Binding of Adenosine Diphosphate to G-Actin*

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ABSTRACT: The binding of adenosine diphosphate (ADP) by G-actin was studied under various conditions. Binding constants were determined by measuring either the rates of denaturation of G-actin solutions in the presence of varying nucleotide concentrations, or the concentration of free nucleotide in G-actin solutions at equilibrium. The binding constant of G-actin for ADP at 0° was 2–5 $\times$ 10^6 M^{-1}; at 25° it was 4 $\times$ 10^4 M^{-1}, whereas at 0° ATP bound to G-actin with an affinity constant of 2.4 $\times$ 10^7 M^{-1}. Changing the actin-bound cation from magnesium to calcium did not appear to alter ADP binding. Free magnesium at a concentration of 0.2 mM reduced the rate of G-actin denaturation by one-third but there was no change in the affinity of G-actin for ADP.

It was concluded that the mechanism by which free magnesium stabilizes G-actin depends on a prolonged lifetime of nucleotide-free G-actin molecules without change in the properties of G-actin containing bound nucleotide.

In view of the possible role of actin-bound nucleotide in energy-transfer processes leading to muscle contraction, studies of the properties of actin-bound nucleotide are important. Several recent publications have presented conflicting evidence on the extent to which ADP–G-actin dissociates in solution to form ADP and native G-actin. Higashi and Oosawa (1965) derived a binding constant of G-actin for ADP of 1.3 $\times$ 10^4 M^{-1} from studies of changes in absorbance at 230 mμ after addition of ADP to ADP–G-actin solutions. Thus, according to these data, at 1.5 mg/ml of ADP–G-actin in the absence of added ADP only 20% of the nucleotide was actually bound to the protein. Seidel *et al.* (1967) determined the amount of native actin containing no bound nucleotide and estimated that the binding constant of G-actin for ADP at 4° was 4 $\times$ 10^5 M^{-1}. In contrast, Hayashi and Rosenbluth (1964) inferred that less than 5% of the total nucleotide present was free after studies of removal of free nucleotide by Dowex 1 from 1 mg/ml of ADP–G-actin solutions at 0°. West *et al.* (1967a) studied the kinetics of ADP hydrolysis by apyrase and found that only 7% of the nucleotide was dissociated in 1.5 mg/ml of ADP–G-actin solutions at 0°. If the free nucleotide in these studies was equivalent to that found at equilibrium these results imply a binding constant greater than 7 $\times$ 10^6 M^{-1}, a value 500-fold greater than that calculated by Higashi and Oosawa (1965) and 20-fold greater than that determined by Seidel *et al.* (1967).

In view of these divergent results the binding of nucleotide to actin was reevaluated. It seemed possible that differences in the methods of preparation of G-actin by the various authors could have altered the nucleotide binding characteristics of G-actin. Thus Higashi and Oosawa (1965) studied G-actin containing bound calcium whereas the actin employed by other investigators normally contained bound magnesium. Hayashi and Rosenbluth (1967) added millimolar $MgCl_2$ to their ADP–G-actin preparations since they found that an excess of magnesium at 0° retarded thermal denaturation of ADP–G-actin. It seemed possible that such a stabilizing effect of free $MgCl_2$ could have been mediated by an increased affinity of G-actin for ADP.

Our results show tighter ADP binding to G-actin at 0° than found in previous studies. A marked temperature dependence for ADP binding to G-actin was found. Nucleotide binding was not altered if calcium was substituted for actin-bound magnesium, nor did addition of free magnesium alter nucleotide binding. The mechanism by which free magnesium retards thermal denaturation of G-actin is discussed.

Materials

Actin was extracted from rabbit muscle by preparing an acetone dried powder (Feuer *et al.*, 1948). This was then extracted with 0.2 mM ATP in 1 mM Tris-HCl (pH 8.0) at 0° (Drabikowski and Gergely, 1962) and purified by polymerization and ultracentrifugation (Mommaerts, 1951). ADP–G-actin solutions were prepared by homogenizing pellets containing no free ATP (West *et al.*, 1967b) in 1 mM Tris-HCl (pH 8.0) plus a stabilizing agent: 0.2 mM $MgCl_2$ or $CaCl_2$ and 10 μM ADP for most apyrase studies or 0.1 mM ADP for the studies on rate of actin denaturation. All G-actin solutions were clarified by centrifugation at 100,000*g* for 15–20 min. ATP–G-actin solutions were similarly prepared but were dialyzed at 4° for 3 days in 0.2 mM ATP and 1 mM Tris-HCl (pH 7.6) prior to clarification. When needed [^{14}C]nucleotide labeled actin was prepared (West *et al.*, 1967a). Free nucleotide was removed just prior to studies by treatment with 20% v/v of a 50% suspension of Dowex (Cl) 1-X2 for 4 min at 0° and the Dowex was removed by filtration through a Millipore filter 0.45 μ pore size. To prepare ATP–G-actin the Dowex 1 treatment was performed twice, 10% v/v for 3 min each time. The Dowex 1 was prepared by washing for 20 min in 4 N HCl followed by washing until chloride free with glass-distilled water. The Dowex was washed just prior to use with Tris-HCl (pH 8.8) until the effluent reached pH 8.3.

