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Direct Evidence for ADP–P_i–F-Actin as the Major Intermediate in ATP–Actin Polymerization. Rate of Dissociation of P_i from Actin Filaments

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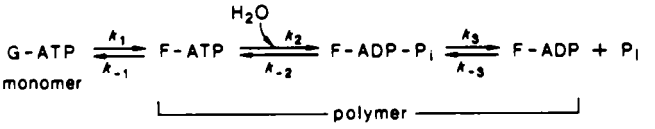
ABSTRACT: The sequence of reactions involved in the polymerization of ATP-actin and accompanying hydrolysis of ATP has been investigated by using a new glass-fiber filter assay. The assay allows the rapid separation of filaments from monomeric actin, and therefore the straightforward identification of the nucleotide bound to F-actin in the time course of polymerization, using double-labeled [γ-^{32}P,^{3}H]ATP. The data bring a direct confirmation of the existence of the previously proposed ATP–F-actin intermediate in the time course of polymerization. Moreover, comparison of the hydrolyzed ATP (i.e., acid-labile [^{32}P]P_i) and of ^{32}P bound to F-actin provides direct evidence for the second intermediate ADP–P_i–F-actin in the polymerization process. This latter species is the major transient in the polymerization of ATP-actin, its lifetime being of the order of minutes.

Actin filaments are dynamic structures that play a crucial role in cell motility. Recently it was discovered that hydrolysis of the tightly bound ATP,[1] which accompanies actin self-assembly, is not coupled to polymerization (Pardee & Spudich, 1982; Pollard & Weeds, 1984; Carlier et al., 1984a). As a consequence, the presence of either ATP or ADP on the terminal subunits of the filament greatly affects its kinetic behavior (Carlier et al., 1984a, 1985; Pantaloni et al., 1985) and, as in the case of microtubules (Carlier et al., 1984b), provides a particularly dynamic response of the polymer (F-actin) to changes in monomer concentration.

In these previous works, the evaluation of the uncoupling between polymerization and ATP hydrolysis was derived from the absence of tight correlation between the time courses of polymerization monitored by light scattering or fluorescence, and ATP hydrolysis followed by extraction in acid solution, of the [^{32}P]phosphomolybdic complex formed following the splitting of [γ-^{32}P]ATP. These kinetic studies thus provided indirect evidence for the existence of the transient ATP–F-actin species in the expected sequence of reactions in ATP-actin polymerization, as shown in Scheme I.

The postulated F–ADP–P_i species (Korn, 1982) could not be observed by using the [^{32}P]P_i acid-extraction technique because the actin–P_i bond is acid-labile. Therefore a method allowing direct measurement of nucleotides bound to F-actin in the time course of polymerization was sought. A glass-fiber filter assay was found convenient. By use of double-labeled [γ-^{32}P,^{3}H]ATP, direct evidence is shown for the transient ATP–F-actin and ADP–P_i–F-actin species preceding the formation of ADP–F-actin. Release of P_i in the medium appears much slower than hydrolysis, with a half-time of several minutes.

Scheme I



Materials and Methods

All reagents were analytical grade. Dithiothreitol, ATP, EGTA, and NaN_3 were from Sigma; NBD-Cl came from Molecular Probes; [^{3}H]- and [γ-^{32}P]ATP came from New England Nuclear; GFF glass-fiber filters (25-mm diameter) came from Whatman.

