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THE ADENOSINE TRIPHOSPHATE SPLITTING OF ACTIN MODIFIED WITH SALYRGAN

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SUMMARY

The polymerization and the ATP splitting of actin modified with salyrgan were investigated by physicochemical methods. When actin was modified with salyrgan at the molar ratio of salyrgan to actin of about 3:1, a steady ATP splitting occurred after polymerization by the addition of 3 mM $MgCl_2$ and 60 mM KCl at slightly alkaline pH. At this condition, about one-third of the actin was polymerized and the rate of the steady ATP splitting was almost proportional to the amount of polymers. When modified G-actin was polymerized with $CaCl_2$, instead of $MgCl_2$, no ATP splitting was coupled with polymerization. After polymerization, however, steady ATP splitting was induced by replacement of Ca^{2+} by Mg^{2+} in the presence of EGTA. The addition of an excess amount of cysteine stopped the steady ATP splitting and increased the viscosity.

INTRODUCTION

In a salt-free solvent, actin is in the state of monomer, G-actin containing bound ATP. This ATP is split into ADP and inorganic phosphate during polymerization to F-actin induced by the addition of salts. One mole of ATP is split per mole of actin polymerized[1]. At an optimal salt concentration, F-actin containing bound ADP is stable and cannot split more ATP. Asakura[2] found, however, that under sonic vibration F-actin continues to split ATP in the solution. This phenomenon was interpreted as an enzymic nature of F-actin associated with some cyclic change in the polymer structure of F-actin[3], although later it was shown by Kasai and the present author that the polymerization–depolymerization cycle at the ends of short fragments of F-actin made a larger contribution to the ATP splitting under sonic vibration[4]. Steady ATP splitting in the solution of F-actin was also found at high temperatures above 50 °C by Asai and Tawada[5]. On the other hand, Hatano showed that actin extracted and purified from plasmodium has an amino acid composition similar to muscle actin[6], but it forms a polymer which shows steady ATP splitting at room temperature in the presence of magnesium ions[7].

The present study was undertaken to examine whether a polymer of muscle actin with steady ATP splitting activity can be obtained by means of chemical modification.

The polymerization of G-actin to F-actin can be performed even without participation of nucleotides[8]. The binding of ATP or the splitting of ATP is not absolutely necessary for polymerization. However, if we start with G-actin containing bound ATP, the splitting of those ATP is obligatorily coupled with polymerization. In other words, it is possible to make F-actin containing bound ADP or no bound nucleotides, but it is impossible to make a stable form of F-actin containing ATP. Therefore, investigations were made to examine whether, in the modified actin, the polymerization and the ATP splitting can be uncoupled.

In the present work, an organomercurial, salyrgan, has been used for modification. It was reported by Drabikowsky and Gergely[9] that salyrgan inhibited polymerization of actin but the loss of polymerizability occurred more rapidly than the loss of bound ATP. Here, modification by salyrgan was carried out to a moderate degree; so that the modified actin was found to be polymerizable on the addition of divalent cations. The polymer obtained exhibited a steady ATP splitting in the presence of magnesium ions, while in the presence of calcium ions only, polymerization took place without ATP splitting.

MATERIALS AND METHODS

Actin was prepared from the acetone-dried powder of rabbit skeletal muscle mince by the method of Straub[10], after careful removal of the new proteins, discovered by Ebashi and co-workers[11–13]; this was carried out by incubation of the myosin-extracted minced muscle in distilled water for 12 h at room temperature. For further purification of actin extracted from the muscle powder, the cycle of polymerization at 30 mM KCl (pH 8.0) and depolymerization in the salt-free condition of pH 8.0 was repeated. Finally, a solution of the pellet of F-actin was dialysed against 100 vol. of a solution of 0.05 mM ATP and 1 mM Tris–HCl (pH 8.0) for 30 h at 2 °C with stirring.

The concentration of G-actin was determined by the biuret reaction, standardized by the micro-kjeldhal determination of nitrogen. The concentration of active actin was also determined by measuring the flow birefringence after polymerization[14,15]. The molecular weight of G-actin was assumed to be about 45000 (refs 16, 17).