Actin containing bound calcium was prepared by adding 0.2 mM $CaCl_2$ to ADP–G-actin pretreated for 4 min at 0°

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with Dowex 50-X2. Subsequently the actin was polymerized with 0.1 M KCl and stored as pellets of Ca–F-actin. Dowex 50 was prepared by washing the Dowex for 30 min in 3 N HCl followed, after several rinses in distilled water, by washing for 30 min in 3 N KOH. Thereafter the Dowex 50 was washed free of chloride ion with distilled water and rinsed with Tris-HCl (pH 8.0) until the effluent reached pH 8.1. Ca–ADP–G-actin from the pellet of Ca–F-actin was prepared in 1 mM Tris-HCl (pH 8.0) and 0.1 or 0.2 mM $CaCl_2$ and 10 μM ADP or in 1 mM Tris-HCl (pH 8.0) and 0.1 mM ADP. The results on nucleotide binding were not affected by the different modes of preparation. Actin so treated had the polymerization characteristics of calcium–G-actin (Mihashi and Ooi, 1965). Atomic absorbance measurements were performed on Ca–G-actin prepared from Ca–F-actin by homogenization in 1 mM Tris-HCl (pH 8.0) and 0.1 mM ADP after treatment of the G-actin with Dowex 50 for 4 min at 0° to remove free cation. A Perkin-Elmer Model 303 atomic absorbance spectrophotometer equipped with a triple head burner was utilized. Calcium was found equimolar to the actin whereas magnesium was detected at a concentration less than one-eighth the actin concentration. In contrast G-actin prepared without added calcium had a magnesium content equimolar to the actin while calcium was detected at a concentration less than one-tenth the actin concentration.

Tropomyosin was felt to be absent from our G-actin preparations since ATP–G-actin solutions from 1 to 4 mg per ml had a reduced viscosity of from 0.06 to 0.1. Drabikowski and Gergely (1962) found similar values for tropomyosin-free ATP–G-actin solutions. ADP–G-actin solutions were checked for the presence of myokinase activity by measuring viscosity at 0° after addition of 0.1 M KCl, 1 mM $MgCl_2$, and 1 mM ADP. No appreciable change in viscosity occurred over a 3-hr period. If ATP was formed by action of myokinase an increase in viscosity would have been expected since added ATP at a concentration of 10^{-6} M or higher produced a substantial increase in viscosity. The possibility of contamination of the G-actin solutions by AMP deaminase was evaluated in view of high concentrations of that enzyme in myosin preparations (Lowey and Luck, 1969). AMP (0.1 mM) was added to G-actin solutions (1.0 mg/ml) utilizing the split compartment technique described under methods. No absorbance change indicating conversion of AMP into IMP (Lowey and Luck, 1969) could be detected over an 18-hr period at 25°.

^{14}C-Labeled ATP and ADP were obtained from New England Nuclear Corp. Apyrase (ATP diphosphohydrolase, EC 3.6.1.5) was purified from potato by the method of Szekely (1951). It hydrolyzed 0.3 μmole of ADP/min per mg of protein at 0°. ATP and ADP were obtained from P-L Biochemicals.

Methods

The binding of nucleotide to actin was evaluated by two methods. The first was a modification of the technique employed by Asakura (1961) to determine the binding constant of G-actin for ATP. In this method the binding constant was calculated from measurements of the initial rates of inactivation of G-actin solutions in the presence of varying concentrations of added nucleotide. The rate of G-actin inactivation was determined by measuring either (a) the viscosity of polymerized aliquots of the G-actin solution taken at intervals after addition of nucleotide or (b) the rate of change of ultraviolet absorbance at 258 mμ of G-actin solutions. A second technique for evaluating binding of nucleotide to actin depended on a measurement of the amount of free nucleotide which dissociated in G-actin solutions after removal of all initially free nucleotide by Dowex 1. This technique employed the enzyme apyrase to hydrolyze free nucleotide to AMP while leaving the G-actin-bound nucleotide largely intact (West *et al.*, 1967a).

Effect of Added Nucleotide on Rate of Actin Denaturation. ADP–G-actin solutions were treated with Dowex 1 immediately prior to the study, appropriate concentrations of nucleotide were added and the preparations were diluted and adjusted to the correct temperature at the start of the study. The denaturation rate of actin preparations run at 25° was determined by measuring the rate of absorbance change at 258 mμ with a Gilford recording spectrophotometer Model 2000. Readings were obtained every 20 sec on three samples against a reference of bovine serum albumin and the result was recorded on an expanded scale of 0–0.1 absorbance unit. All preparations were maintained at 25 ± 0.2° with a water-jacketted cell compartment. The absorbance change of actin preparations at 0° was too slow for accurate determination and viscosity measurements were employed instead. Aliquots were removed from the actin preparation and polymerized at appropriate intervals by addition of 0.1 M KCl, 1 mM $MgCl_2$, and 0.2 mM ATP. Viscosity was measured at 25 ± 0.1° 50–60 min after salt addition, using Ostwald viscometers with outflow times of about 30 sec.

Actin concentration was calculated from the molar concentration of the bound nucleotide after removal by Dowex 1 of all free nucleotide. The bound nucleotide concentration was determined by measuring the ultraviolet absorbance of a 0.6 M perchloric acid extract of the actin, using an extinction coefficient of 14.9×10^3/mole at 257 mμ (Pabst, 1956). The protein concentration computed by this technique was always close to that calculated from the biuret technique assuming a molecular weight for G-actin of 60,000.

