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REMOVAL OF NUCLEOTIDES FROM F-ACTIN*

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SUMMARY

A partially nucleotide-free F-actin was obtained by a prolonged dialysis of a normal F-actin having bound ADP, but which maintained its polymer structure. The removal of ADP seemed to happen with equal probability everywhere in the F-actin. There was no appreciable difference in optical and rheological properties between the nucleotide-free F-actin and the normal F-actin. The nucleotide-free F-actin could not rebind nucleotide added to the solvent. However, repolymerizable G-actin was obtained from the nucleotide-free F-actin by depolymerization in the presence of ATP. The site for nucleotide binding in F-actin was not denatured by the removal of ADP. This was suggested also by the fact that the nucleotide-free F-actin had the same ATPase activity in sonic field as the normal F-actin and during this ATPase action ADP was incorporated again into F-actin. The activation of myosin ATPase (EC 3.6.1.4) at low salt concentration in the presence of Mg^{2+} was caused by the nucleotide-free F-actin similarly to the normal F-actin.

The nucleotide-free F-actin could be obtained only from F-actin having bound Ca^{2+}. F-actin having bound Mg^{2+} was destroyed in parallel with the removal of ADP. This qualitative difference came from the species of divalent cations incorporated into the structure of F-actin, not from the cations in the dialysing solvent.

Some discussion is presented concerning the possible role of nucleotides in F-actin, suggested by the present experiment.

INTRODUCTION

G-actin in a salt-free solvent binds ATP on itself, 1 ATP molecule for every 60000 molecular weight[1-7]. This ATP, which is necessary for maintaining the polymerizability of G-actin, is exchangeable with ATP in the solvent[8]. During the polymerization by salts, the ATP is split into ADP and inorganic phosphate[1,5]. The ADP, which remains tightly bound to the polymer structure of F-actin, is not normally exchangeable and cannot be removed by simple washing or sedimentation[1,5,8]. It is not reactive with creatinekinase (EC 2.7.3.2) or apyrase (EC 3.6.1.5)[1-3,9]. These facts suggest the importance of ADP in forming and maintaining the structure of F-actin.

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ADP seems to take part in the binding between the G-actins constituting F-actin. It is also probable, however, that ADP in F-actin may be nonreactive because of a steric hindrance of the polymer structure[10].

Prolonged dialysis against a salt solution free from nucleotides was found to remove ADP from F-actin very slowly[5]. At first it was shown that the F-actin solution keeps its high viscosity in spite of the partial removal of ADP (ref. 5). In another case, however, viscosity dropped in parallel to the removal[11]. Therefore we have undertaken systematic studies on the course of removal of ADP. Results showed that if we started with F-actin having bound Ca^{2+}, we could always obtain F-actin at least partially free from nucleotides.

Various physical and chemical properties of this F-actin, free from ADP, have been compared with those of the normal F-actin. Results forced us to suppose that ADP tightly bound to F-actin does not take part directly in the binding between G-actins, although it plays an important role in forming the bond. In other words, nucleotides seem to be pure rate regulators of the bond-formation process.

EXPERIMENTAL

Materials

Crude G-actin extracted from the acetone-dried muscle powder was purified by polymerization by 60 mM KCl (ref. 13). The pellet of F-actin ultracentrifuged out was dissolved in water containing 500 μM ATP and dialysed against a solvent of 100 μM Na_2CO_3 and 100 μM ATP. After a few days, the solution was centrifuged at 100000 $\times g$ for 1 h. The supernatant was our original G-actin solution. (According to the electric birefringence measurement, some tropomyosin, less than a few percent of actin, is occasionally contained in this G-actin solution[14,15].) The G-actin was polymerized into F-actin under various salt conditions. The dialysis to remove ADP from F-actin was carried out in ice water against solvents of various salt compositions. The solvents, the volumes of which were a hundred times that of the F-actin solution, were exchanged every 3 days.