The concentration of inorganic phosphate was determined by the method of Taussky and Shorr[18]. Deproteinization was carried out using 12 % trichloroacetic acid. Colorimetry was done in a Carl Zeiss spectrophotometer at a wavelength of 700 nm.

Viscosity measurements were carried out in an Ostwald-type viscometer in a thermo-controlled bath. The flow time of water was 40 s at 23 °C. The degree of flow birefringence was measured by a homemade Rao-type apparatus at low shear rates. Sedimentation experiments were made in a Spinco Model E ultracentrifuge.

The procedure of chemical modification by salyrgan was as follows. To a solution of G-actin (concn about 1 mg/ml), containing 5 mM Tris–HCl buffer (pH 7.7) and 1 mM ATP, was added salyrgan of a molar concentration nearly equivalent to, or a few times higher, than that of the actin. After incubation for 24 h at 0 °C, salts were added and then polymerization and ATP splitting were followed. Before

salt addition, it was checked that actin was in the state of monomer and no ATP splitting took place.

RESULTS

(1) *Effect of salyrgan on polymerization and ATP splitting of actin*

After incubation of G-actin in salyrgan of various concentrations, polymerization was induced by the addition of 3 mM $MgCl_2$, 60 mM KCl and 5 mM Tris–HCl buffer (pH 7.7). In the absence of salyrgan, G-actin was polymerized completely and the total number of moles of ATP split during this polymerization was nearly equal to that of actin polymerized. No splitting of ATP was observed after polymerization. However, as shown in Fig. 1, with increase in the amount of salyrgan added, the degree of polymerization measured by flow birefringence decreased, whereas the amount of ATP split increased, exceeding the total amount of actin. Moreover, the ATP splitting was found to continue after the flow birefringence had attained a stationary value. This steady ATP splitting showed a maximum at a molar ratio of added salyrgan to actin of about 3:1. At higher concentrations of salyrgan, the flow birefringence was reduced and the amount of split ATP decreased. The viscosity of these solutions is shown in Fig. 2; viscosity decreased

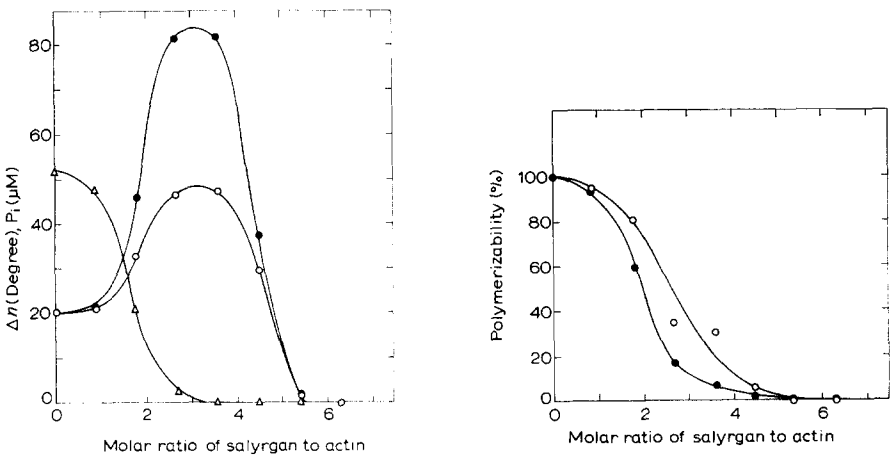

Fig. 1. Effect of salyrgan on polymerization and ATP splitting of actin. To a solution of G-actin various amounts of salyrgan were added and G-actin was modified to different degrees, as described in the text. After the addition of $MgCl_2$ and KCl to the modified G-actin solutions they were incubated in a thermostat at 24 °C and the degree of flow birefringence (△) and the amount of ATP split (○, ●) were measured after 1 (○) or 2 (●)h. The degree of flow birefringence did not change for 1 h. Actin concentration, 0.9 mg/ml. Solvent conditions: 10 mM Tris–HCl buffer (pH 7.7), 60 mM KCl, 3 mM $MgCl_2$ and 1 mM ATP.

