Adenosine Triphosphate Cleavage during the G-Actin to F-Actin Transformation and the Binding of Adenosine Diphosphate to F-Actin*  


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Since the discovery by Straub and Feuer (1) and by Laki, Bowen, and Clark (2) that adenosine triphosphate bound to G-actin is transformed to adenosine diphosphate and inorganic phosphate during polymerization of actin, it has become increasingly clear that the chemical changes in the nucleotide are related to the change in the physical state of the protein. Bárány, Biro, Molnar, and Straub have shown that highly purified actin preparations, free of any enzyme which would use ATP, ADP, or AMP as a substrate, still hydrolyze ATP during polymerization, and thus support the original idea that the ATP to ADP transformation is related to the globular to fibrous transformation of the actin protein itself (3). Mommaerts (4) was the first to show that the ADP formed during polymerization remains bound to F-actin, and Ulbrecht et al. (5), while extending Mommaert's finding on exhaustively purified actin preparations, have shown that the P_1 formed during polymerization is not bound to F-actin.

The stoichiometry of the cleavage and the tightness of binding of the ADP lead inevitably to questions regarding the position of bond breaking during the hydrolysis and the nature of the forces involved in the tight binding of ADP to F-actin. To aid in clarifying these problems, this study with ^18O isotope was initiated.

**EXPERIMENTAL PROCEDURE**

In a typical experiment, 40 g of acetone-dried muscle powder (6) were extracted with 1 liter of distilled water with stirring at room temperature. The crude actin solution, 840 ml, was polymerized in 0.1 M KCl and purified by centrifugation in the Spinco preparative ultracentrifuge with the No. 30 rotor for 3 hours (7). The F-actin pellets were rinsed two to three times with distilled water to remove any traces of KCl from their surface, and then the pellets were allowed to drain in the refrigerator for about 40 hours. This draining reduced the water content of the pellets to a large extent and thus increased their original protein concentration of about 10% to almost 50%. To convert F-actin to G-actin, the pellets were homogenized in a Potter-Elvehjem-type tissue grinder in 200 ml of 0.5 mM ATP dissolved in H_2^18O (1.45 atom % excess) and brought to pH 8.4 by addition of solid Tris buffer. After the G-actin solution, 6.83 mg per ml, was allowed to stand in the cold for 30 minutes, free ATP was removed by stirring with 20 mg of Dowex 1 X8 (200 to 400 mesh) per ml at room temperature for 3 minutes (8).

After centrifugation, the G-actin supernatant was polymerized by the addition of solid anhydrous MgCl_2 (final concentration, 2 mM) and was kept stirring at room temperature for 1 hour. Samples were removed for protein, nucleotide, and P_1 determination. The remaining F-actin solution was transferred to polyethylene bottles and stored in the deep freeze.

Nine such experiments were performed in a period of 1 month. The average nucleotide content was 0.98 mole per 62,000 g of actin, and the average P_1 liberated during polymerization was 0.87 mole per 62,000 g of actin.

Before ^18O analysis on the ADP bound to F-actin and the P_1 liberated, the native state of the actin protein was tested. It was found that F-actin solutions, stored in the deep freeze for 4 months, contained about 50% of precipitated actin. This precipitation might be caused by an increase in the Mg^{2+} concentration (3) during freezing. The mixture of dissolved F-actin and of precipitated F-actin in 2 mM MgCl_2 was brought to a final concentration of 0.1 M KCl (9) and homogenized, and its activating effect on the L-myosin ATPase was determined in the presence of Mg^{2+} at low ionic strength (10). No difference was found between the activating effect of F-actin which was stored in the deep freeze for 4 months and that of the freshly prepared F-actin, the specific activities were 0.94 and 0.38 μmole of P_1 per mg of actomyosin per minute at 25°, respectively. Since there is a strict relationship between the bound nucleotide content of actin and its activating effect on the L-myosin ATPase (10), the complete activation effect of the deep freeze-stored actin indicates that the ADP was maintained bound to the protein in the frozen state.