Myosin was prepared by the method described by PERRY[16], with a slight modification. $^{45}CaCl_2$ was supplied by the Institute of Atomic Energy in Japan.

Methods

Protein concentration was determined by the biuret method, using for the absorbancy the value of 0.070 at 540 mμ for 1 mg/ml protein[17]. Nucleotide content was determined by ultraviolet absorption at 260 mμ after deproteinization by adding 1 ml of 60% perchloric acid to 5 ml of sample solution. Inorganic phosphate concentration was measured by the MARTIN–DOTY method[18]. Sulfhydryl groups were titrated with PCMB according to BOYER'S procedure[19]. Mg^{2+} and Ca^{2+} concentrations were determined by YANAGISAWA'S method[20].

The degree of flow birefringence was measured by an apparatus of Rao type described in a previous paper[21], with a shear rate about 6 sec^{-1}. The viscosity was measured by an Ostwald viscometer having a flow time for water of about 20 sec. The apparatus for electric birefringence measurement and that for ultraviolet dichroism measurement were described in other papers[14,22]. Sonic vibration was given by the same apparatus as used in a previous paper[11].

RESULTS

Removal of ADP

F-actins which had been made under various conditions of salts were dialysed for a very long period against salt solutions of various compositions. If the dialysing solvent contained ATP and a suitable concentration of salts, *e.g.*, 100 mM KCl, the long dialysis did not denature F-actin. Neither the amount of F-actin nor the amount of bound ADP changed during the dialysis. In the absence of nucleotide in the dialysing solvent, however, the amount of ADP in the F-actin solution gradually decreased. For example, in the case of F-actin polymerized in and dialysed against 100 mM KCl and 5 mM Tris–HCl (pH 8.0), the nucleotide content decreased to about 30% after 20 days dialysis. (100% nucleotide corresponds to 1 ADP per 60000 molecular weight F-actin.) The time course of the decrease of ADP is expressed in a simple exponential formula $\exp(-t/\tau)$; in the above example the life time τ was about 16 days. This result means that the rate of ADP removal was proportional to the remaining ADP content; thus the probability of bound ADP being removed is constant everywhere in the F-actin filament. It is improbable that the removal took place only at the ends of the F-actin filament. When the dialysing solvent contained Ca^{2+} or Mg^{2+}, the removal of ADP became slower and could not be expressed by a simple exponential formula. It seemed that a two-step mechanism (F–ADP $\rightarrow$ F*–ADP $\rightarrow$ F′ + ADP) could explain the experimental results, although the numerical data were not reproducible enough for quantitative analysis. Divalent cations slowed down the second step.

Survival of F-action during ADP removal

During the removal of ADP, we followed the change of physical properties of the F-actin solution to estimate the total amount of surviving F-actin. An example is

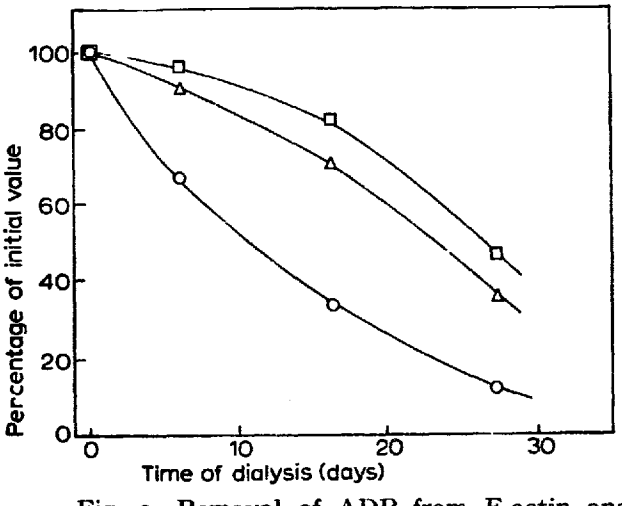

Fig. 1. Removal of ADP from F-actin and change of physical properties during the prolonged dialysis against a solvent of 0.1 M KCl and 5 mM Tris–HCl (pH 8.0) in ice water. F-actin had been made from Ca–G-actin by KCl. ○, bound ADP; △, viscosity; □, degree of flow birefringence. F-actin concentration 3.0 mg/ml. Initial values were 43 μM (ADP), 250° ($\Delta n/C_p$), and 7.5 (η_{sp}/C_p).