Fig. 2. Effects of salyrgan on polymerizability. After $MgCl_2$ and KCl were added to the G-actin solution, modified with increasing amount of salyrgan, the solutions were left for about 1 h at 24 °C and the viscosity of the solution was then measured. Analytical centrifugation of the solution was carried out at 29 500 rev./min with the Spinco Model E centrifuge using a synthetic boundary cell at 24 °C. After 40–60 min, where the slow and fast peaks were separated completely, the concentration of polymers was estimated by subtracting the area of the slow peak from the total. Both viscosity (●) and the amount of polymers (○) are expressed on the ordinate as the fraction of the total actin. Actin concentration, 3.0 mg/ml. Solvent conditions were the same as in Fig. 1.

with increase in the amount of salyrgan added, almost in parallel to the flow birefringence.

The amount of polymer was also measured by ultracentrifugation. In the centrifugal pattern of modified actin after the addition of salts, two peaks appeared, one had a very large sedimentation constant, about 50 S, and the other, about 5 S, which corresponds to unpolymerized actin or small polymers. By subtracting the area of the slow peak, the amount of large polymers was estimated. As shown in Fig. 2, this decreased with increase in the amount of salyrgan. However, at a molar ratio of about 3:1, about one-third of the actin formed polymers which gave only small flow birefringence.

(2) Dependence on concentration of actin

Various concentrations of G-actin, modified at the molar ratio of salyrgan to actin of 3.5:1, were polymerized at the optimal salt condition; 60 mM KCl, 3 mM $MgCl_2$, 1 mM ATP and 5 mM Tris–HCl buffer (pH 7.7) at room temperature. As shown in Fig. 3, the final viscosity increased in proportion to the concentration of actin. The rate of steady ATP splitting also showed a linear increase. On the addition of 0.5 mM $MgCl_2$ only, the viscosity increased when the actin concentration exceeded a critical value of about 1 mg/ml. Above this concentration the viscosity increase occurred in parallel to that at the optimal salt condition. This situation is very similar to the case of native actin and thus polymerization can be regarded as a kind of condensation phenomena.

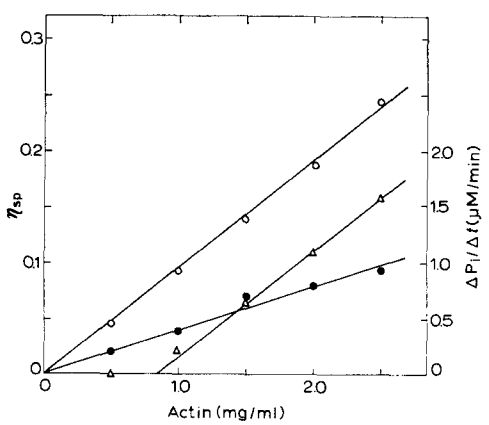

Fig. 3. Polymerizability and the rate of ATP splitting of various concentrations of modified actin. After $MgCl_2$ and KCl were added to the solution of various concentrations of G-actin, modified with 3.5 times its concentration of salyrgan, the solutions were incubated at 24 °C for about 1 h. Then the viscosity (○, △) and the rate of ATP splitting (●) were measured. Solvent conditions: 5 mM Tris–HCl buffer (pH 7.7), 1 mM ATP, 60 mM KCl, and 3 mM $MgCl_2$ (○, ●) or 0.5 mM $MgCl_2$ (△).

The results in Figs 1, 2 and 3 suggest that a certain fraction of actin was modified by salyrgan to lose polymerizability. This fraction increased with increase in the amount of salyrgan. The remaining fraction of actin maintained polymerizability. The structure of polymers formed by this actin, however, is different from normal F-actin, depending on the molar ratio of salyrgan to actin. The polymers

were able to be spun down by ultracentrifugation, but gave a low degree of flow birefringence and low viscosity, and showed remarkable ATP splitting activity.

