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The effect of the replacement of ADP with a photoaffinity ATP analogue, 2-azido-ADP, in F-actin on its function

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2-Azido-ATP, a photoaffinity ATP analogue, was incorporated into actin and the influence of the incorporation on the actin function was studied. The replacement of ADP with 2-azido-ADP in F-actin both before and after photocross-linking decreased appreciably the actin-activated S1-ATPase activity. Photocross-linked 2-azido-ADP-F-actin could be depolymerized by dialysis against a solution containing 0.1 mM $CaCl_2$, 0.1 mM ATP and 1 mM Tris-HCl (pH 8.0). However, once it depolymerized, it lost very quickly the ability to polymerize even in the presence of a sufficient amount of ATP and Ca^{2+}.

Actin; ATP analogue, photoaffinity; 2-azido-ATP

1. INTRODUCTION

At low ionic strength, actin exists as a monomeric form (G-actin). One mole of G-actin binds one mole of ATP (affinity constant of 7.5×10^9 M^{-1} [1]) which is easily exchanged with other nucleotides in solution. During polymerization, the terminal phosphate is removed and the bound ADP in F-actin is no longer readily exchangeable. The role of bound nucleotide in actin is not clear but without ATP, G-actin easily denatures. The nucleotide-binding site is located in the cleft region between N-terminal and C-terminal domains of the actin molecule [2]. Many amino acids are involved in the nucleotide-binding site including residues 115-118, 121-124 [3], Lys-336, Trp-356 [4] and Tyr-306 [5].

2-Azido-ATP, a photoaffinity ATP analogue, has hindered rotation around the glycosidic bond due to the bulky group at the C-2 atom of the purine ring and consequently it is always in the anti-configuration. Under irradiation with near ultraviolet light, the azido group is activated and becomes cross-linked to Tyr-306 [5]. To understand the role of the nucleotide in actin, we have characterized the effects of replacing bound ATP with 2-azido-ATP with and without photocross-linking.

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2. MATERIALS AND METHODS

Actin, S1, tropomyosin and troponin were prepared as described previously [6]. Synthesis of 2-azido-ATP was carried out according to the method of Czarnecki [7]. Absorption was measured with a Philips PU8800 and fluorescence spectra were measured with an SLM 8000. ATPase activity was measured using a radiometer pH-stat as previously reported [6]. Viscosity was measured at 25°C with an Ostwald viscometer having an outflow time of 52 s for water. Protein concentrations were determined from the absorption as described previously [6] and F-actin concentration was determined using the Biuret method.

2.1. *Preparation of 2-azido-ADP-F-actin*

F-actin pellet was washed with 60 mM KCl and 10 mM Tris-HCl (pH 8.0) and then homogenized in the same buffer solution. The sample was ultracentrifuged at 100000 × *g* for 90 min. The pellet was homogenized in 1 mM Tris-HCl (pH 8.0)

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and 0.1 mM $CaCl_2$ (actin concentration was about 2 mg/ml). The solution was sonicated using a heat system W-10 probe sonicator at 0°C for 1 min. 0.5 mM 2-azido-ATP was immediately added and again the sample was sonicated for 1 min at 0°C. 1/10 vol. of Dowex-1 was added and, after a 5 min incubation at 0°C, removed by filtration. 0.5 mM 2-azido-ATP was again added and the sample was incubated for 10 min at 0°C. 2-Azido-ATP-G-actin was polymerized in 60 mM KCl, 2 mM $MgCl_2$ and 10 mM Tris-HCl (pH 8.0) (buffer-F) and after 1 h incubation at room temperature, ultracentrifuged at 100000 × *g* for 90 min. The pellet was homogenized in buffer-F. Half the sample was photolyzed on ice with a 450 W medium pressure Hg lamp for 20 × 15 s with 15 s breaks.

3. RESULTS

2-Azido-ADP-F-actin, with and without photolysis, was denatured with 5% perchloric acid and the precipitates were removed by ultracentrifugation at 100000 × *g* for 15 min. The supernatant was passed through a 0.45-μm filter. The absorption spectrum of 2-azido-ADP has a peak at 272 nm (ϵ = 14 200). After photolysis, most of the bound nucleotide was not released since 2-azido-ADP was covalently cross-linked to Tyr-306 in actin [5]. From the difference in the absorption at 272 nm, the ratio of cross-linked 2-azido-ADP to actin was calculated to be 0.82.