Monomeric actin (G-actin) was purified from rabbit muscle by the usual procedure (Spudich & Watt, 1971; Eisenberg & Kielley, 1974). NBD-labeled actin was prepared as described by Detmers et al. (1983). The NBD derivative was chosen to follow actin polymerization rather than the pyrenyl deriv-

[1] Abbreviations: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; NBD, 4-nitro-2,1,3-benzoxadiazole; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole; P_i, inorganic phosphate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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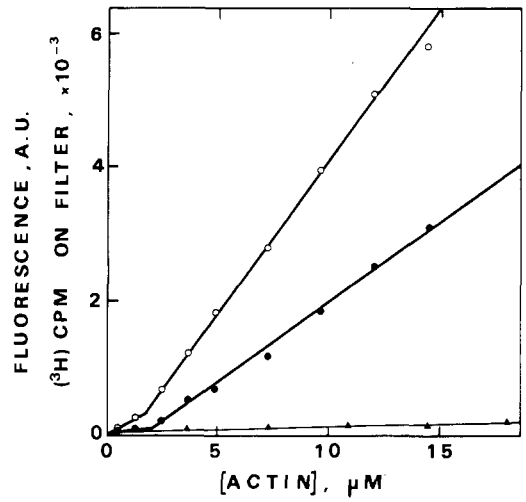

FIGURE 1: Selective adsorption of F-actin on GFF glass-fiber filters and correlation between filter assay and fluorometry to determine critical concentration for actin polymerization. The ^{3}H-labeled ATP–G-actin complex (15 μM) was prepared as described under Materials and Methods, polymerized upon addition of 0.1 M KCl, and diluted to different concentrations with buffer F_0 (5 mM Tris-HCl, 0.1 mM $CaCl_2$, 0.2 mM dithiothreitol, 0.01% sodium azide, 0.1 M KCl). When equilibrium was reached, the fluorescence of each solution was measured (○). Aliquots of 50 μL of each sample were diluted 80-fold in quenching buffer Q and processed for glass-fiber filter assay (see Materials and Methods). Radioactive label trapped in the filters was eluted with 1 mL of 0.1 M NaOH and counted in 10 mL of Aquasol (●). Controls were done with solutions of G-actin in the same range of concentrations (▲). The same critical concentration of 1.9 μM was derived from both the filter assay and fluorescence measurements.

ative because its fluorescence is independent of the nature of the bound nucleotide (Carlier et al., 1984a). Polymerization of actin was monitored fluorometrically in a MPF 44 A Perkin-Elmer spectrofluorometer. Conversion of Ca–G-actin into Mg–G-actin was achieved by adding 50 μM $MgCl_2$ and 0.2 mM EGTA to a 20–40 μM Ca–G-actin (NBD-labeled) solution in buffer G_0, consisting of 5 mM Tris-HCl, pH 7.8, 0.2 mM dithiothreitol, 0.1 mM $CaCl_2$, and 0.01% sodium azide; the reaction was followed by observing the accompanying 13% increase in NBD fluorescence previously reported (Carlier et al., 1986b).

Glass Filter Assay. A preliminary experiment showed that when 500 μL of a 25 μM F-actin solution in 0.1 M KCl was filtered through a 25-mm diameter GFF filter, the concentration of actin in the filtrate was 1 μM. In contrast, 22 μM actin was found in the filtrate when the same experiment was done with G-actin.

This result indicated that actin filaments could be selectively trapped on GFF filters. The assay was further developed on a microscale in analogy with the assay successfully used on microtubules by Wilson et al. (1982) as follows: A 10% NBD-labeled G-actin (20 μM) solution in buffer G was incubated with [^{3}H]ATP on ice for 90 min, a time known to ensure complete equilibration with the radiolabeled ATP (Frieden, 1982). The [^{3}H]ATP–G-actin 1:1 complex then was isolated free of unbound ATP by Dowex 1 treatment (Mockrin & Korn, 1980), polymerized by addition of 0.1 M KCl, and diluted to different final concentrations in F_0 buffer (5 mM Tris-HCl, pH 7.8, 0.2 mM dithiothreitol, 0.1 mM $CaCl_2$, 0.01% azide, and 0.1 M KCl). After 30 min, the fluorescence of each sample was measured and 50-μL aliquots of each sample were diluted in 4 mL of quenching buffer Q consisting of 5 mM Tris-HCl, pH 7.8, 0.1 mM dithiothreitol, 0.1 mM $CaCl_2$, 0.1 M KCl, 7 M glycerol, 0.5% glutaraldehyde, and 0.1 μM cytochalasin D and filtered through 25-mm GFF

