INTERACTION OF RABBIT MUSCLE ACTIN AND CHEMICALLY MODIFIED ACTIN WITH ATP, ADP, AND PROTEIN REACTIVE ANALOGUES; ROLE OF THE NUCLEOTIDE

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1. Introduction

The polymerization mechanism of actin differs from that of many other proteins that aggregate to defined fibres, in that ATP is hydrolyzed to ADP and P_i in a stoichiometric reaction during the process. Mg^{2+} is best suited to induce the typically cooperative process [1]. Apparently, only tubulin is polymerized to microtubuli by a similar reaction, using GTP hydrolysis. The role of the nucleotide in these cases is not known, as actin freed from nucleotide in the monomeric form may polymerize upon addition of salt [2]. Moreover, fibrillar F-actin can be depolymerized to monomeric G-actin by the two ATP analogues adenylyl-imidodiphosphate (AMP-PNP) and adenylyl-methylenediphosphate (AMP-PCP), and will repolymerize without hydrolysis of the nucleotide [3].

We have found that actin covalently labelled by an ADP analogue in its F form will not depolymerize to G-actin under the usual experimental conditions. The measurement of the exchange rate of the nucleotide in momomeric- and F-actin seems to strengthen our hypothesis that the ADP in F-actin may serve the purpose of stabilizing the polymer form in muscle fibres, and that the energy of ATP is used for the depolymerization rather than the polymerization reaction.

2. Materials and methods

Actin was prepared from rabbit striated muscle after the method of Spudich [4]. It was purified by two repolymerization steps. For all experiments, the G-actin after the last depolymerization was reacted with a 5-fold excess of *N*-ethylmaleimide. Previous experiments had shown [5], that only a single SH group on the molecule becomes modified by this reagent, while the polymerization or myosin binding reactions are not influenced. In addition, this treatment seems to retard the slow denaturation of actin during storage. [^{14}C]ATP (specific activity 52 μCi/μmole), [^{14}C]ADP (specific activity 10–60 μCi/μmole) and [^{36}Cl]sodium (specific activity $>$ 3 mCi/g) were obtained from Amersham-Buchler Co. ^{32}P-Pyrophosphate was obtained from New England Nuclear Co. [^{14}C]Barium cyanamide was obtained from Farbwerke Hoechst. *S*-Dinitrophenyl-6-mercaptopurine riboside triphosphate, labeled with ^{32}P at the β and γ phosphate to a specific activity of 240 μCi/μmole was synthesized as described previously [6]. A new route has also been applied in this synthesis, however. Starting with isopropylidated 6-Cl-purine riboside, either the monophosphate or the triphosphate were synthesized, before the substance was converted to the *S*-carboxynitrophenyl-6-mercaptopurine derivative [7] by reaction with 4-carboxy-3-nitrothiophenol at pH 8.5–9.5. [^{14}C]5-Amino-1-H-tetrazole was synthesized from barium cyanamide, and sodium azide by a procedure described elsewhere [5]. Continuous-flow dialysis was run in a chamber very similar to the apparatus described by Colowick and Womack [8]. The cylindrical vessel, 4 cm in diameter, had a total height of 1.0 cm, and was divided into two equal chambers by a sheet of dialysis membrane easily permeable to nucleotides (Iris 3069, Rhône-Poulenc

Co., Paris). A peristaltic pump from a Technicon Auto-Analyzer system with a flow of 0.42 ml/min in both tubes was used for the continous perfusion of the lower chamber, stirred by a magnetic bar, while the upper chamber, containing the protein solution, was stirred by a similar bar suspended from a stirring motor. Viscosity measurements were taken in Ostwald-type viscosimeters with a flow time for water of approximately 55 sec.

3. Results and discussion

In model reactions, the ATP analog (fig. 1) was found to react with cysteine SH groups, but with none of the other physiological amino acid side chains. In this reaction, a new stable thioether bond between the nucleotide and the cysteine is formed [7]. Since by neighbouring effects the reactivity of amino acid side chains may be changed to a large extent, the covalent attachment of the analogue could not be, by itself, a strong indication of an SH group in the vicinity of the ATP binding site. However, it appeared necessary to modify the one superficial, and non-functional, SH group before labeling experiments.