Calculation of Equilibrium Constant. The following equation appears to account for the inactivation process (Grubhofer and Weber, 1961)

$$\begin{array}{c} I \\ \uparrow \\ \text{GADP} \rightleftharpoons \text{G} + \text{ADP} \end{array} \qquad (1)$$

where G-ADP represents the undissociated monomeric form of actin and G the dissociated but not yet denatured form. It is assumed for reasons outlined in the discussion that the equilibrium is rapid and the inactivation process whereby G is converted into I is relatively slow. The dissociation constant for the first reaction is

$$K_d = \frac{(\text{G})(\text{ADP})}{(\text{GADP})} \qquad (2)$$

At zero time the rate of the inactivation process can be expressed as

$$\frac{d(\text{I})}{dt} = \alpha(\text{G}) \qquad (3)$$

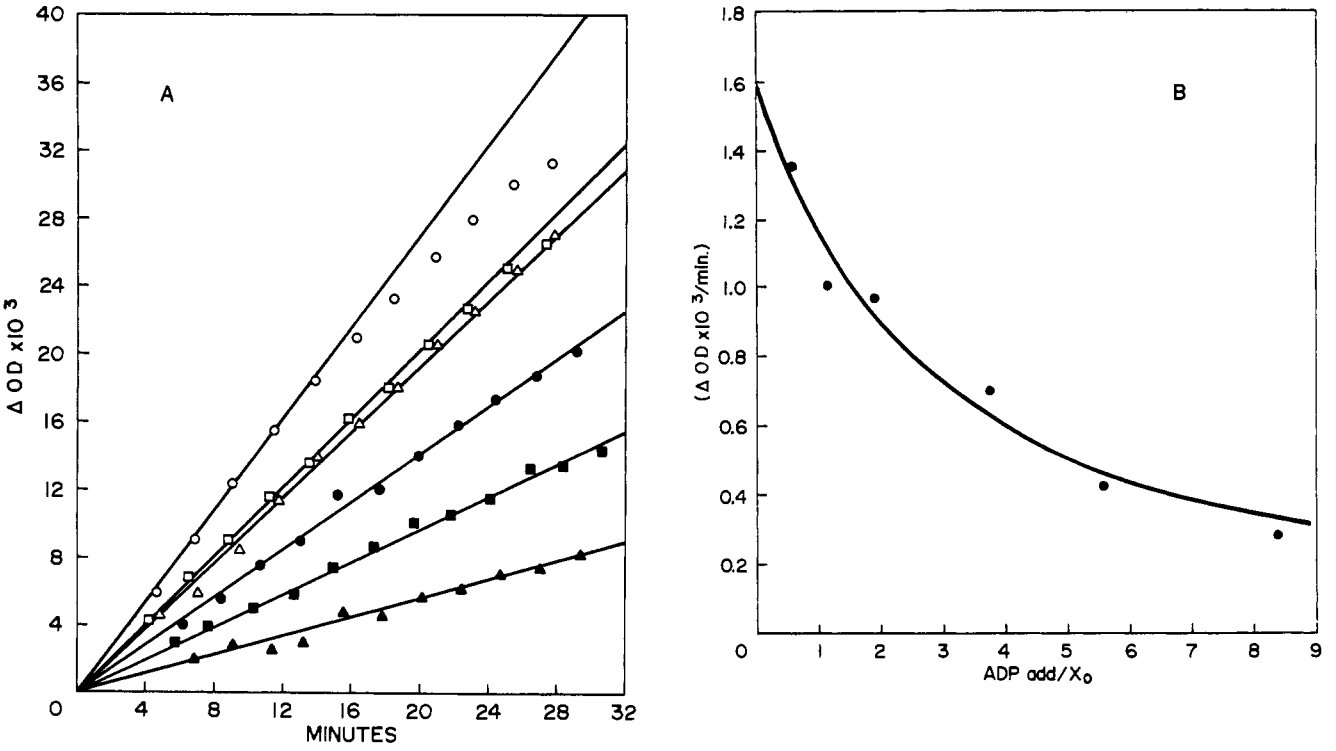

FIGURE 1: Effect of added nucleotide on rate of absorbance change of actin solutions at 25°. Absorbance change was read at 258 mμ with the use of an expanded scale (0–0.1 optical density unit) and recorded as optical density × 10^3. The initial absorbance of each sample was zeroed against a blank of bovine serum albumin. Measurements were obtained at 20-sec intervals and each reading was averaged over a 5-sec period. Every fifth measurement is illustrated. Samples were diluted and warmed to 25° at zero time and maintained at 25 ± 0.2°. All solutions contained Dowex 1 pretreated ADP-G-actin 1.1 mg/ml in 1 mM Tris-HCl (pH 8.0) and added ADP at concentrations (× 10^{-5} M) of (○) 1.04, (□) 2.08, (△) 3.47, (●) 6.90, (■) 10.3, and (▲) 15.4. Initial rates of ADP-G-actin inactivation at 25° *vs.* the concentration of free ADP. Initial rates of inactivation were calculated from the slopes of the lines in part A expressed as optical density × 10^3/min. The abscissa indicates the ratio, Z, of the concentration of added ADP to the initial concentration of active actin, (X_0). The line was the computed best fit to these points by the method of least-mean squares and corresponds to a K_d of 2.6 × 10^{-5} mole/l.

When ADP is added to a solution of ADP–G-actin, eq 2 can be written as

$$K_d = \frac{(G)[(\text{ADP add}) + (G)]}{(GADP)} \quad (4)$$

where (ADP add) is the molar concentration of the added nucleotide. If (X_0) is defined as the molar concentration of G-actin initially containing bound nucleotide, and Z = ADP add/(X_0) then, assuming that G-actin in excess of the molar nucleotide concentration denatured before the start of the study, eq 4 can be written as

$$K_d = \frac{(G)[(X_0)Z + (G)]}{(X_0) - (G)} \quad (5)$$

Solving this equation for G and substituting in eq 3 yields the initial rate of inactivation

$$\frac{d(I)}{dt} = \frac{\alpha}{2}\{[((X_0)Z + K_d)^2 + 4K_d(X_0)]^{1/2} - [(X_0)Z + K_d]\} \quad (6)$$

Consequently, the equilibrium constant K_d and the binding constant of G-actin for ADP, $K_b = 1/K_d$, can be computed by determining the curve that best fits a plot of initial rates of inactivation *vs.* Z, the ratio of added nucleotide to initial bound nucleotide (Figure 1). The curve fits were obtained by the method of least squares with the help of an IBM 1130 computer. Values of K_d and α were found which would minimize the sum of the squared differences between the left- and right-hand sides of eq 6. In performing the nonlinear fitting procedure, the least-squares solution for the linear parameter, α, was first obtained by differentiation as a function of the nonlinear parameter, K_d. A one-dimensional search for the minimum value of the squared residuals was then used to find the required solution for K_d.