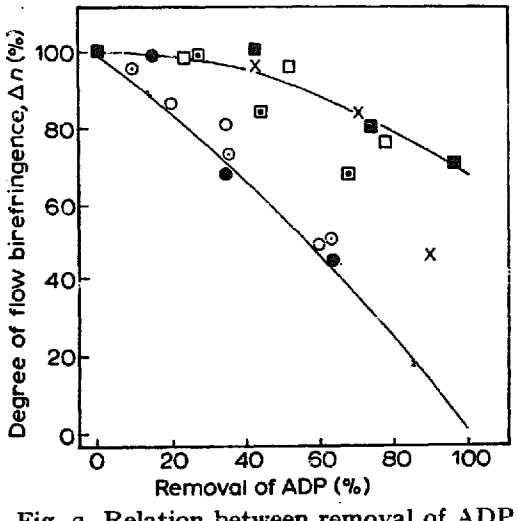

Fig. 2. Relation between removal of ADP and degree of flow birefringence during a prolonged dialysis of F-actin solutions against solvents of 0.1 M KCl and 5 mM Tris–HCl (pH 8.0). × ● ■, no divalent cations; ○ □, 3 mM $CaCl_2$; ⊙ ⊡, 3 mM $MgCl_2$. ×, F-actin made from G-actin by KCl only; ○ ⊙ ●, F-actin made from G-actin by 0.1 M KCl and 3 mM $MgCl_2$; □ ⊡ ■, F-actin made from G-actin by 0.1 M KCl and 3 mM $CaCl_2$. F-actin concentration 3.0 mg/ml.

shown in Fig. 1. A solution of F-actin which had been made from the original G-actin by 100 mM KCl without divalent cations, was dialysed against 100 mM KCl. Parts of the solution in the dialysing tube were taken up at suitable intervals and their viscosity and flow birefringence were measured. They were also ultracentrifuged at 100000 × *g* for 2 h. In the supernatant we found no nucleotides.

With prolonged dialysis, the decrease of degree of flow birefringence was very much slower than the decrease of bound ADP. The viscosity, also, remained high. The relation between the degree of flow birefringence and the remaining content of ADP is given in Fig. 2. For instance, when half the bound ADP was removed, about 90 % of F-actin still remained. Consequently, in this case we had F-actin which bound about half the ADP in normal F-actin.

It must be remarked here that the extinction angle of flow birefringence was kept very low. The supernatant after centrifugation was nonviscous and nonbirefringent. The total amount of sediment was proportional to the degree of flow birefringence before the centrifugation. These facts mean that the solution during dialysis contained extremely long F-actin filaments and monomers, or very short polymers. The former contributed to flow birefringence and had ADP molecules partially bound. The latter did not contribute to birefringence and had no nucleotides. There was no appreciable amount of polymers of intermediate lengths, just as in the case of the normal F-actin before dialysis[21,23].

Comparison between Ca–F-actin and Mg–F-actin

After polymerization, divalent cations tightly bound to actin, for every 60000 molecular weight, become nonexchangeable, just like nucleotides[24-26]. Therefore, it was thought probable that the property of F-actin depended on the species of these cations. In the experiment described above we started with an F-actin solution made by adding only KCl to G-actin. As reported in other papers, in this case, F-actin has bound Ca^{2+} and no Mg^{2+} (Ca–F-actin). F-actin having bound Mg^{2+} instead of Ca^{2+} (Mg–F-actin) was obtained either by adding KCl plus $MgCl_2$ to G-actin, or by placing Ca–F-actin in a sonic field for about 0.5 h in the presence of $MgCl_2$[25,26]. In the former case, about 80 % of divalent cations bound to F-actin were replaced by Mg. In the latter, 100 % replacement was realized.