By ultracentrifugation the unpolymerizable actin was separated from polymers. The pellets which were redissolved into a salt solution showed ATP splitting, while actin in the supernatant had no effect on the ATP splitting of the redissolved polymers. Thus, it is very likely that the unpolymerizable actin did not participate in ATP splitting in the original solution before centrifugation.

(3) Effect of divalent cation

Salts of various compositions were added to actin, modified by 3.5 moles of salyrgan per mole of actin, and the change of viscosity and the ATP splitting were followed. As shown in Fig. 4, monovalent salts did not cause the viscosity increase. Divalent cations were required for polymerization of the modified actin. The final viscosity was larger in the case of calcium ions than magnesium ions. However, the splitting of ATP was observed only in the presence of magnesium ions.

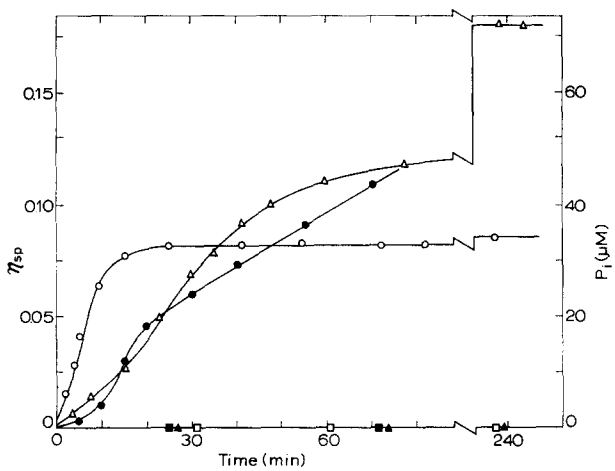

Fig. 4. Time course of polymerization and ATP splitting in various conditions. After various salts were added to the solution of G-actin, modified with 3.5 times its concentration of salyrgan, the increase of viscosity and the amount of ATP split were measured in a thermostat at 24 °C. Actin concentration, 0.9 mg/ml. Solvents for the polymerization were as follows: 3 mM $MgCl_2$ and 60 mM KCl (○, ●); 3 mM $CaCl_2$ and 60 mM KCl (△, ▲); 60 mM KCl only (□, ■); each contains 5 mM Tris–HCl buffer (pH 7.7) and 1 mM ATP. Filled symbols show the amount of ATP split and open symbols show the viscosity.

The final viscosity of the solutions of modified actin at various concentrations of divalent cations is shown in Fig. 5. The ATP splitting rate in the presence of magnesium ions was almost proportional to the viscosity.

If induced by calcium ions, polymerization of modified actin took place without splitting of ATP. Therefore, the polymers formed were expected to contain bound ATP. When $MgCl_2$ was added to a final concentration of 3 mM to the solution of modified actin, which was fully polymerized in 2 mM $CaCl_2$, no ATP splitting was found. However, when $MgCl_2$ and the chelating reagent for calcium ions, ethyleneglycol bis(aminoethyl)tetraacetic acid (EGTA), were added to the solution,

ATP splitting began to occur, as shown in Fig. 6. The splitting became steady soon after the addition of $MgCl_2$ and EGTA and the rate reached the same level as that reached in the presence of $MgCl_2$ only. On the other hand, the steady ATP splitting of actin polymerized by $MgCl_2$ was found to be stopped by a small amount of $CaCl_2$. Thus, the ATP splitting reaction in the polymer of modified actin can be switched on or off by the replacement of divalent cations.