The effects of photolyzed and non-photolyzed 2-azido-ADP-F-actin on the S1 Mg^{2+}-ATPase activity were measured in 30 mM KCl, 2 mM $MgCl_2$, 2.5 mM ATP and 1 mM Tris-HCl at pH 8.0 (buffer A) and 25°C. The concentration of S1 was 0.05 mg/ml. Fig.1 shows the double-reciprocal plot of the ATPase activity of S1 versus the concentrations of 2-azido-ADP-F-actin with and without photolysis. The results indicate that the replacement of ADP with 2-azido-ADP with and without photolysis reduced appreciably the actin activation of S1 Mg^{2+}-ATPase activity. This decrease is mainly due to a change in V_{max}. The effects of regulated 2-azido-ADP-F-actin (i.e. in the presence of tropomyosin and troponin) on the Mg^{2+}-ATPase activity of S1 were also measured in buffer A and 0.05 mM $CaCl_2$ or buffer A and 2 mM EGTA. The concentrations of S1, actin, tropomyosin and troponin were 0.05 mg/ml, 0.25 mg/ml, 0.086 mg/ml and 0.087 mg/ml, respectively. Strong inhibition of actin-activated S1-ATPase activity was observed in the case of regulated normal F-actin (85–92%), regulated 2-azido-ADP-F-actin without photolysis (84%) and regulated 2-azido-ADP-F-actin with photolysis (70%) in buffer A and 2 mM EGTA. No inhibition was observed in buffer A and 0.05 mM $CaCl_2$.

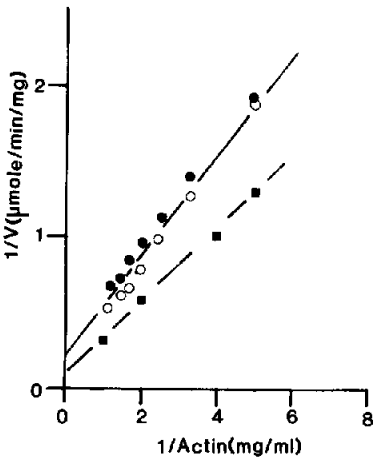

Fig.1. Double-reciprocal plot of the ATPase activity of S1 vs the concentration of 2-azido-ADP-F-actin with (●) and without (○) photolysis, and of normal ADP-F actin (■).

The ability of 2-azido-ADP-F-actin to depolymerize and to repolymerize was studied. 2-Azido-ADP-F-actins with and without photolysis were dialyzed against 0.2 mM ATP, 0.1 mM $CaCl_2$ and 2 mM Tris-HCl at pH 8.0 and 4°C. After 3 days dialysis, 2-azido-ADP-F-actin both with and without photolysis depolymerized completely, judging from the viscosity measurements. 2-Azido-ADP in F-actin without photolysis was replaced with ATP during dialysis but 2-azido-ADP in F-actin with photolysis was not replaced. The repolymerization of depolymerized samples was measured. Fig.2 shows that G-actin with bound 2-azido-ADP covalently lost its ability to polymerize.

The fluorescence spectra of native ATP-G-actin, or 2-azido-ADP-G-actin in buffer-G or in buffer-G and 2 mM EDTA were measured. Excitation wavelength was 295 nm. The emission maxima were at 330 nm for native ATP-G-actin and 337 nm for denatured G-actin in the presence of 2 mM EDTA. The fluorescence spectrum of 2-azido-ADP-G-actin in buffer-G was similar to that of G-actin which was denatured in the presence of 2 mM EDTA. 2-Azido-ADP-G-actin seems to denature quickly even though it binds nucleotide covalently.