Solutions of Ca–F-actin and Mg–F-actin were dialysed against three kinds of salt solutions, KCl only, KCl plus $MgCl_2$, and KCl plus $CaCl_2$. Results are summarized in Fig. 2. When we started with Ca–F-actin, the decrease of degree of flow birefringence was very much slower than that of ADP content. On the other hand, when we started with Mg–F-actin, the degree of flow birefringence decreased in parallel with that of ADP content. The composition of dialysing solvents did not change the behavior so much. (In the case of Ca–F-actin dialysed against a solvent containing Mg^{2+}, an intermediate behavior is observed. This may be due to a slow exchange of bound Ca with Mg during dialysis.)

Thus we could obtain from Ca–F-actin an F-actin almost completely free from nucleotides, while we could not obtain it from Mg–F-actin. This qualitative difference was certainly reproducible. The difference came from the different species of divalent cations nonexchangeably bound to F-actin. The divalent cations in the dialysing solvents could change the rate of the removal of ADP from F-actin as described above, but it could not change the stability of the structure of F-actin during the ADP

removal. These findings on the difference between Ca–F-actin and Mg–F-actin clarified the cause of the apparent contradiction in past experiments; in one case the dialysis experiment was carried out using Ca–F-actin and in another case using Mg–F-actin[5,11].

We could prepare F-actin free from divalent cations by placing F-actin in a sonic field in the presence of EDTA[25]. This F-actin (0–F-actin) was dialysed against a KCl solution to remove ADP. The removal was much faster than in the case of Ca– or Mg–F-actin. The degree of flow birefringence was decreased in parallel with the removal of ADP.

We tried various kinds of F-actin and dialysing solvents. Up to the present the only case where F-actin free from ADP was obtained was from the slow dialysis of Ca-F-actin against salt solvents.

Physical properties of F-actin free from ADP

In every case of F-actins dialysed, the degree of flow birefringence and the amount of sediment after ultracentrifugation were proportional to each other, giving the total amount of surviving F-actin. We started with three kinds of F-actins, Ca–F-actin, Mg–F-actin and 0–F-actin. After 7 days and 13 days dialysis, we measured the ratio of degree of flow birefringence to amount of sediment, which can give the birefringence of unit weight of F-actin oriented by flow. The ratio was compared with that of F-actin solutions stored without dialysis for the same periods. These F-actin samples dialysed or nondialysed had different amounts of bound nucleotides and different species and amounts of divalent cations. For instance, the ADP content of Ca–F-actin after 13 days dialysis was about half of the nondialysed one. In spite of such differences, the birefringence of unit weights of F-actins agreed with each other within a very small deviation from the average value. The ratio had a value, $(1.32 \pm 0.02) \cdot 10^{-5}$/mg/ml in absolute units of birefringence, common to all of the above samples of F-actin solutions. This meant that the optical anisotropy of F-actin was not changed by the change of amount and species of bound nucleotides and divalent cations.

Other properties, absorption spectrum and flow dichroism in ultraviolet region, of these F-actin solutions were also compared. As reported elsewhere, there was no remarkable difference among different kinds of F-actins[22]. In electric birefringence measurements also, the ADP-free F-actin dissolved into a 1 mM $MgCl_2$ solution after ultracentrifugation showed a behavior similar to the normal F-actin[14].

As seen in Fig. 1, the ratio of the viscosity to the degree of flow birefringence or the viscosity per unit weight of F-actin decreased a little with removal of ADP from Ca–F-actin. In the case of Mg–F-actin where the remaining F-actin always had 100% bound ADP, such a decrease of viscosity of unit weight of F-actin was not observed. This result suggests that the structure of partially ADP-free F-actin is more fragile than the normal F-actin. Various rheological properties, such as viscoelasticity under slow oscillation and decay of orientation in flow, were examined; however, no qualitative and reproducible difference was found between the ADP-free F-actin and the normal F-actin.