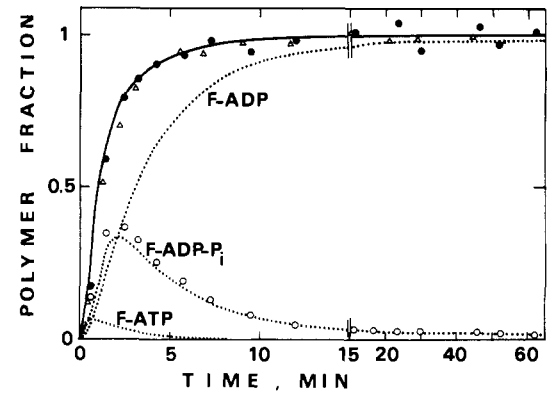

FIGURE 2: Evidence for ATP–F-actin, ADP–P_i–F-actin, and ADP–F-actin intermediates in polymerization of Mg–ATP–actin. The ATP–G-actin 1:1 complex (23.5 μM) was prepared as described under Figure 1, except that ATP was double labeled (γ-^{32}P,^{3}H). Conversion of Ca–actin to Mg–actin was achieved by a 3-min preincubation in the presence of 0.2 mM EGTA and 50 μM $MgCl_2$. Polymerization was started by addition of 0.1 M KCl. Several parameters were followed simultaneously on the polymerizing sample, as follows. (1) Polymerization was monitored both by the increase in NBD fluorescence (solid line) and by the amount of ^{3}H nucleotide on the filters (●). The amount of ^{3}H bound to filters at t = 0 (subtracted) represented less than 7% of the ^{3}H bound at equilibrium. (2) ATP hydrolysis (△) was monitored by acid extraction of [^{32}P]P_i (see Materials and Methods). (3) The amount of ^{32}P retained on the filter (○) represents the sum ATP–F-actin + ADP–P_i–F-actin (^{32}P specific radioactivity has been normalized to the same value as that of ^{3}H). The dotted curves represent the evolution with time of the amounts of the consecutive intermediates ATP–F-actin, ADP–P_i–F-actin, and ADP–F-actin as indicated. ATP–F-actin is obtained by the difference between the polymerization curve (—, ●) and the ATP hydrolysis curve (△), ADP–P_i–F-actin as the difference between the total F-actin-bound ^{32}P (○) and ATP–F-actin, and ADP–F-actin as the difference between the polymerization curve (—, ●) and the F-actin-bound ^{32}P (○).

glass-fiber filters under gentle suction. A 4-mL wash of Q buffer was applied to the filters, which were then cut into fourths and deposited in scintillation vials. Radioactive label was eluted with 1 mL of 0.1 N NaOH and counted in 10 mL of Aquasol. The same amount of ^{3}H was found on the filters at different times in the range 30 s–15 min following dilution of filaments in Q buffer (a time of 30 s is necessary for dilution, filtration, and washing), which showed that actin filaments were stable in Q buffer, depolymerization being prevented by cross-linking of subunits by glutaraldehyde. The concentration of glutaraldehyde was optimized. Concentrations higher than 0.5% resulted in the gradual loss of ^{3}H retained on the filter; 30% loss occurred in a 15-min incubation in Q buffer containing 1% glutaraldehyde. Presumably extensive cross-linking caused the deformation of the F-actin and subsequent dissociation of the nucleotide. The presence of cytochalasin D further prevented depolymerization of filaments by blocking the barbed ends (Brown & Spudich, 1979; Brenner & Korn, 1979; Carlier et al., 1986a). Finally, glycerol prevented any aggregation and retention of G-actin on the filter and also slowed down the depolymerization of filaments.