When F-actin at a concentration of 12 mg/ml was incubated with a 100-fold molar excess of the ^{32}P-labeled ATP analogue at pH 7.4, rapid depolymerization of the actin gel was observed. The incubation of the resulting G-actin was continued for 4–6 hr, before repolymerization was started by the addition of $MgCl_2$ to a final concentration of 2 mM, and KCl to a final concentration of 50 mM. The rate of polymerization, measured as the increase in specific viscosity, was equal to the rate observed under normal ATP hydrolysis. The F-actin formed was centrifuged down, and washed several times with 0.05 M KCl–2 mM $MgCl_2$ solution with centrifugation. The final F-actin was subjected to depolymerization conditions by cautious homogenizing in a 10 mM ATP solution buffered to pH 7.4 with 1 mM Tris–HCl. It was found that only a small amount of the protein, at most 20%, could be brought to the monomeric or oligomeric state under these conditions, at least 80% remained in the polymer state, and could be characterized as F-actin after centrifugation by the usual methods [6].

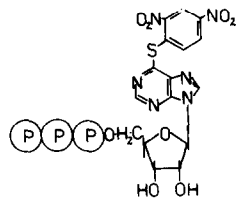

Fig. 1. Structural formula of *S*-dinitrophenyl-6-mercaptopurine riboside triphosphate.

Table 1
Analogue and inorganic phosphate contents

	Protein spot on paper chromatograms	Inorganic phosphate spot on paper chromatograms	Dialyzed protein
Incubation mixture of F-actin with ATP analogue	0.75	0.65	0.67
Centrifuged G-actin solution	0.79	0.72	0.82
Repolymerized solution of F-actin	0.81	1.67	0.79
Washed F-actin	0.87	0.02	0.88
F-Actin pellet after attempt at depolymerization with ATP	0.92	0.03	0.87
TCA-precipitated F-actin	–	–	0.92

Expressed as μmoles of ^{32}P-labeled inorganic phosphate, ATP or ADP analogues per μmole of actin monomer.

At all stages of the experiment, the amount of nucleotide bound per molecule was determined after prolonged dialysis, or after precipitation of the protein in 10% trichloroacetic acid solution, and washing. The amount of free nucleotide and of inorganic phosphate was determined by subjecting an aliquot of the solutions to paper chromatography in isopropanol–0.5 M ammonium acetate buffer, pH 6, 5:2 (v/v), and counting the spots identified by control strips. The results are summarized in table 1.

From these figures we concluded that the analogue was specifically bound to the actin ATP binding site, as approximately one molecule was covalently attached per molecule of the protein, and the repolymerization was accompanied by the liberation of a stoichiometric amount of inorganic phosphate. The specificity of the label could then be further demonstrated by protection of the protein by ATP. Thus, the incubation of F-actin with the analogue in a solution practically free of ATP, as described above, achieved a covalent labeling of at least 80% of the protein, as the analogue in this case is in competition with ADP on the protein only. When the same amount of the analogue was mixed with ATP to a final concentration of 0.02 M, the covalent label was found on only 50% of the protein, and when the competiting ATP concentration was raised to 0.1 mM, only 10–20% of the actin carried the covalently attached analogue after incubation for 6 hr.

To complete the necessary evidence for specific labeling, it was shown that the analogue was attached to a single peptide in a tryptic digest fingerprint and autoradiograph, as reported previously [6].

In view of the depolymerization and repolymerization of actin with the aid of non-hydrolyzable ATP analogues, as AMP-PMP and AMP-PCP [3], it seems evident that the energy released upon hydrolysis of the triphosphate is not used to bring the protein into a conformation suitable for polymerization, which we may call 'F-conformation'. On the other hand, the binding of a nucleoside triphosphate strongly favours the shift of the protein conformational equilibrium towards 'G-conformation'. The binding of a nucleoside diphosphate shifts this equilibrium to the 'F-conformation', as the covalent attachment of the analogue diphosphate even blocks depolymerization completely under relatively high ATP concentrations. Salt concentration also influences the equilibrium, and this always has been used for the preparation of actin. Thus, one might say that the energy of ATP, for which actin has a higher affinity than for ADP, goes into the creation and stabilization of the 'G-conformation' at low salt concentration, from which it is released at higher salt concentration in a hydrolysis to ADP. This hydrolysis is not very important for the salt-induced shift to the 'F-conformation', but helps to produce ADP, which in turn stabilizes F-actin.