Measurement of Nucleotide Dissociation from G-Actin Utilizing Apyrase. Dowex 1 pretreated [^{14}C]ADP–G-actin solutions containing 0–0.5 mM $MgCl_2$ or 0–0.2 mM $CaCl_2$ were prepared and apyrase 21 μg/ml was added. Aliquots were removed at appropriate intervals and the reaction was stopped by addition of perchloric acid, 0.6 M final concentration. The precipitated protein was removed by centrifugation and the solution was neutralized with KOH. The proportion of ^{14}C present as [^{14}C]ADP, [^{14}C]AMP, or other products was determined with a chromatograph scanner after separation of the nucleotides by paper chromatography (West *et al.*, 1967a). The rate of hydrolysis of [^{14}C]ADP was determined from a semilogarithmic plot of the per cent of

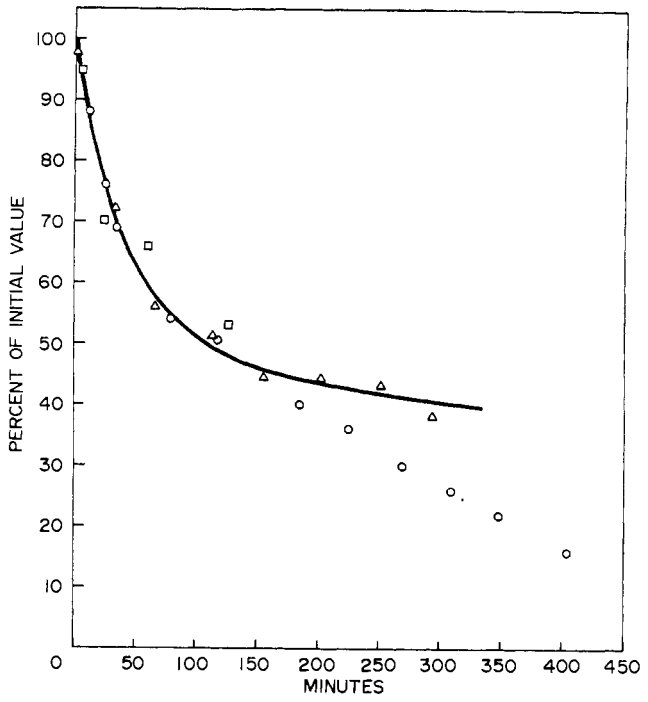

FIGURE 2: Comparison of the rate of loss of polymerizability and bound nucleotide of ADP–G-actin at 25° with the rate of absorbance change at 258 mμ. ADP–G-actin 1.27 mg/ml in 1 mM Tris-HCl (pH 8.0) was brought to 25° at zero time. (△) Logarithm of the relative viscosity after polymerization at the times indicated by addition of 0.1 M KCl, 1 mM $MgCl_2$, and 0.5 mM ATP. Viscosity measurements were made 90 min after the addition of salt and are expressed as a percentage of the initial value. The line illustrated was fitted to the viscosity values. (□) Bound nucleotide was determined by measuring the optical density at 257 mμ of 0.6 M perchloric acid extracts of G-actin solutions which had been treated with Dowex 1 (Cl) 10% v/v at the times indicated on the abscissa. Nucleotide concentration is expressed as a per cent of the initial value. (○) Absorbance change at 258 mμ expressed as a percentage of the total change in optical density and subtracted from 100%. The total change in optical density at 258 mμ (0.073/mg of actin) was determined by measuring the change in absorbance 15 min after addition of 1 mM EDTA at 25° (West *et al.*, 1967b).

^{14}C present as ADP *vs.* time. Since the free nucleotide was rapidly hydrolyzed while the bound nucleotide was only slowly hydrolyzed according to first-order kinetics, the intercept at zero time was taken as a measure of the free ADP initially present (West *et al.*, 1967a). Protein concentration was determined by the biuret technique, standardized by micro Kjeldahl determination of actin nitrogen content. A factor of 6.25 was used to correct milligrams of nitrogen to milligrams of protein (Kuehl and Gergely, 1969).

Studies of the Actin Difference Spectra. Difference spectra were measured with a Beckman Model DK-1 or DK-2 spectrophotometer in a thermostated cuvet holder at 0°. A four-compartment arrangement was utilized, either using two 1-cm split compartment cuvets ((Yankeelov, 1963) or, for measurements at 230 mμ, four 1-mm quartz cuvets. The reference cuvets contained G-actin and nucleotide in separate compartments while the sample cuvets contained the same solutions mixed in one compartment and buffer in the second compartment. Protein concentrations were determined by the biuret technique and were adjusted to 1–2 mg/ml.