Summarizing these results, we can say that in the case of Ca–F-actin the main polymer structure of F-actin was well maintained even when most of the bound ADP was removed.

In addition we investigated the behavior of divalent cations and sulfhydryl groups in F-actin during removal of ADP. To follow the removal of bound Ca, by using ^{45}Ca-labelled F-actin, we measured the change of specific activity of ^{45}Ca during dialysis against a solvent of KCl only. The Ca removal was found to be a little faster than that of ADP; for example, after 14 days dialysis the specific activity became less than 20%, while the ADP content remained about 50%. Therefore, the ADP-free F-actin dialysed against KCl only is free of divalent cations.

2 moles of –SH per 60000 molecular weight actin in the normal F-actin are rapidly titratable by PCMB (ref. 27). By the addition of EDTA, the number of rapidly titratable sulfhydryl groups is increased to about 4 without depolymerization of F-actin. In the ADP-free F-actin, this number is 3–4 without EDTA. In this respect, the ADP-free F-actin behaves like the normal F-actin in EDTA.

Recovery of native G-actin

If the site of F-actin for nucleotide binding was kept native even after the removal of ADP by dialysis, we could expect, upon the addition of ATP to solvent, the recovery of the ADP-free F-actin to a normal F-actin. During this recovery process, added ATP would be adsorbed to the ADP-free actin and then split into inorganic phosphate and tightly bound ADP. Repeated experiments, however, showed only a little splitting of ATP added to the ADP-free F-actin. Sometimes, we could see the splitting, the amount of which was, however, considerably smaller than the amount of ADP-free sites in F-actin. In Fig. 3, the ATP splitting or the recovery of bound ADP happened in 20% of vacant sites. This result seemed to suggest that the nucleotide-binding site of F-actin was denatured irreversibly with removal of ADP by dialysis, although the polymer structure was conserved. However, the next experiment showed that this interpretation was wrong.

A solution of partially ADP-free F-actin was ultracentrifuged and the pellet was

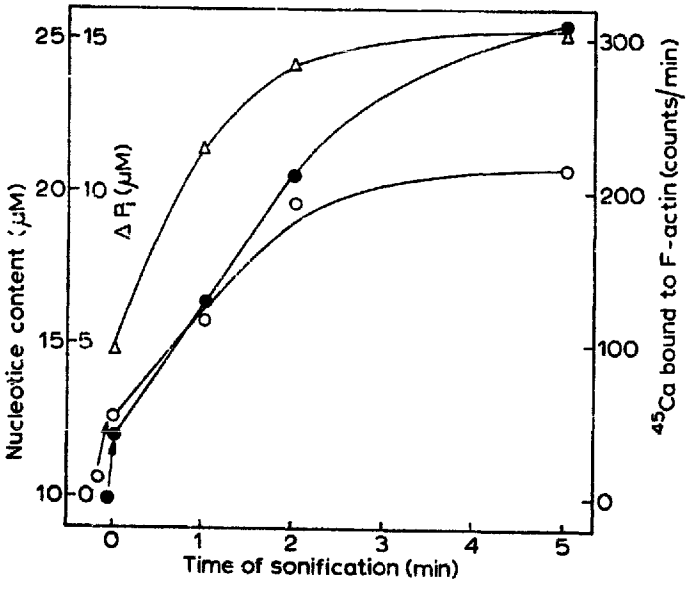

Fig. 3. ATP splitting and rebinding of ADP and Ca to ADP-free F-actin. F-actin (4.1 mg/ml) polymerized in 0.1 M KCl and 5 mM Tris–HCl (pH 8.0) was dialysed for 19 days in 0.1 M KCl and 5 mM Tris–HCl. Sonification was done under the solvent condition: 0.1 M KCl, 5 mM Tris–HCl (pH 8.0), 20 μM ATP, 60 μM $^{45}CaCl_2$. Actin concentration 1.5 mg/ml. Initial increases show the increases before sonification. ●, ATP splitting; ○, ADP rebinding; △, Ca rebinding.

