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PROTECTION OF ONE OF THE TWO REACTIVE THIOL GROUPS IN F-ACTIN BY ATP AND PHALLOIDIN

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SUMMARY: In F-actin, $[A(SH)_5]_n$, prepared from rabbit skeletal muscle, two thiol groups react with 2,4-dinitrophenyl-glutathionyl-disulfide, DNPSSG, to form $[A(SH)_3(SSG)_2]_n$. One of the two thiol groups reacts fast, (20 min), while the reaction of the second is slow (200 min). The fast reacting group has been identified as cysteine-373.

In the presence of approximately one equivalent of ATP, only one of the thiol groups is reactive. The reaction product is $[A(SH)_4SSG]_n$. In comparison, the shielding effect of ADP is about 2 to 3 times smaller than that of ATP, while AMP is ineffective.

The mushroom toxin phalloidin, which binds to polymeric actin, exhibits a similar protective activity as ATP and shields one thiol group from reaction with DNPSSG.

We conclude from these data that in F-actin a second low affinity binding site for adenosin-nucleotides exists, which can be monitored by the reactivity of one of the two reactive thiol groups.

INTRODUCTION

In polymeric actin, each monomer binds one molecule of nucleotide, preferentially ADP. The exchange of bound nucleotide with free nucleotide is very slow (1); therefore, it is reasonable to assume that such exchange occurs only at the terminal protomers of the filaments or at the monomers in equilibrium with the polymer. This can be concluded from the observation that agents like the mushroom toxin phalloidin, which stabilize the polymeric form (2, 3, 4, 5) and hence decrease the critical concentration of monomers, also decrease the exchange of the nucleotide which is tightly bond to F-actin (5).

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Besides the high-affinity-binding site for adenosin-nucleotides, in polymeric actin another site with low affinity has been previously suggested. For example, it has been described by Sleigh and Burley that in the presence of ATP, the reaction of F-actin with a sulfenyl spin-label was at another thiol group than in the absence of ATP (6). In other experiments, it was shown that denaturation of F-actin was slower in the presence of ATP (7). In the present study, we substantiated the interaction of F-actin with a second molecule of adenosine nucleotides by studying the reactivity of the two thiol groups reactive in F-actin. In the presence of equimolar amounts of ATP the reaction of one of these two thiol groups with an activated glutathione derivative is completely suppressed. A similar effect was detected with the mushroom poison phalloidin.

MATERIALS AND METHODS

Glutathione, ATP, ADP, and AMP were from Boehringer, Mannheim, FRG. N-ethylmaleimide (NEM) was from Schuchardt, München, FRG. Phalloidin was prepared in our own laboratory.

Actin was prepared from rabbit skeletal muscle using a modification of the method described by Löw and Dancker (8). By one additional homogenizing-centrifugation step, the protein was purified from excess ATP. The final ratio of adenosine nucleotide to actin was 0.9 : 1.0. F-actin solutions were obtained by homogenizing the pellet in 0.1 M KCl, 1 mM Tris/HCl pH 7.4, or, alternatively, in 0.1 mM $MgCl_2$, 1 mM Tris/HCl pH 7.4. Before use, occasional turbidity was removed by a 5 min centrifugation at 40.000 g.

The preparation of 2,4-dinitrophenyl-glutathionyl-disulfide (DNPSSG) and the conditions of its reaction with actin have been described recently (9). The mono-NEM-derivative of actin was prepared according to Bender et al. (10).

RESULTS

When F-actin $[A(SH)_5]_n$ was reacted with an excess of 2,4-dinitrophenyl-glutathionyl-disulfide (DNPSSG), two thiol groups of the protein formed disulfides with the glutathione moiety. The product, $[A(SH)_3(SSG)_2]_n$, was formed in both buffers previously described; therefore, the reaction was independent of the presence of potassium or magnesium ions. As Figure 1 shows, the two thiol