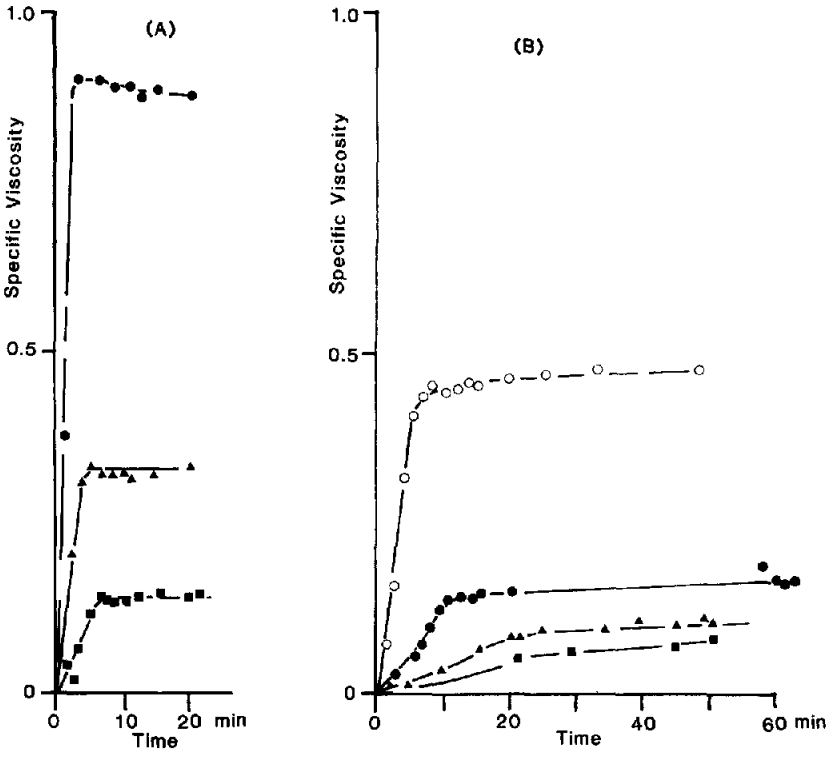

Fig.2. Time course of the repolymerization of depolymerized 2-azido-ADP-F-actin with (B) and without (A) photolysis at 25°C. The polymerization was initiated by the addition of 10-fold concentrated buffer-F solution at time zero. The concentrations of actin were 2.2 mg/ml (○), 1.0 mg/ml (●), 0.5 mg/ml (▲) and 0.25 mg/ml (■).

4. DISCUSSION

Recently, Hegyi et al. [8] replaced the bound ADP in F-actin with 8-bromo-ADP. They found that the replacement reduced the actin activation of myosin S1-ATPase activity. 8-Bromo-ADP, substituted at the C-8 atom of the purine ring by the bulky bromide, has hindered rotation around the glycosidic bond. On the other hand, 2-azido-ADP also has hindered rotation around the glycosidic bound due to its bulky group at the C-2 position of the purine ring or its covalent cross-linking to Tyr-306 in F-actin. 2-Azido-ADP-F-actin, both with and without photolysis, activated the S1-ATPase activity less effectively than normal F-actin. The results suggest that a free rotation around the glycosidic bond in the bound nucleotide is important for the actin function as suggested by Hegyi et al. [8]. 2-Azido-ADP-G-actin loses very quickly the ability to polymerize. It is possible that during depolymerization, a conformational change occurs [4] and cross-linked nucleotide may not allow it to revert to a proper conformation of G-actin and then a denaturation may occur.

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REFERENCES

[1] Neidl, C. and Engel, J. (1979) Eur. J. Biochem. 101, 163–169.
[2] Kabsch, W., Mannherz, H.G. and Suck, D. (1985) EMBO J. 4, 2113–2118.
[3] Barden, J.A. and Kemp, B.E. (1987) Biochemistry 25, 5793–5798.
[4] Hegyi, G., Szilagyi, L. and Elzinga, M. (1986) Biochemistry 25, 5793–5798.
[5] Kuwayama, H. and Yount, R.G. (1986) Biophys. J. 49, 454a.
[6] Miki, M. (1987) Biochim. Biophys. 164, 229–235.
[7] Czarnecki, J.J. (1984) Biochim. Biophys. Acta 800, 41–51.
[8] Hegyi, G., Szilagyi, L. and Belagyi, J. (1988) Eur. J. Biochem. 175, 271–274.