For ^18O analysis, 11,750 mg of F-actin in 1,766 ml of H_2^18O containing 185 μmoles of bound ADP and 164 μmoles of P_1 were used. After a sample had been taken for the determination of ^18O content of the medium, solid trichloroacetic acid was added to a final concentration of 1% with stirring. One minute after addition of trichloroacetic acid, the actin precipitate was filtered through Whatman No. 42 paper into Buchner flasks kept in ice. To about 1.5 liters of the combined, cooled filtrates, carrier-
free $^{32}$P (total counts, $1.5 \times 10^9$) was added, and samples were removed for determination of radioactivity. Then approximately 1 g of BaBr$_2$ was added with stirring, and the pH was adjusted to 8 with solid KOH. The precipitate, containing Ba$_3$(PO$_4$)$_2$ and also Ba$_5$(ADP)$_3$, was allowed to settle in an ice bath for 1 hour and was collected by centrifugation in a Servall refrigerated centrifuge at 6000 r.p.m. for 5 minutes. Determinations for radioactivity and optical density were performed on the supernatant. The radioactivity determinations showed that only 5% of the original $P_i$ remained in the supernatant, and the optical density determinations showed that about 50% of the original ADP remained in the supernatant. This remaining ADP was then adsorbed on charcoal.

After filtration the supernatant showed essentially no remaining ADP. The mixed barium precipitate was dissolved in dilute HBr and subjected to a charcoal treatment to separate the ADP from the inorganic phosphate. After filtration the charcoal residue was washed with 1% HBr to effect complete separation. Determination of $^{32}$P on the supernatant showed a recovery of 72.5% of the original $P_i$, whereas the ADP content was <0.1% of the original ADP. This solution was treated with Dowex 50 in the K$^+$ form, then the pH was adjusted to 4, and the solution was evaporated to dryness in the rotary evaporator. The residue was dissolved in a minimal amount of H$_2$O and KH$_2$PO$_4$ precipitated with the addition of 2 to 3 volumes of 95% ethanol. The KH$_2$PO$_4$ sample gave difficulty in analysis and was, therefore, converted to Ba$_3$(PO$_4$)$_2$, which was analyzed by a modification of the CO method of Cohn and Drysdale (11, 12).

The 2 charcoal residues were combined and heated with 1 N HCl in a boiling water bath for 15 minutes to hydrolyze ADP to AMP and $P_i$. The resulting supernatant was treated with approximately 100 mg of BaBr$_2$ 2H$_2$O and the pH was adjusted to approximately 8. The Ba$_3$(PO$_4$)$_2$ precipitate was removed by centrifugation, and the supernatant was checked for ADP, $^{32}$P, and $P_i$. The ADP content was found to be <0.2% (0.13 $\mu$ mole as compared with 185 $\mu$ moles of the original ADP), the $^{32}$P content <0.1%, and the $P_i$ content 3.1%.

The Ba$_3$(PO$_4$)$_2$ was dissolved in dilute HBr and found to contain 35.2% (61 moles) of the original ADP as $P_i$ and 2.6% of the $^{32}$P. The precipitate was treated and analyzed as described previously (12).

### RESULTS

The results of the $^18$O experiments are given in Table I. It is clear that the predominant pathway of cleavage is between the terminal phosphorus atom and its bridge oxygen. The corrected $P_i$ values indicate that essentially all of the reaction goes by this pathway.

Although the corrected figures indicate a single pathway, the correction factors in the case of ADP in Experiment 2 are substantial and deserve some comment. The uncorrected observed $^{18}$O content of the $P_i$ isolated from ADP was 0.05 atom % excess, which is higher than usual for a zero value, viz. 0.005 atom % excess. Some of this (0.02 atom % excess) was caused by the failure to separate all the $P_i$ from the ADP, and this correction was easily made with the $^{32}$P content of the ADP (7%). The remainder, however, was above the usual experimental error, and therefore samples of unlabeled KH$_2$PO$_4$ were put through the same procedure as the ADP except that H$_2$O was used throughout. Observed values of 0.02 and 0.05 atom % excess were obtained even though analysis of reagent KH$_2$PO$_4$ gave values of only 0.005 atom % excess. Evidently some impurity, either in the resin or the reagents, gave a material of mass 30 in the mass spectrometer. Since separate tests on KH$_2$PO$_4$ treated with resin and reagents gave an average blank value of 0.03 atom % excess, it seems clear that the observed value for ADP is the result of this impurity and not an alternate pathway of hydrolysis. This is supported by the low value for ADP observed in Experiment I, which was performed at an earlier date with a different supply of reagents and resin.