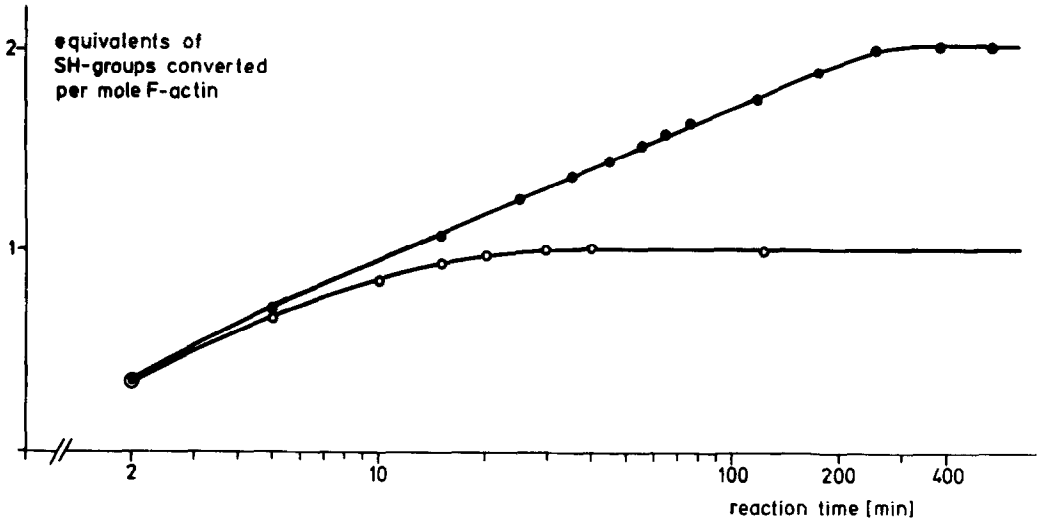

Figure 1 Reaction kinetics of 2,4-dinitrophenylgluthathionyl-disulfide with F-actin in the presence of (o) or in the absence of (●) ATP.

groups reacted at different rates. While the reaction of one thiol group was complete after 20 min, the other one took more than 200 min for full reaction with DNPSSG.

The addition of ATP completely inhibited the reaction of one of the thiol groups. For example, in the presence of 1.1 equivalents of ATP only one thiol group in a 2×10^{-5} M F-actin solution was converted to the disulfide $[A(SH)_4SSG]_n$. This was a fast reaction which reached completion after 20-30 minutes. When 1.1 equivalents of ADP were used instead of ATP, the protection was less effective. To achieve the protective effect of ATP, 2-3 equivalents of ADP were necessary. AMP had no protective activity at all.

In order to identify the most reactive thiol group, we prepared the mono-NEM-derivative of actin, $[A(SH)_4SNEM]_n$, which, according to Bender et al. (10) and Faust et al. (11), is substituted at the cysteine-373 moiety. As expected, there was no reaction with the reagent in the presence of ATP, however, when ATP was omitted, one thiol group reacted at the slow rate. These results substantiate that the highly reactive thiol group was, under both conditions, that of cysteine-373.

When the titration was run in ATP-free buffer in the presence of 1 equivalent of the mushroom toxin phalloidin, again only one thiol group of F-actin was reactive. Again, this reaction was complete after 20-30 min, indicating that the effects of ATP and phalloidin on the reactivity of the thiol groups in F-actin were very similar. This was supported by the fact, that NEM-substituted F-actin did not react with DNPSSG in presence of the toxin, as was the case in the presence of ATP.

DISCUSSION

The present study provides evidence that ATP interacts with polymeric actin at a second site of the protein, different from that of the tightly bound nucleotide. Otherwise, the protective effect of ATP on the reaction of one of the thiol groups of actin with the activated glutathione moiety cannot be understood. The interaction can be characterized by a K_D value $\leqslant 2 \times 10^{-5}$M, because at that concentration of ATP and actin the protection is complete. The interaction of ATP with the reagent DNPSSG can be excluded by the fact that phalloidin, which is known to interact with F-actin, has the same effect.

The interaction with ATP shields only the slow-reacting thiol group of actin and leaves the fast-reacting thiol group unaffected. By studying the (Cys-373)NEM-derivative of F-actin, which in the presence of ATP shows no reaction at all, the fast-reacting thiol group was identified as that cysteine residue located at the next to last position of the C-terminus (12). It is, probably, also this thiol group which reacts first when ATP is absent.

To understand the effect of ATP on the reactivity of the thiol groups in actin we assume that ATP either shields one thiol group directly, or, suppresses a conformational change of F-actin, by which a second thiol group becomes exposed. Provided that conformational change is slow, it would determine the rate of the second disulfide formation.

This explanation seems more reasonable, because a similar immobilisation of the conformation as with ATP is likely to occur

with phalloidin. The mushroom toxin binds to F-actin with an apparent K_D of 2×10^{-8}M, and decreases the critical concentration of actin monomers in equilibrium with the polymer (5). The resulting stabilization of the actin filaments and the low reactivity of the second thiol group may therefore be related processes.

The shielding of the slow-reacting thiol group is also achieved by ADP. From the higher concentrations needed we conclude that the affinity of ADP to the postulated binding site is at least 2-3 times lower than that of ATP. AMP has no protective activity, even at high concentrations.

The complex of phalloidin with F-actin cannot be recognized by electron microscopy (13). Therefore, phalloidin-F-actin is usually characterized by the stability of the filaments under various depolymerizing conditions (for a review see 14). The stoichiometry of the titration of F-actin with activated disulfides of glutathione and its regulation by phalloidin may now provide another assay for the phalloidin-actin-complex.

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