by seeking those values of K_T and K_D for which the best agreement between calculations and measurements was achieved. K_T turned out to be 2.6, and K_D was found to be 1.5. On the basis of these values, the equilibrium constant for exchange of monomer-bound ATP for ADP (K) can be calculated to be ~2 ($K = K_T/K_D$) (eq 7).

$$K = \frac{K_T}{K_D} = \frac{c_D T}{c_T D} \tag{7}$$

This value is in good agreement with a previous report in which it has been demonstrated that at physiologically relevant salt concentrations ATP has a slightly higher affinity for actin monomers than ADP (Wanger & Wegner, 1983). The ratio of the concentrations of ATP monomers and ADP monomers was adjusted by the concentrations of ATP and ADP. The nucleotides were applied in an excess over actin so that the free nucleotide concentrations (T, D) were practically equal to the total concentrations.

ATP Hydrolysis by Actin. Actin-bound ATP is known to be hydrolyzed following incorporation of an ATP monomer into a filament (Straub & Feuer, 1950; Asakura & Oosawa, 1960). The phosphate resulting from ATP hydrolysis remains bound to filament subunits and is subsequently released in a slow reaction (Carlier & Pantaloni, 1988; Korn et al., 1987). It is conceivable that ATP is hydrolyzed at the subunits near the ends of actin filaments so fast that the equilibrium distribution of terminal ATP-carrying and ADP-carrying subunits is perturbed by the conversion of subunit-bound ATP into ADP. We, therefore, measured the rate of ATP hydrolysis of polymeric actin solutions and compared this rate to the rate of binding of actin monomers to filament ends. The rate of ATP hydrolysis of a 3 μM polymeric actin solution produced by dilution of 20 μM polymerized actin was found to be as low as 0.5–0.6 μM/h. The rate (v) of binding of monomeric ATP–actin to filament ends at the critical monomer concentration was calculated from the half-lifetime ($t_{1/2}$) of polymerization of monomeric ATP–actin onto 3 μM polymeric actin. The rate of monomer binding is given by (Ruhnau et al., 1989)

$$v = k_b^+ \bar{c}_1 c_p = \bar{c}_1 \frac{\ln 2}{t_{1/2}} \tag{8}$$

where k_b^+ is the rate constant for binding of monomeric ATP–actin to barbed filament ends and c_p is the concentration of barbed filament ends. The half-lifetime of polymerization, $t_{1/2}$, was determined to be 135 s. Thus, the rate of monomer binding can be calculated to be about 2.2 μM/h. As the rate of monomer binding is considerably faster than that of ATP hydrolysis, the distribution of nucleotides at the subunits near the end of filaments is expected not to be substantially affected by ATP hydrolysis. The distribution of ATP-carrying and ADP-carrying subunits near the ends of filaments can be approximately treated as an equilibrium. It is possible that under conditions different from ours subunits with bound ADP and phosphate may play an important role in the polymerization reaction of actin. It is known that experimental conditions (ions, temperature) affect the mechanism of actin polymerization (Frieden 1983; Gershman et al., 1984; Grazi et al., 1984; Colombo et al., 1991). We attempted to render our experimental conditions as similar to physiological salt concentrations as possible (1 mM $MgCl_2$, 100 mM KCl, pH 7.5).

Evaluation of the Critical Concentrations. The critical concentrations of copolymers of ATP–actin and ADP–actin were evaluated by using eq 6 and 7. The equilibrium constants for binding of ATP monomers to an ATP end (K_{TT}) and for binding of ADP monomers to an ADP end (K_{DD}) can be easily extracted from the experimental data. These equilibrium constants are equal to the reciprocal critical concentrations of pure ATP–actin solutions or pure ADP-actin solutions, respectively (eq 6). According to Figure 1, K_{TT} turns out to be 8.3×10^6 M^{-1} (1/0.12 μM), and K_{DD} can be evaluated to be 0.9×10^6 M^{-1} (1/1.1 μM). The shape of the plot of the critical concentration versus the molar fraction of ATP–actin and ADP–actin depends on the value of the third equilibrium parameter, $K_{DT}K_{TD}$. In Figure 1, critical concentrations were calculated for various values of $K_{DT}K_{TD}$. Good agreement of the calculated curves with the measurements was achieved if copolymerization was assumed to be uncooperative ($K_{DT}K_{TD} = K_{TT}K_{DD}$). A slightly better agreement was obtained if heterologous interactions between ATP-carrying and ADP-carrying subunits were assumed to be 4-fold more favored than

homologous interactions (Figure 1). The significance of this fit is illustrated in Figures 1 and 3 where also curves calculated for stronger or weaker interactions are depicted. According to the analysis of critical concentrations, ATP–actin and ADP–actin copolymerize essentially randomly in an uncooperative manner. If any cooperative interactions are involved in this copolymerization, then heterologous interactions are slightly favored over homologous interactions.

Recently, it has been demonstrated that the structures of filaments formed by polymerization of ATP–actin and of ADP–actin reveal major differences (Janmey et al., 1990). Actin filaments formed by polymerization of ADP–actin are more flexible and more disordered than polymerized ATP–actin. This structural difference could be observed although the chemical composition of polymerized ATP–actin in which most of the ATP molecules are hydrolyzed is the same as that of ADP–actin. The flexible disordered ADP–actin filaments have been reported to be converted into stiff filaments by addition of ATP. It was concluded that actin can occur in two conformations. One of the conformations forms the flexible and the other the stiff filaments. In the experiments reported in this paper, the equilibrium constant (K_{DD}) for formation of flexible filaments (pure ADP–actin) and that for incorporation of ADP–actin into a stiff filament (ADP–actin in a mixture with ATP–actin) appear to be indistinguishable. Thus, our results do not exclude the existence of two conformations. However, they also do not confirm the model of two conformations because our results could be interpreted without assuming two conformations of actin.

Registry No. ATP, 56-65-5; ADP, 58-64-0.

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