The value for the $^{18}$O content of the $P_i$, i.e. 90% of theoretical, is within experimental error of this method. Control samples of Ba$_3$(P$^{18}$O$_4$)$_2$ made from KH$_2$P$^{18}$O$_4$ gave values 10% below the known $^{18}$O atom % excess. In other investigations this consistently low value (perhaps caused by entrapped water in the Ba$_3$(PO$_4$)$_2$) can be avoided by converting to KH$_2$PO$_4$. However, in these studies the need for a stoichiometric quantity of actin made it necessary to analyze the minimum amount of $P_i$ and the Ba$_3$(PO$_4$)$_2$ precipitate proved superior to KH$_2$PO$_4$ in this regard, because of the unexpected difficulty in assaying KH$_2$PO$_4$ mentioned in "Experimental Procedure." It is, therefore, reasonable to conclude that within experimental error the theoretical amount of oxygen corresponding to the cleavage of one P—O bond occurs during the hydrolysis.

### DISCUSSION

The conclusions to be derived from the $^18$O data can be most readily deduced by considering the possible pathways by which cleavage of ATP would occur in the G-actin to F-actin conversion. Previous studies on the major protein of muscle, myosin, have shown that cleavage occurs by attack at the terminal phosphorus atom (13), and extensive study of other ATPases (14) has shown that these cases also proceed with cleavage at the terminal phosphorus atom. A priori, therefore, such cleavage might be expected in any ATP cleavage leading to inorganic phosphate and ADP. The actin polymerization is significantly different from these previous examples in two major ways. First, the G-actin to F-actin conversion is not catalytic but stoichiometric, 1 molecule of $P_i$ being released for each molecule of actin which is polymerized. In the second place, the extremely tight binding of ADP is without parallel in the other reactions catalyzed by the ATPases. Moreover, since there is no theoretical reason to exclude attack on the middle phosphorus atom, a mechanism involving such an attack (as shown in Equations 1 and 2) would provide a reasonable explanation for the tight binding of ADP. Such an attack might lead either to a final covalent ADP-actin bond as indicated in Equation 1 or, via a transient covalent...
ADP-actin intermediate, to the tightly bound actin-ADP as shown in Equation 2 (a dash will be used in these mechanisms to indicate a covalent attachment, and a dot to indicate an association stabilized by noncovalent forces). Since all of these mechanisms would produce P₁, which does not contain any ³¹O, the experimental results exclude all of these mechanisms and any of numerous variations on them which involve at any stage an attack on the middle phosphorus atom. Despite its significant difference from the ATPases, therefore, actin apparently causes a similar cleavage of the terminal phosphorus-oxygen bond.

It remains to consider the type of binding involved in the F-actin-ADP interaction. G-actin has a dissociation constant in the range of 10⁻⁶ (15, 16); polymerization of actin by the addition of salts decreases the dissociation constant of the resulting F-actin-ADP by several orders of magnitude since no removal of nucleotide is observed with F-actin upon 4-fold ultracentrifugation or magnesium precipitation (5), or by 3-fold treatment with charcoal (10). The two most likely explanations for the tight binding seem to be a conformational change concomitant with ATP hydrolysis or a covalent bond between protein and ADP. Since the liberation of ADP from the F-actin was accomplished in our experiments by trichloroacetic acid in the presence of H³¹O, cleavages of the type shown in the latter part of Equation 2 would be obtained if an ADP intermediate were formed. The lack of ³¹O in the inorganic phosphate isolated for ADP and its presence in P₁ produced from ATP do exclude such an intermediate. Hence, the conformational change appears to be the reason for the tight binding of ADP. The suggestion by Grubhofer and Weber (17) that ADP is buried between protein units during the polymerization is one attractive model for such a conformational change.

SUMMARY

Polymerization of actin in H³¹O shows that the nucleophilic attack during polymerization is on the terminal phosphorus atom of adenosine triphosphate. This, together with previous data, essentially eliminates the possible existence of a covalent bond in the actin-ADP complex. The tightness of binding of adenosine diphosphate in F-actin is, therefore, probably a function of the conformation of the protein.

REFERENCES

Adenosine Triphosphate Cleavage during the G-Actin to F-Actin Transformation and the Binding of Adenosine Diphosphate to F-Actin
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