Results

Effect of Added Nucleotide on the Rate of Actin Denaturation. Linear rates of absorbance change were observed in ADP–G-actin solutions containing various concentrations of free ADP, during the first 10–15 min after warming to 25° (Figure 1A). Since the rate of change in absorbance at 258 mμ was found to parallel the rate of decrease in viscosity of polymerized aliquots of the solution (Figure 2), absorbance change at 258 mμ was taken as a measure of the initial rates of G-actin inactivation. The binding constant of ADP to G-actin at 25° could be computed from the initial rates of inactivation as described above (Figure 1B). For six preparations of ADP–G-actin with a bound nucleotide content from 1.04×10^{-5} to 2.08×10^{-5} M the binding constant was found to be 3.9×10^4 (Table I). Similar curves were obtained for ADP–G-actin at 0° using viscosity as a measure of the initial rate of inactivation (Figure 3). For three preparations with a bound nucleotide content near 3×10^{-5} M the binding constant for ADP was 2.2×10^6 M^{-1} (Table I). Addition of 0.2 mM $MgCl_2$ retarded the inactivation of ADP–G-actin by a factor of three (Figure 4). The reduced rate of G-actin inactivation in the presence of added $MgCl_2$ did not appear to be attributable to aggregation of the G-actin since there was no significant change in the viscosity of the G-actin solution after $MgCl_2$ addition.

Measurement of Nucleotide Dissociated from G-Actin Utilizing Apyrase. The binding constant of ADP to G-actin was also computed from the percentage of free nucleotide which dissociated from ADP–G-actin, as determined from the intercept of the curve of hydrolysis of ADP by apyrase (Figure 5). The free nucleotide found was assumed to be in equilibrium with the G-actin. The binding constant calculated from these determinations was 4.9×10^6 M^{-1} (Table I), which is not greatly different from that calculated from the initial rate of G-actin inactivation at 0°. The percentage of free nucleotide found in ADP–G-actin solutions was not affected by preparing the actin with bound calcium in place of bound magnesium or by raising the free magnesium concentration to 0.5 mM (Table I). The binding constant of G-actin for ATP at 0°, as determined by the apyrase technique, was estimated to be 2.4×10^7 M^{-1}, a value approximately fivefold greater than that for ADP at 0°. Asakura (1961) previously reported a binding constant of 6.7×10^7 M^{-1} for ATP by G-actin at 6°.

Studies of the Actin Difference Spectrum. No appreciable absorbance changes were detected at 232 mμ or other regions of the ultraviolet spectrum when ADP was added to ADP–G-actin at 0° in contrast to the observations of Higashi and Oosawa (1965) at room temperature. Measurements of absorbance change at 25° using the four-cell technique described by Higashi and Oosawa (1965) were not reliable because of change in absorbance of ADP–G-actin in the reference cell. Even at high concentration ADP–G-actin without added ADP denatures at an appreciably greater rate at 25° than does G-actin with added ADP (Figure 1). The absorbance changes determined with the four-cell technique when ATP or ADP were added to ADP–G-actin at 25° gradually enlarged with time at all wavelengths from 300 to 230 mμ, and resembled the absorbance changes expected with denaturation of ADP–G-actin in the reference cuvet (West *et al.*, 1967b; Higashi and Oosawa, 1965).

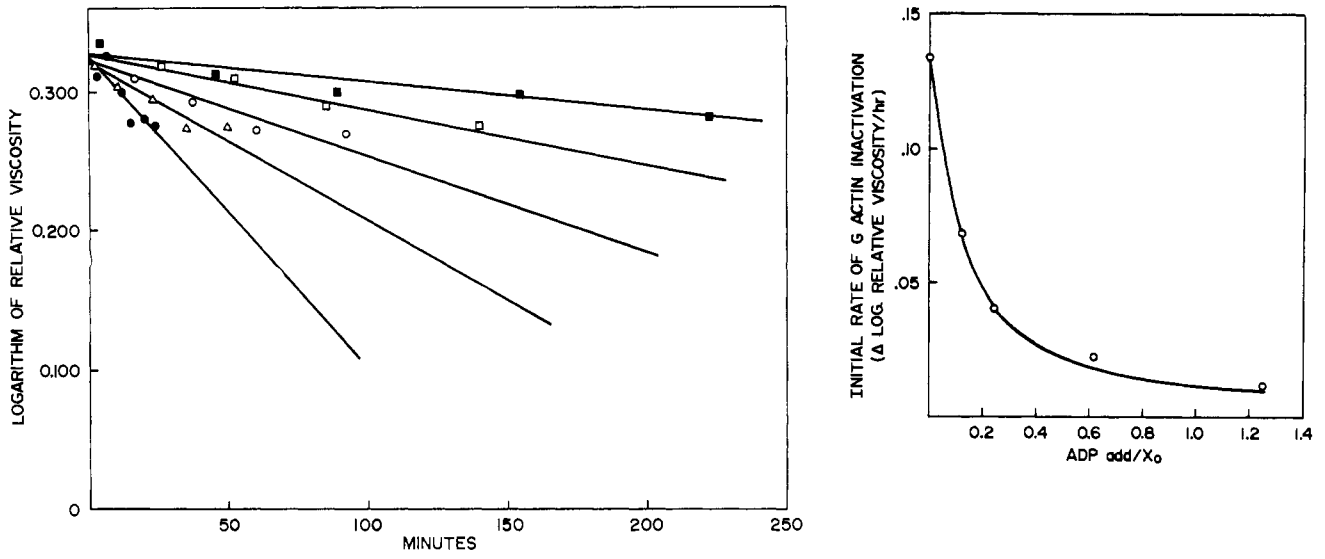

FIGURE 3: Effect of added nucleotide on rate of denaturation of ADP–G-actin solutions at 0°, as was used by viscosity. Aliquots were polymerized at the times indicated on the abscissa by addition of 0.1 M KCl, 1 mM $MgCl_2$, and 0.5 mM ATP and the viscosity at 25° was determined 60 min later. G-actin samples were maintained at 0° prior to polymerization. All solutions contained Dowex 1 pretreated ADP–G-actin which was diluted to 1.8 mg/ml in 1 mM Tris-HCl (pH 8.0) at zero time. Solutions contained added ADP at concentrations ($\times 10^{-5}$ M) of (●) 0, (△) 3.74, (○) 7.50, (□) 18.8, and (■) 38.4. (B, right) Initial rates of ADP–G-actin inactivation at 0° *vs.* the concentration of free ADP. Initial rates of inactivation were calculated from the slopes of the lines in Figure 3A expressed as logarithm of relative viscosity per hour. The abscissa indicates the ratio, Z, of the concentration of free ADP and the initial concentration, (X_0), of active actin. The solid line, computed as in Figure 1B, corresponds to a K_d of 2.12×10^{-7} mole/l.