We also checked the ability of the GFF filter to trap short filaments as follows: A solution of F-actin polymerized with [^{3}H]ATP was sonicated for 1 min, conditions under which filaments are about 0.2 μm long. At time zero, sonication stopped, and the filter assay was carried out at different time intervals during the length redistribution to longer filaments (Carlier et al., 1984c). The increase in length was measured simultaneously by the decrease in filament number by using the elongation assay (Pantaloni et al., 1984). We found that the 25-fold increase in average length was accompanied by a 20% increase only in the amount of ^{3}H polymer retained on

the filter. This result shows the efficiency of the filter in trapping short filaments.

For the measurement of ATP hydrolysis and P_i dissociation from F-actin in the time course of polymerization, the same assay was used, with double-labeled [γ-^{32}P,^{3}H]ATP initially bound to actin. Aliquots were taken off the polymerizing solution at different times of the polymerization process of labeled ATP–actin, diluted in Q buffer, and immediately processed for GFF filtration. The amount of polymerized actin was derived from the amount of ^{3}H nucleotide retained on the filter, representing the sum of ATP–F-actin + ADP–P_i–F-actin + ADP–F-actin, while the amount of ^{32}P bound to the filter represents the sum of ATP–F-actin + ADP–P_i–F-actin. In the ADP–P_i–F-actin species, ATP is split but P_i is still bound to F-actin. Within the assumption that the actin–P_i bond in this complex is acid-labile, monitoring the reaction of ATP hydrolysis by the usual method of extraction of the phosphomolybdate complex in acid medium gives a measurement of the time course of formation of ADP–P_i–F-actin + ADP–F-actin. The evolution of the individual subsequent intermediates can be easily derived from the three simultaneous kinetic measurements of total F-actin, ^{32}P bound to the filter, and ATP hydrolysis.

We should note that, while the ability of Q buffer to stabilize F-actin has been assessed, as described above, we have no evidence for its quenching the ATP hydrolysis reaction. However, the ability of Q buffer to quench the release of P_i can be estimated by measuring the ratio of ^{32}P/^{3}H retained on the filter at different times following dilution in Q buffer of an aliquot taken off the polymerizing solution at an early time of the assembly process. In such an experiment, we observed that the ratio ^{32}P/^{3}H declined from 0.84 (30 s after dilution) to 0.75 after 8 min in Q buffer (experiment done on Ca–actin, six measurements between 30 s and 8 min); these values correspond to an 11% loss in the ratio ^{32}P/^{3}H in Q buffer, while a decrease of 50% was obtained during the same period of time in the polymerization buffer (see Figure 3b). We conclude that Q buffer is a satisfactory quenching buffer also for the dissociation of P_i from F-actin, which validates the assay.

Results

Determination of the Critical Concentration for Actin Polymerization by the Glass-Fiber Filter Assay. Serial dilutions were made from a 15 μM 10% NBD-labeled F-actin solution prepared by polymerization of the [^{3}H]ATP–G-actin complex (in its Ca form) by addition of 0.1 M KCl. Figure 1 shows the critical concentration plots derived from NBD fluorescence reading of the F-actin samples and from the glass-fiber filter assay as described under Materials and Methods. Although only about 80% of actin filaments were retained on the filter, the same critical concentration was derived from the two methods. The selectivity of the GFF filter assay for actin filaments is assessed by the 20-fold lower amount of ^{3}H nucleotide found on the filter when the same concentration of G-actin was assayed.