dissolved into salt-free water containing ATP. The dissolution gave a non-viscous and non-flow birefringent solution, in which most of actins were in the monomeric state whether they might be denatured or not. Then an optimal concentration of salts was added to induce repolymerization. If the nucleotide-binding sites had been denatured during the dialysis, the amount of repolymerizable actin would be equal to the amount of ADP which had remained in F-actin before depolymerization, and not equal to the

TABLE I

REPOLYMERIZABILITY OF AN ADP-FREE F-ACTIN

F-actin (5 mg/ml) polymerized in 85 mM KCl and Tris–HCl (pH 8.0) was dialysed against the same solvent for 20 days in ice water. The remaining F-actin concentration was determined by flow birefringence and centrifugation at 100000 × *g* for 2 h.

F-actin	*Concentration of F-actin*		*Bound nucleotides (μM)*	*Dissolved solvent after centrifugation*	*Degree of flow birefringence (Δn) after repolymerization in 0.1 M KCl and 3 mM $MgCl_2$, 5 mM Tris-HCl (pH 8.0)*	
	mg/ml	*μM***			*Δn*	*$Δn/C_p$*
Dialysed F-actin	3.9 (3.6)*	65	16.9	*Distilled water containing* 0.4 mM ATP	220	200
				0.1 M KCl + 0.4 mM ATP	310	280
Non-dialysed F-actin	5.0 (5.0)*	83	(83)	*Distilled water containing* 0.4 mM ATP	376	263
				0.1 M KCl + 0.4 mM ATP	440	300

* Determined by degree of flow birefringence.
** Calculated from the protein concentrations by assuming molecular weight 60000.

amount of F-actin which had survived during dialysis. In Table I is given the ratio of the degree of flow birefringence of repolymerized actin to the amount of F-actin before depolymerization. As compared with the nucleotide content (26 %), the value of this ratio for the partially ADP-free F-actin is found not to be so different from that for the normal F-actin. That is, repolymerizable G-actin can be obtained not only from F-actin having ADP but also from most of F-actin having no ADP.

By the way, when the pellet of the partially ADP-free F-actin is dissolved into a salt solution, almost all of the pellet can be recovered again in the state of F-actin.

Thus, it is very likely that in spite of the removal of ADP, the polymer structure of F-actin and the ability of rebinding of nucleotides were not lost. The failure of the previous experiment to show the direct rebinding of ADP or ATP added to the solvent might be due to the situation that the nucleotide-binding sites in F-actin were not exposed outside and the rebinding needed some structural loosening of F-actin. If the sites were exposed outside, then irreversible denaturation might have occurred during the dialysis just as in the case of G-actin.

ATPase of the F-actin under sonic vibration

F-actin in ordinary conditions has no ATPase activity because bound ADP is nonreactive to external agents. However, when the F-actin is placed in a sonic vibration field it catalyses the splitting of ATP, while maintaining its polymer structure[12]. Can the ADP-free F-actin have such an ATPase activity under sonic

vibration? We compared three kinds of F-actins; a normal F-actin freshly prepared, a normal F-actin stored without dialysis, and an ADP-free F-actin obtained by dialysis, the nucleotide content of which was 26 % of the normal F-actin. The concentrations of these F-actin solutions were adjusted to have the same value of degree of flow birefringence which means the same F-actin concentration. The concentration of bound ADP of the third sample was about one-fourth of the others.