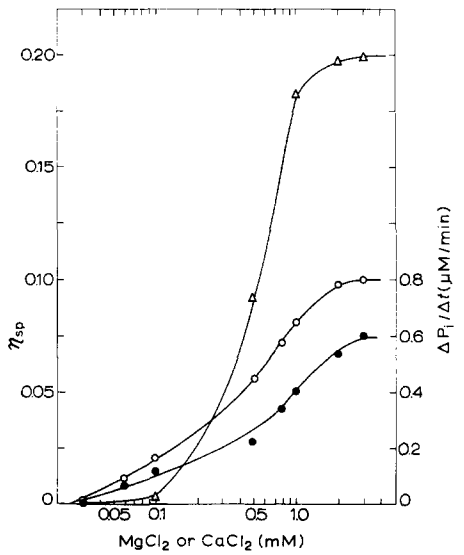

Fig. 5. Polymerizability and the rate of ATP splitting in various concentrations of salts. Increasing amounts of $MgCl_2$ or $CaCl_2$ were added to G-actin, with 3.5 times its concentration of salyrgan. The procedure for measurements was the same as in Fig. 3. Actin concentration, 0.9 mg/ml. Solvent conditions: 5 mM Tris–HCl buffer (pH 7.7), 1 mM ATP, 60 mM KCl and $MgCl_2$ (○, ●) or $CaCl_2$ (△). Filled circles show the rate of ATP splitting in the presence of $MgCl_2$.

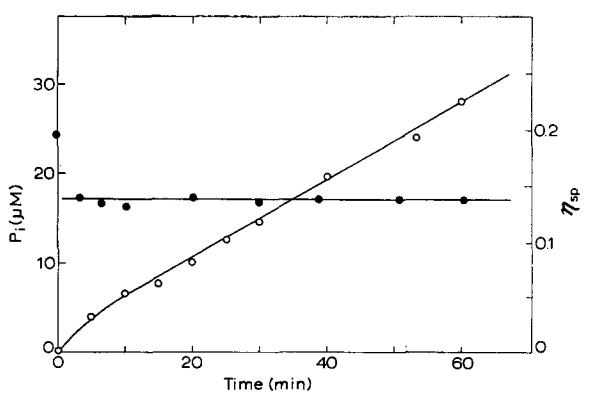

Fig. 6. Time course of ATP splitting induced by the replacement of $CaCl_2$ with $MgCl_2$. To the solution of G-actin, modified with 3.5 times its concentration of salyrgan, 3 mM $CaCl_2$ and 60 mM KCl, 5 mM Tris–HCl buffer (pH 7.7) and 1 mM ATP were added and left for about 1 h. At this stage, no ATP was split. Then, 3 mM $MgCl_2$ and 8 mM EGTA were added and the amount of ATP split (○) and the viscosity (●) were measured. Actin concentration, 0.8 mg/ml.

(4) Effects of cysteine

To examine the reversibility of the effect of salyrgan, cysteine was added to a solution of modified actin which showed steady ATP splitting. As shown in Fig. 7, the addition of an excess amount of cysteine immediately inhibited steady ATP splitting and induced a large increase in viscosity. Flow birefringence was also increased by the addition of cysteine. The increase of viscosity and birefringence is not due to the increase of polymerizability, because it did not couple with the ATP splitting. It is more probable that the structure of actin polymers was changed by the removal of salyrgan. The structure giving low viscosity has steady ATP splitting activity and that giving large viscosity corresponds to normal F-actin having no steady ATP splitting activity.

The addition of salyrgan to a solution of normal F-actin induced no ATP splitting and only a slight decrease of viscosity.

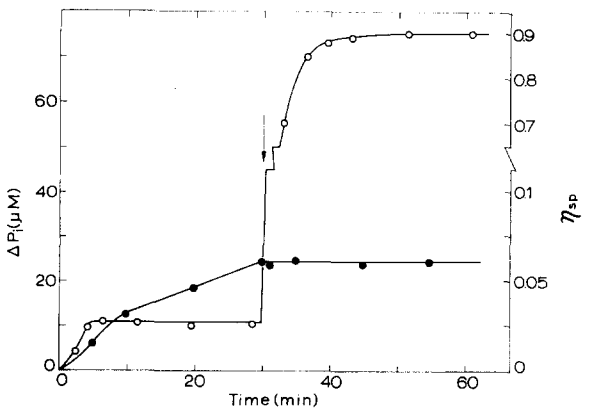

Fig. 7. Effects of cysteine on ATP splitting and the polymerizability. To a solution of G-actin, modified with 3.5 times its concentration of salyrgan, $MgCl_2$ and KCl were added at zero time in the figure. The viscosity (○) and the amount of ATP split (●) were measured. The arrow in the figure shows the time of the addition of 10 mM cysteine. Actin concentration, 0.9 mg/ml. Solvent conditions: 7 mM Tris–HCl buffer (pH 7.5), 60 mM KCl, 3 mM $MgCl_2$ and 1 mM ATP. Temperature, 20 °C.