Kinetic Steps in the Polymerization of ATP–Actin. The above result let us anticipate that the filter assay could be used to monitor the time course of actin polymerization. Moreover, with double-labeled [γ-^{32}P,^{3}H]ATP, the nature of the different nucleotides bound to F-actin during polymerization could be directly measured (see Materials and Methods). The experiments have been performed by using both Mg–actin (Figure 2) and Ca–actin (Figure 3), because previous experiments (Carlier et al., 1986b) have shown that ATP is hydrolyzed at a faster rate on Mg–F-actin than on Ca–F-actin; in addition,

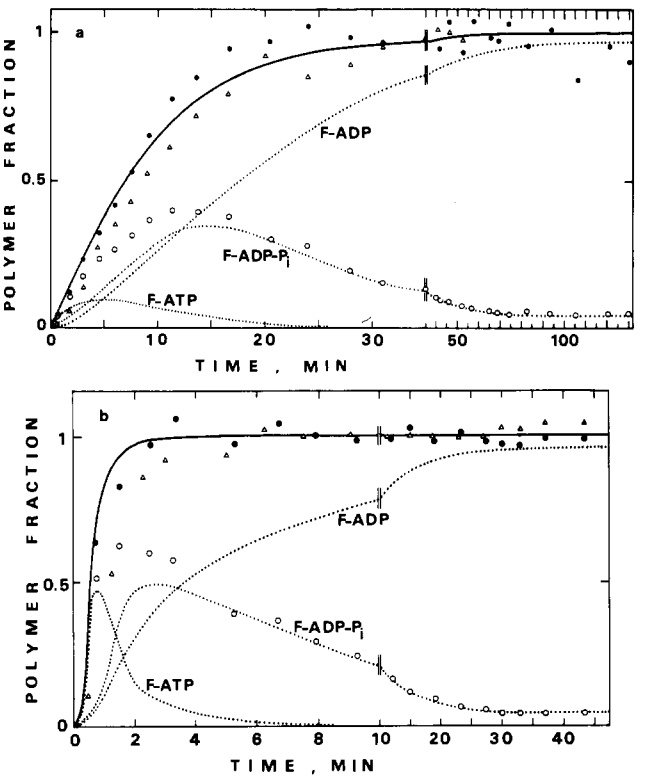

FIGURE 3: Evidence for ATP–F-actin, ADP–P_i–F-actin, and ADP–F-actin as intermediates in the polymerization of Ca–ATP–actin. The γ-^{32}P, ^{3}H double-labeled ATP–G-actin 1:1 complex (40 μM) was prepared as described in Figure 2, in its Ca form. Polymerization was started by addition of 0.1 M KCl. In addition, in (a) 10 μL of a solution of 15 μM F-actin under sonication was added as seed to the 2.7-mL sample; the final concentration of filaments was 1.1 nM. In (b) the sample was maintained under sonication for 10 s following the addition of KCl, to accelerate polymerization; the final concentration of filaments was 20 nM. A larger uncoupling between polymerization and hydrolysis was therefore observed in case b, where polymerization was achieved in only 2 min. Symbols represent the same parameters as in Figure 2. Evolution with time of the different kinetic intermediates is derived from the data as explained under Figure 2.

the mechanism of ATP hydrolysis is different on these two actin isoforms in that, on Mg–actin, the rate of ATP hydrolysis is greatly enhanced on an ATP subunit interacting with an ADP subunit at the next deeper position in the filament, which results in a vectorial process (Pantaloni et al., 1985).

Figures 2 and 3 clearly show that the time courses for spontaneous polymerization, followed by the change in NBD fluorescence and the increase in the amount of ^{3}H nucleotide bound to the filters, were superimposable curves. This result further assesses the validity of the filter assay, as designed, to monitor actin polymerization.

The difference between the polymerization curve and the ATP hydrolysis curve represents the evolution with time of the ATP–F-actin intermediate. As previously shown (Carlier et al., 1986b), this intermediate transiently exists in larger amounts on Ca–F-actin than on Mg–F-actin, due to the slower rate of ATP hydrolysis on Ca–actin. In addition, the striking point documented on Figures 2 and 3 is that the amount of ^{32}P bound to F-actin on the filter is always much larger than the expected amount of ATP–F-actin, and its decrease follows much slower kinetics than ATP–F-actin. For example, Figure 3b shows that at time 5 min, when 95% of ATP has been hydrolyzed, 40% of the polymerized actin still has ^{32}P bound. These data indicate that the intermediate of F–ADP–P_i that is formed following the hydrolysis of ATP on F-actin has a long lifetime. By combination of all the kinetic data, the time