For a physiological role of the mechanism, one might postulate, that the exchange rate of the nucleotide in F-actin should be distinctly smaller than in G-actin. This would lead to a very well stabilized F-actin polymer in the muscle fibre. Therefore, we undertook the measurement of these processes, as described in Methods. The protein solution between 1 and 7 mg/ml was placed in the upper chamber of the apparatus, and the lower chamber was perfused with buffer containing the same nucleotide as the G- or F-actin solution, and at the same concentration. At zero time, [^{14}C]ATP or [^{14}C]ADP was added to the protein solution to a concentration less than 1/20 of the cold nucleotide concentration and its appearance in the lower chamber was measured. Evidently, the higher viscosity of the F-actin solution will effect a retardation of dialysis of the nucleotides. Therefore, the curve for $^{36}Cl^-$ was also measured, and used to correct for the viscosity of the protein solutions at various concentrations. It soon became evident, moreover, that the dialysis rate was a function of the salt concentration. In order to obtain comparable curves for G- and F-actin, we also used G-actin modified with diazonium tetrazole at tyrosine 53. Due to this single side chain modification, the polymerization reaction of rabbit muscle actin is blocked selectively, while the myosin binding and ATP binding sites are still intact [5]. The modification was checked by the application of ^{14}C-labeled diazonium tetrazole. With this material, nucleotide dialysis rates could be measured at the same salt concentrations as used for F-actin.

Examples of the curves obtained are shown in fig. 2. Following the deductions of Colowick [8], the relationship of the peak heights is a function of the dissociation constants of the nucleotide–protein combination. The ascending and descending parts of the curves, on the other hand, are functions of the rate of exchange of the nucleotide. A high rate of exchange,

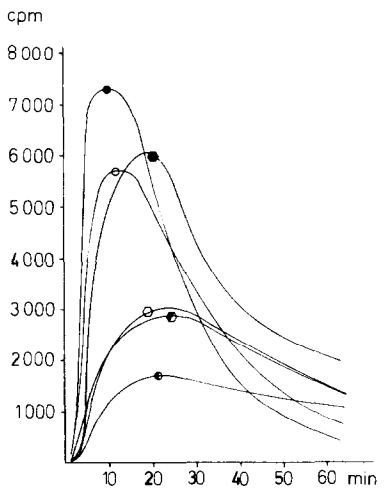

Fig. 2. Examples of dialysis rate curves for the actin–nucleotide system: (•) $^{36}Cl^-$ in 0.01 mM ATP, 1 mM Tris, 0.2 mM ascorbate buffer, pH 7.5, in both chambers. (●) $^{36}Cl^-$ in 0.01 mM ATP, 1 mM Tris, 0.2 mM ascorbate buffer and 50 mM KCl–2 mM $MgCl_2$ in both chambers. (○) $^{36}Cl^-$ in 0.01 mM ATP, 1 mM Tris, 0.2 mM ascorbate buffer and 50 mM KCl–2 mM $MgCl_2$ and F-actin (7 mg/ml) in the upper chamber. (●) [^{14}C]ATP in 0.01 mM ATP, 1 mM Tris, 0.2 mM ascorbate buffer and 50 mM KCl–2 mM $MgCl_2$. (⊖) [^{14}C]ATP in 0.01 mM ATP, 1 mM Tris, 0.2 mM ascorbate buffer and 50 mM KCl–2 mM $MgCl_2$ and F-actin (7 mg/ml) in the upper chamber. (⊖) [^{14}C]ATP in 0.01 mM ATP, 1 mM Tris, 0.2 mM ascorbate buffer and 50 mM KCl–2 mM $MgCl_2$ and diazonium tetrazole modified G-actin (1.5 mg/ml) in the upper chamber.

for example, would lead to a slower increase of the amount of labeled nucleotide in the dialysis fluid, and to a protracted curve.

From these experiments it could be concluded that F-actin indeed shows an exchange rate for the pysiological nucleotides at least 5–7 times smaller than the rate for G-actin or modified G-actin, even after correction for the higher viscosity of these solutions. This gives rise to the speculation that the ADP in F-actin may be located in a crevice of the protein which is partially covered up during the polymerization process, a subject for future investigations.

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