DISCUSSION

The number of SH groups per mole of actin was estimated to be about 5 (refs 19, 20). In the present experiment, the groups modified by salyrgan were not identified. It was not certain whether all monomers were modified to the same degree. It is more likely that at a constant molar ratio of salyrgan to actin, monomers were modified to different degrees and the solution was a mixture of these monomers. The molar ratio only gave the average value. The unpolymerizable actin may correspond to the monomers modified to a larger degree.

In the present experiment the solution of actin modified by salyrgan in an intermediate range of the molar ratio of salyrgan to actin showed the steady ATP splitting. The rate of splitting was 0.02–0.04 mole per mole of G-actin per min. This value is about 20–40 times larger than that in the case of the dynamic equilibrium between G- and F-actin reported by Asakura and Oosawa[21], and about one-tenth

of that of sonicated F-actin[4]. It might be possible that modified actin monomers showed ATP splitting activity in the dispersed state. It was shown, however, that the unpolymerizable fraction of monomers in $MgCl_2$ did not exhibit ATP splitting nor increased the splitting activity of polymerized actin. In the experiment of Fig. 5, carried out at varied concentrations of $MgCl_2$, the ATP splitting rate was nearly proportional to the amount of polymers formed. At the optimal salt concentration the viscosity and the ATP splitting rate were proportional to the total concentration of actin. At this condition the amount of polymerizable actin in the state of monomers must be very small, if present. Fig. 3 suggested that, at low concentrations of salts, the polymerization of modified actin took place as a kind of condensation phenomena, similar to that in the case of normal actin, where polymers and polymerizable monomers coexist in equilibrium. Nevertheless, the ATP splitting rate is smaller than that at the optimal salt concentration. Therefore, it is improbable that the depolymerization–polymerization cycle was the main cause of the ATP splitting. Instead, polymers themselves are considered to possess ATP splitting activity. The fact that the removal of salyrgan by cysteine transforms the polymer structure to normal F-actin and stops the steady ATP splitting supports the above interpretation.

The ATP splitting of polymers of modified actin was found only in the presence of magnesium ions. It must be noted here that the ATP splitting of the polymers of modified actin is very similar to that of actin from plasmodium[7]. Plasmodium actin forms two kinds of polymers, one is normal F-actin formed in monovalent salts and the other is the polymer formed in the presence of magnesium ions[22]. Only the latter polymer shows the steady ATP splitting. Electron microscopic observations and other physical analyses suggested that this polymer is more flexible than normal F-actin and the fluctuation in the polymer structure may be related to the ATP splitting[23].

The modified actin used in the present experiment was observed under an electron microscope after negative staining; F-actin-like polymers, large globular polymers and some flexible polymers were observed. No remarkable difference was found between polymers formed in the presence of magnesium ions or in the presence of calcium ions. It was difficult to identify which structure was responsive to the ATP splitting.

However, it was found that actin modified by salyrgan to a certain degree can form polymers showing steady ATP splitting activity. This actin can discriminate between magnesium ions and calcium ions, and the ATP splitting activity is exhibited only in the presence of the former. Polymers formed in the presence of calcium ions retained ATP, and magnesium ions released the ATP splitting reaction. The tight coupling between polymerization and ATP splitting was removed.

Recently, Mihashi[24] modified actin with iodoacetate at a high urea concentration and obtained two kinds of actin polymers after removal of urea. This artificial alteration of the chemical structure of actin changed its properties. This kind of study is useful for understanding the structural origin of the function of actin. In addition, it gives valuable suggestions for the comparative study of actins from different sources. For further investigation of the relationship between structure and function, more quantitative analyses are required of the chemical structure of actin after its modification.

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