TABLE I: Binding Constants and Dissociation Constants of G-Actin for Nucleotide under Various Conditions.

Character of Free and Bound Nucleotide and Cation	Method	Temp (°C)[a]	No. of Expt	Dissocn Constant, K_d (moles/l.)	95% Confidence Limits of K_d	Binding Constant, K_b (M^{-1})	Av % Free Nucleotide[b]
ATP–Ca	Apyrase[c]	0	5	4.2×10^{-8}	$2.4\text{–}6.0 \times 10^{-8}$[d]	2.4×10^{7}	4.0
ADP–Mg	Apyrase[c]	0	6	2.1×10^{-7}	$0.3\text{–}3.3 \times 10^{-7}$[d]	4.8×10^{6}	8.7
ADP–Mg with $2\text{–}5 \times 10^{-4}$ M $MgCl_2$	Apyrase[c]	0	7	2.0×10^{-7}	$1.2\text{–}2.8 \times 10^{-7}$[d]	4.9×10^{6}	8.6
ADP–Ca	Apyrase[c]	0	5	2.5×10^{-7}	$1.3\text{–}3.7 \times 10^{-7}$[d]	4.0×10^{6}	9.5
ADP–Mg	Rate of G-actin inactivation	25	6	2.6×10^{-5}	$0.8\text{–}7.1 \times 10^{-5}$[e]	3.9×10^{4}	58.8
ADP–Mg	Rate of G-actin inactivation	1	3	4.6×10^{-7}	$2.4\text{–}8.8 \times 10^{-7}$[e]	2.2×10^{6}	12.9

[a] Temperature was regulated to within ±0.2°. [b] Calculated for a G-actin concentration of 1.5 mg/ml (mol wt 60,000) with no added nucleotide. [c] The binding constants were calculated assuming a molecular weight for G-actin of 60,000. The binding constant is approximately 20% lower if the molecular weight of actin is taken as 50,000. [d] Confidence limits were computed as the means plus or minus two standard errors. [e] Confidence limits were computed for the estimated parameters, K_d and α, assuming that differences between the two sides of eq 6 are normally distributed with variance independent of z. The calculations were based on the asymptotic relation between the log likelihood function and the chi-square distribution (Rao, 1965).

Discussion

This study, utilizing two different techniques, indicates a binding constant of G-actin for ADP of $2\text{–}5 \times 10^{6}$ M^{-1} at 0°, a value tenfold greater than that (4×10^{5}) reported by Seidel *et al.* (1967) at 4° and more than 200-fold greater than that found by Higashi and Oosawa (1955). The latter authors apparently performed their study at room temperature but even at 25° we found a binding constant of 3.9×10^{4} M^{-1}, a value somewhat larger than their result. Higashi and Oosawa's results were based upon absorbance changes at 232 mμ in ADP–G-actin solutions after nucleotide addition. However, we did not find absorbance changes at 232 mμ or elsewhere at 0° when ADP was added to ADP–G-actin perhaps because

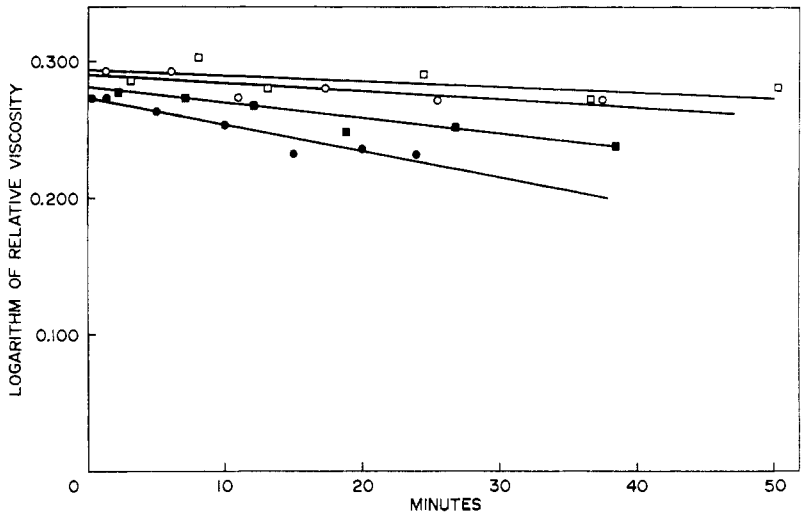

FIGURE 4: Effect of $MgCl_2$ on the rate of inactivation of ADP–G-actin solutions at 0°. Aliquots were polymerized and measured as in Figure 3A. All solutions contained Dowex 1 pretreated ADP–G-actin 1.5 mg/ml in 1 mM Tris-HCl (pH 8.0). (●) No added ADP; (○) no added ADP, 0.2 mM $MgCl_2$; (■) 4 μM ADP; and (□) 4 μM ADP, 0.2 mM $MgCl_2$.

of the much tighter binding of ADP to G-actin at that temperature. At 25°, some of the absorbance change at all wavelengths appeared to be due to denaturation of the ADP–G-actin reference solution which was not protected from the denaturing effect of elevated temperature by added nucleotide. In addition, a variety of nonspecific factors appreciably influence the absorbance of ultraviolet light at 232 mμ. Despite these considerations, Higashi and Oosawa's (1965) result for the binding constant of ADP to G-actin is not greatly different from that found at 25° in this study.