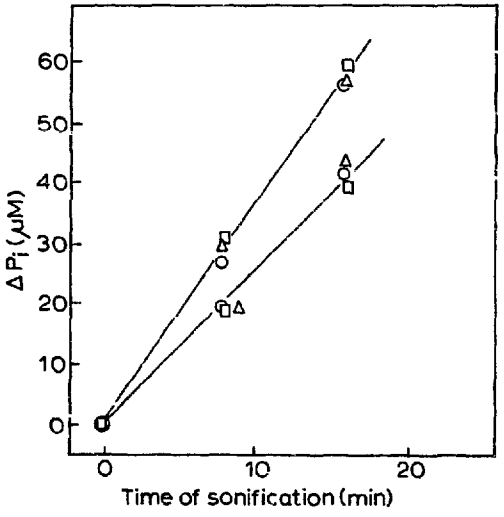

Fig. 4. F-actin ATPase in the sonic field. ○, ADP-free F-actin, of which the nucleotide content was one-fourth of a normal F-actin, dialysed against 85 mM KCl and 5 mM Tris–HCl (pH 8.0) for 20 days. □, normal F-actin left standing without dialysis for 20 days. △, fresh F-actin. The F-actin concentration was adjusted to the same value of degree of flow birefringence. The total F-actin concentration was 1.13 mg/ml in the upper curve and 0.74 mg/ml in the lower curve. Sonification was done at about 15°.

As shown in Fig. 4, the three F-actin solutions had exactly the same ATPase activity under sonic vibration. As far as the ATPase activity under vibration was concerned, in the polymer structure of F-actin, actin molecules having had no ADP before vibration could make the same contribution as those having had ADP.

During sonic vibration a local interruption of the polymer structure of F-actin occurred and the bound ADP or the nucleotide-binding site was exposed outside. Then ATP in the solvent replaced the bound ADP or it was bound to the vacant site, and the following reconstruction of the polymer structure was accompanied by splitting of the ATP. The recovery of bound ADP and Ca^{2+} of the partially ADP-free F-actin was followed during sonic vibration in the presence of ATP and Ca^{2+}. As shown in Fig. 3, the splitting of ATP and the incorporations of ADP and Ca^{2+} took place almost in parallel with each other. The amount of bound ADP finally reached the value corresponding to the normal F-actin.

Interaction with myosin

The partially ADP-free F-actin could bind with myosin, as could the normal F-actin. At low salt concentrations, the complex of the partially ADP-free F-actin with myosin could superprecipitate upon the addition of ATP. Under this condition, the ATPase of the complex was compared with that of the complex of the normal F-actin with myosin. Results at various ratios of F-actin to myosin are summarized in Table II. The same concentration of actin means the same value of flow birefringence.

Since the ADP content of the dialysed F-actin in the table was about 26% of the normal one, the complex of this F-actin with myosin in a ratio of 1/4 contained nearly the same concentration of bound ADP as the complex of the normal F-actin with myosin in a ratio of 1/16. The ATPase of the former was clearly larger than the latter.

TABLE II

MYOSIN A ATPASE ACTIVATION BY AN ADP-FREE F-ACTIN

ATPase was measured at 20°. Myosin concentration 0.525 mg/ml. Reaction media: 48 mM KCl, 1.5 mM $MgCl_2$, 8.3 mM Tris–maleate (pH 7.0), and 1 mM ATP.

F-actin	*F-actin:myosin*	*Inorganic phosphate (μM) liberated after addition of ATP*		
	Time (min)	*0.5*	*2*	*10*
F-actin dialysed for 20 days	1/4:1	122	261	444
	1/8:1	66	192	378
	1/16:1	33	142	331
Control, non-dialysed	1/4:1	102	272	466
	1/8:1	79	240	391
	1/16:1	46	187	420
No F-actin	0:1	16.5	22	46

At the same ratio 1/4, the ATPase activities of the complexes of two kinds of F-actins with myosin were nearly equal. Therefore, the part of F-actin from which bound ADP was removed could have the same ability of activation of myosin ATPase as the normal F-actin.