courses of F–ATP, F–ADP–P_i, and F–ADP can be derived and are shown in dotted lines on Figures 2 and 3. It clearly appears that F–ADP–P_i is the major intermediate in ATP–actin polymerization. Dissociation of P_i from this complex, leading to the final ADP–F-actin product, is about 10-fold slower than hydrolysis itself. The data also show that dissociation of P_i is about 3-fold faster from Mg–F-actin than from Ca–F-actin. It is interesting to note that, similarly, dissociation of ATP takes place at a faster rate from Mg–G-actin than from Ca–G-actin (Waechter & Engel, 1975; Frieden, 1982), which suggests that the environment of the γ-phosphate of ATP is different on Ca- and Mg–actin.

Dissociation of P_i from F-actin obeys first-order kinetics, with rate constants of 1.4×10^{-3} (Figure 3a)–2.5×10^{-3} s^{-1} (Figure 3b) for Ca–actin and 5.5×10^{-3} s^{-1} (Figure 2) for Mg–actin. Another experiment (not shown) carried out at a much higher concentration of Mg–actin (65 μM) gave a rate constant of 4.5×10^{-3} s^{-1} for the dissociation of P_i. These values show that the rate of dissociation of P_i varies very little when the rate of polymerization is increased either by an increase of the concentration of actin or by an initial fragmentation of filaments as in Figure 3b. The fact that a 20-fold increase in the number concentration of filaments (Figure 3) causes either none or a less than 2-fold increase in the apparent rate constant for P_i dissociation actually suggests that the dissociation of P_i is independent of the number of ends and occurs mainly randomly on any subunit of the filament according to Scheme I, in a noncooperative fashion. Therefore, although Scheme I may be oversimplified concerning the detailed mechanism of polymerization and ATP hydrolysis, it nevertheless accounts well for the transient evolution of F–ADP–P_i.

Finally, although the second and third equilibria in Scheme I are strongly shifted to the right, the fact that a small but measurable amount of ^{32}P remains bound to F-actin at late times (3% of Ca–F-actin and 1–1.5% of Mg–F-actin) suggests that some reversibility may take place, as has been shown in the case of myosin (Bagshaw & Trentham, 1973) and of dynein (Holzbaur & Jackson, 1986).

Discussion

In this paper we have attempted to get a more direct insight in the chemical reactions associated to the polymerization of ATP–actin. Using a new filter assay that selectively retains actin filaments, we have been able to monitor actin polymerization. This assay has been used in combination with fluorescence measurements of polymerization and associated ATP hydrolysis to show direct evidence for the ADP–P_i–F-actin complex as a major intermediate in actin polymerization. The existence of this complex has been reported for monomeric (Ferri et al., 1979) and cross-linked dimeric actin (Mockrin & Korn, 1981); the present work quantitatively documents the kinetics of formation and disappearance of ADP–P_i–F-actin in the polymerization of ATP–actin. The apparent rate of P_i dissociation from actin following ATP hydrolysis is a slow step, a feature that actin shares with other well-known ATPases (Lymn & Taylor, 1971; Johnson, 1983, 1985). However, the release of P_i from ADP–P_i–F-actin appears very slow as compared to dynein or even myosin, which may indicate that the split nucleotide ADP–P_i is sequestered in the site and that P_i release is rate-limited by an isomerization process, as proposed for the myosin S_1–actin cross-linked complex (Stein et al., 1985). The occurrence of at least three different species of F-actin, whose proportions vary in the time course of polymerization, introduces a new "subtlety" (Wegner, 1985) in actin assembly and complicates the kinetic analysis of polymerization. We have previously shown the role played by the ATP cap at the ends of actin filaments (Pantaloni et al., 1985); now new experiments will have to be designed to investigate the structural and functional differences that may exist between these three different F-actin species. It should be noted that these three actin species represent the minimum number of actual kinetic intermediates in the polymerization process, since the possible isoforms of the F–ADP–P_i transient cannot be discriminated by the filter assay.