The binding constant determination of Seidel *et al.* (1967) depended on their calculation of the molar concentration of F-actin containing no bound nucleotide. This measurement required subtraction of the molar concentration of bound nucleotide from the molar actin concentration. However, determination of actin molarity could be an unreliable procedure in view of (a) discrepancies in reports of the molecular weight of actin (Tsuboi, 1968), and (b) of difficulties in adequately removing α-actinin, denatured actin, tropomyosin, and other impurities from actin (Ebashi and Maruyama, 1965; Rees and Young, 1967). Such difficulties could lead to relatively large mathematical errors in the calculation of the binding constant by Seidel *et al.* (1967), whereas similar errors would have a small effect on the magnitude of the binding constant with our techniques. It is possible that tropomyosin or other impurities could alter nucleotide binding to actin. Such an effect has not been systematically evaluated, although in one study in this laboratory 0.5 mg/ml of tropomyosin prepared by a modification of the Bailey technique (Bailey, 1948; Mueller, 1966), when added to 1.5 mg/ml of ADP–G-actin (final concentrations) did not change and nucleotide binding to G-actin, as determined by the apyrase technique. AMP deaminase has been reported to appreciably contaminate myosin preparations and to alter absorbance readings at 257 mμ (Lowey and Luck, 1969). However, no evidence for AMP deaminase contamination as evaluated by the technique of Lowey and Luck (1969), was found in ADP–G-actin preparations prepared according to our techniques. In addition, if myokinase was present in our ADP–G-actin solutions, some conversion of added ADP into AMP and ATP could have been expected during the studies of the rate of actin denaturation. Appreciable production of ATP would be expected to decrease the measured rate of breakdown of the ADP–G-actin. However, our studies indicated that there was no significant production of ATP in ADP–G-actin over a prolonged period at 0°.

Our techniques for determination of the binding constant of ADP to G-actin depend on the assumptions that equilibrium between free and bound nucleotide is rapidly established and that the rate of actin denaturation is relatively slow. This model was proposed by Grubhofer and Weber (1961) on the basis of their measurements of rates of exchange of bound nucleotide and rates of actin denaturation, and has been supported by additional data since then. Thus Kuehl and Gergely (1969) recently demonstrated a rapid exchange of radio-labeled free nucleotide with actin-bound nucleotide and presented evidence that the exchange occurred *via* dissociation of bound to free nucleotide and not *via* displacement of bound nucleotide by free nucleotide. In addition, West *et al.* (1967a) noted that doubling apyrase concentration doubled the rate of hydrolysis of actin bound nucleotide. This was true up to apyrase concentrations sufficient to hydrolyze all the actin bound nucleotide within 5 min. Since West *et al.* (1967a) found that creatine kinase did not phosphorylate actin bound nucleotide, it is likely that the apyrase also was not directly hydrolyzing bound nucleotide and hence that there was a rapid equilibrium between bound and free nucleotide. Furthermore, the reasonable agreement of the binding constant measurement of this study based on the initial concentration of free nucleotide with that based on rates of actin denaturation supports the concept that rapid equilibration of free and actin-bound nucleotide occurs. It is not clear, however, why Dowex 1 removes actin-bound nucleotide to such a limited extent. The comparatively slow irreversible denaturation of G-actin containing no bound nucleotide was deduced by Grubhofer and Weber (1961) who found that it has a half-life of about 12 min at 0°. Relatively long lifetimes were also obtained by Asakura (1962) who found a half-life of about 38 min at 6° while in our studies with ADP–G-actin the half-life averaged about 25 min at 0°. The scatter in these results may be due to the effect of variable concentrations of magnesium or other ions,

since, as discussed below, added magnesium appreciably altered the rate of G-actin denaturation without affecting the binding constant of G-actin for ADP.

The procedure for the preparation of ADP–G-actin included a treatment of concentrated (4–6 mg/ml) ADP–G-actin solutions with Dowex 1 to remove free nucleotide. Since the actin concentration as calculated from biuret and perchloric acid determinations were normally within 10% of each other, it seems unlikely that appreciable removal of bound nucleotide by the Dowex 1 treatment occurred. It has been assumed in eq 5 that any protein in excess of the free nucleotide largely denatured by the time the study commenced. However, in view of the moderately long lifetime for G-actin not containing bound nucleotide discussed above, some of it may have been present at the start of the studies. Excess G-actin containing no bound nucleotide would shift eq 1 to the left and thus reduce the free nucleotide. Hence the binding constant as found by the apyrase technique may be too high, while a similar error would only slightly alter the binding constant found with the inactivation technique since extra nucleotide was added. Thus in the latter method the actin concentration could be somewhat higher than indicated by the concentration of bound nucleotide, though by no more than 10%, so that the actual binding constant would be less than 10% lower than reported. These effects may explain the somewhat lower binding constant determined with the inactivation technique than that found in the apyrase study.