DISCUSSION

We have succeeded in obtaining F-actin having less bound ADP than the normal F-actin. This partially ADP-free F-actin has the same activities of ATPase in a sonic field and of interaction with myosin as the normal one. It is very likely that the ADP bound to F-actin does not participate directly in the bond between G-actins in F-actin, so that the bond is not broken by the removal of ADP. However, we have to examine another possibility in connection with the helical polymer model of F-actin recently proposed on the basis of ultrastructural[2] and thermodynamic[29-31] analysis. In this model, each G-actin in F-actin is bound with four neighboring G-actins. The separation of G-actin from F-actin needs the simultaneous breaking of four bonds. The destruction of F-actin into two fragments needs the breaking of more than three bonds in definite relative positions. Even if ADP directly participates in each bond and the removal of ADP gives rise to bond breakage, a parallel relation is not necessarily required between the ADP removal and the fragmentation of F-actin. If the breaking of bonds took place everywhere with equal probability, we could expect that with increasing number of broken bonds, the amount of surviving F-actin would decrease first very slowly and then rapidly. Accordingly, the relation in Fig. 2 seems to be explained by the helical polymer model even if the removal of a molecule of ADP means the breaking of a bond between G-actins.

Nevertheless, at present it seems more probable for two reasons that ADP does

not directly participate in the bond. First, we can sometimes obtain F-actin which has an ADP content less than 10 % of the normal one. The second reason is as follows. In spite of the maintenance of the native structure of binding sites, a simple addition of ATP to the ADP-free F-actin did not result in the rebinding of ADP. The rebinding needed depolymerization or agitation in a sonic field. Taking into consideration the nonexchangeability of ADP in F-actin standing quiet, it is reasonable to suppose that ADP groups are hidden behind the bond without participating in the bond itself, as suggested by some of the previous works[10]. Each ADP group is confined in a cage made by the polymer structure. Each bond between G-actins takes a role as a shutter of the cage. The shutter can be opened by depolymerization or sonic vibration[12]. The slow removal of ADP during dialysis might be realized by opening (followed by closing) of the shutter with a very low frequency. It must be remembered that nucleotide is very important in maintaining the polymerizability of G-actin and inducing the polymerization. ATP accelerates the polymerization of G-actin and the reformation of interrupted polymer of actin[30,32,33]. ATP is a rate-regulating factor of bond formation between G-actins. The rate of shuttering the cage is regulated by nucleotide in the cage. The fast shuttering by ATP is accompanied by its splitting. Such an interpretation of the nucleotide role suggests the importance of reexamining the possibility of reversible polymerization without any participation of nucleotides[34].

Of course we have no reason to consider that there is only one bond between two neighboring G-actins. They can interact with each other through more than two kinds of bonds. Nucleotide may be contained in one of these bonds which is exposed during depolymerization or sonic vibration.

A similar situation has been found with the divalent cations tightly bound to F-actin[25,26]. The cations also seem to be confined in the cage which can be opened only by depolymerization or by interruption under sonic vibration. The cations in the cage regulate the rate of bond formation.

As described above, Mg–F-actin behaves differently from Ca–F-actin during dialysis. When F-actins are dialysed against solvents containing no Ca^{2+} or Mg^{2+}, Ca^{2+} or Mg^{2+} bound to F-actin are removed in parallel with or faster than the removal of ADP. Therefore, the cause of the difference between two kinds of F-actins must be in the structure of the F-actins before dialysis. However, physical and chemical analyses have shown no indication of such structural difference[35].

The ADP-free F-actin showed the activation of myosin ATPase to the same extent as the normal F-actin. This does not necessarily mean the inutility of the nucleotide in F-actin in the activation process, because at present we cannot exclude the possibility that during the ATPase action the nucleotide is incorporated into the ADP-free F-actin.

In the present method, it takes a very long time to remove the bound ADP from F-actin. The long dialysis might have various ill effects caused by, for instance, a bacterial growth, oxidation, etc. Although these effects, if present, give no essential change in the qualitative conclusion in the present experiment, it is desirable to find an improved method of removing ADP faster. We have tried various treatments, for instance, the addition of chemical reagents, the sonic vibration in the presence of resins, etc. However, in these treatments the faster removal of ADP was always associated with the parallel destruction of F-actin.

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