The finding that the lifetime of the F–ADP–P_i transient, under physiological conditions, is compatible with the time scale of other motile events taking place during the cell cycle may have biologically important consequences. It will be of obvious interest to investigate whether ADP–P_i–F-actin and ADP–F-actin interact differently with proteins that bind to actin filaments; reciprocally F-actin binding proteins may modify the kinetics of ATP hydrolysis and P_i dissociation to serve some important function in the cell motility.

References

Bagshaw, C. R., & Trentham, D. R. (1973) *Biochem. J. 133*, 323.

Brenner, S. L., & Korn, E. D. (1979) *J. Biol. Chem. 254*, 9982.

Brown, S. S., & Spudich, J. A. (1979) *J. Cell Biol. 83*, 657.

Carlier, M. F., Pantaloni, D., & Korn, E. D. (1984a) *J. Biol. Chem. 259*, 9983.

Carlier, M. F., Hill, T. L., & Chen, Y. D. (1984b) *Proc. Natl. Acad. Sci. U.S.A. 81*, 771.

Carlier, M. F., Pantaloni, D., & Korn, E. D. (1984c) *J. Biol. Chem. 259*, 9987.

Carlier, M. F., Pantaloni, D., & Korn, E. D. (1985) *J. Biol. Chem. 260*, 6565.

Carlier, M. F., Criquet, P., Pantaloni, D., & Korn, E. D. (1986a) *J. Biol. Chem. 261*, 2041.

Carlier, M.-F., Pantaloni, D., & Korn, E. D. (1986b) *J. Biol. Chem. 261*, 10785.

Detmers, P., Weber, A., Eleinga, M., & Stephens, R. E. (1983) *J. Biol. Chem. 258*, 99.

Eisenberg, E., & Kielley, W. W. (1974) *J. Biol. Chem. 249*, 4742.

Ferri, A., Magri, E., & Grazi, E. (1979) *Biochem. J. 183*, 475.

Frieden, C. J. (1982) *J. Biol. Chem. 257*, 2882.

Holzbaur, E. L. F., & Johnson, K. A. (1986) *Biochemistry 25*, 428.

Johnson, K. A. (1983) *J. Biol. Chem. 258*, 13825.

Johnson, K. A. (1985) *Annu. Rev. Biophys. Biophys. Chem. 14*, 161.

Korn, E. D. (1982) *Physiol. Rev. 62*, 672.

Lymn, R. W., & Taylor, E. W. (1971) *Biochemistry 10*, 4617.

Mockrin, S. C., & Korn, E. D. (1980) *Biochemistry 19*, 5359.

Mockrin, S. C., & Korn, E. D. (1981) *J. Biol. Chem. 256*, 8228.

Pantaloni, D., Carlier, M. F., Coué, M., Lal, A. A., Brenner, S. L., & Korn, E. D. (1984) *J. Biol. Chem. 259*, 6274.

Pantaloni, D., Hill, T. L., Carlier, M.-F., & Korn, E. D. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7207.

Pardee, J. D., & Spudich, J. A. (1982) *J. Cell Biol. 93*, 648.

Pollard, T. D., & Weeds, A. G. (1984) *FEBS Lett. 170*, 94.

Spudich, J. A., & Watt, S. (1971) *J. Biol. Chem. 246*, 4866.

Stein, L. A., Greene, L. E., Chock, P. B., & Eisenberg, E. (1985) *Biochemistry 24*, 1357.

Waechter, F., & Engel, J. (1975) *Eur. J. Biochem. 57*, 453.

Wegner, A. (1985) *Nature (London) 313*, 97.

Wilson, L., Snyder, B. K., Thompson, W. C., & Margolis, R. L. (1982) *Methods Cell Biol. 24*, 159.