The measurement of the binding constant of ADP to G-actin at 25° depended on the accuracy of the spectrophotometric method as an indicator of the time course of G-actin denaturation. Measurement of absorbance change at 258 mμ was performed since absorbance change at that wavelength appears to reflect loss of G-actin-bound nucleotide (West *et al.*, 1967b) and hence should be an accurate indicator of the rate of actin denaturation. There was not exact agreement between the viscosity and spectrophotometric methods of measuring G-acetin denaturation (Figure 2). The difference could be due either to incorporation of denatured actin into the F-actin mesh producing falsely high viscosity values in the presence of a considerable proportion of denatured actin, or to late absorbance changes resulting from delayed unfolding of the actin molecule after denaturation has occurred. However, the binding constant determination depended on the initial rate of actin inactivation and discrepancies of the two techniques for measuring actin denaturation appeared only after prolonged exposure of G-actin to 25°. Our results indicate a 250-fold decrease in the binding of ADP to G-actin at 25° compared with that at 0°. In contrast, Asakura (1961) found only a 30-fold decrease in the binding of ATP to G-actin between 6 and 30°. Thus binding of ADP to G-actin appears to be markedly temperature dependent.

The apparent identity of nucleotide binding by actin containing either bound calcium or bound magnesium is of interest in view of the postulated role of bound cation in nucleotide binding by G-actin (Strohman and Samorodin, 1962). These findings suggest either that magnesium and calcium act equally well in ADP binding or that bound cation does not play an essential role in nucleotide binding to actin. The latter does not appear to be true since studies in this laboratory indicate a considerable reduction in the binding constant of G-actin for ADP and especially ATP when the actin contains no bound cation (J. J. West, 1969, unpublished data). Although addition of 0.2 mM magnesium was found to decrease the rate of inactivation of ADP–G-actin by one third, it did not appear to influence ADP binding to G-actin. If the model of actin denaturation described in eq 1 is correct then the reduction of the rate of actin inactivation could be ascribed to one of three modes of action: (A) the cation could induce aggregation of G-actin; (B) the cation could shift the equilibrium to the left, *i.e.*, increase the affinity of G-actin for ADP; and (C) the cation could increase the stability of G-actin containing no nucleotide and thereby decrease the α of eq 3. Grant *et al.* (1964) found that ADP–G-actin does not aggregate when low concentrations of $MgCl_2$ are added at 0°, and no significant change in viscosity was demonstrated in this study upon addition of 0.2 mM $MgCl_2$

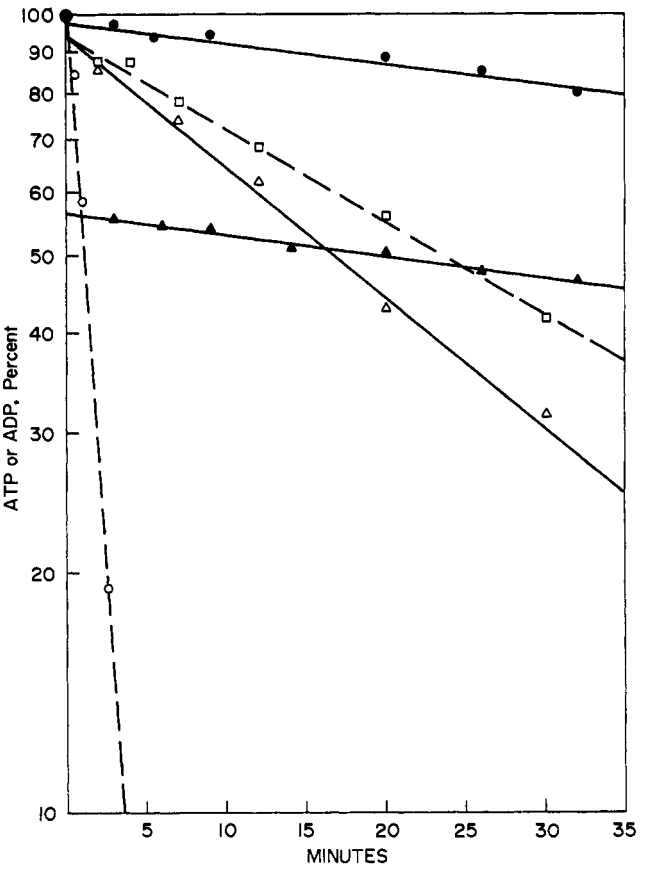

FIGURE 5: Rate of hydrolysis of free and actin-bound [^{14}C]nucleotide by apyrase at 0°. For details of chromatographic separation and determination, see West *et al.* (1967a). The ordinate is the per cent of [^{14}C]nucleotide remaining as ADP (or ATP). The per cent values were normalized to 100% ADP (or ATP) at zero time. ADP–G-actin preparations consisted of about 95% ADP prior to addition of apyrase, while ATP–G-actin preparations were initially about 90% ATP. (●) Dowex 1 pretreated [^{14}C]ATP–G-actin 2.66 mg/ml (4.4×10^{-5} M) in 1 mM, Tris-HCl (pH 7.5). Apyrase (23 μg/ml) was added at zero time. (▲) ATP–G-actin as above plus added [^{14}C]ATP with a total nucleotide concentration of 6.9×10^{-5} M. Apyrase (23 μg/ml) was added at zero time. (□) Dowex 1 pretreated Mg-ADP–G-actin (1.6 mg/ml) in 1 mM Tris-HCl (pH 7.5). Apyrase (21 μg/ml) was added at zero time. (△) Same as in □ except that actin contained bound Ca instead of bound Mg. (○) [^{14}C]ADP (7.9×10^{-6} M) in Tris-HCl (pH 7.5). Apyrase (21 μg/ml) was added at zero time.

to ADP–G-actin solutions. Our data indicate that G-actin had the same affinity for ADP in the presence or absence of added $MgCl_2$ (Table I). Since the rate of G-actin inactivation with added magnesium was one-third the rate in the absence of added cation, a ninefold change in the binding constant would have been expected. Such a binding constant change should have been readily detectable by the apyrase method. Consequently, it appears that added magnesium stabilizes G-actin solutions by slowing the rate of denaturation of nucleotide-free G-actin molecules.

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