Binding of Nucleotide to Cation-Free G-Actin*

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ABSTRACT: After addition of EDTA to G-actin solutions at 0° absorbance changes occur at many wavelengths including a prominent absorbance increase at 256 mμ. The rate of change in absorbance at 256 mμ was shown to correlate with the rate of loss of bound nucleotide following EDTA addition, whereas a more rapid series of absorbance changes, particularly at 270 mμ, appear to relate to the rapid removal of actin-bound cation by EDTA. Late absorbance changes, particularly at 287 mμ, appear to be a reflection of the gradual denaturation of the actin molecule subsequent to nucleotide loss. The absorbance change at 256 mμ following EDTA addition was used to determine the binding constant of cation-free G-actin for nucleotide. The binding constant of cation-free actin for ATP and ADP was near 3×10^5 M^{-1}, a value approximately $^1/_{1000}$th that of the binding constant for ATP in the presence of bound cation. Since, when cation is present, binding of ATP is appreciably higher than ADP, this result suggests that cation binds primarily to the two terminal phosphates of ATP while nucleotide binding to actin in the absence of bound cation does not depend on the terminal phosphate group. The interaction of ITP and UTP with cation-free actin was also studied.

Since actin-bound nucleotide and cation may have an important role in the biochemical events leading to contraction, it seems important to learn more about their biochemical properties. The role of actin-bound cation has not been entirely clear, although Strohman and Samorodin (1962) and others have suggested that metal plays an important role in nucleotide binding partly based on inference from the role of cation in nucleotide binding in other proteins (Williams, 1959) and the observation that bound nucleotide was no longer detectable in the absence of bound metal (Grubhofer and Weber, 1961).

In proteins other than actin, bound cation appears to contribute to nucleotide binding by interaction with the terminal phosphates (Williams, 1959). However, since actin binds different nucleotides to a different degree (Martinosi and Gouvea, 1961; Iyengar and Weber, 1964), it would appear that nucleotide binding is influenced by the adenine ring or other portions of the nucleotide molecule. Consequently, when metal is removed by the addition of chelating agents such as EDTA, some nucleotide should remain bound to the G-actin although previous workers have found little remaining bound nucleotide after removal of bound cation (Strohman and Samorodin, 1962; Gergely *et al.*, 1968). In contrast, in previous studies of absorbance changes in actin solutions after addition of EDTA (West *et al.*, 1967), it appeared that much of the nucleotide remained bound after cation removal. This conclusion was based on the assumption that the slow rate of absorbance changes at 256 mμ after EDTA addition implied slow loss of actin-bound nucleotide. An alternative explanation was that the absorbance changes at 256 mμ and elsewhere were largely due to unfolding of the actin molecule, and not loss of bound nucleotide (Gergely *et al.*, 1968). If, in fact, appreciable amounts of nucleotide remain protein bound after bound cation is removed, then a study of the role of cation bound to actin and of actin without bound cation in nucleotide binding would be feasible. This communication reports studies of the significance of absorbance changes after addition of EDTA to G-actin solutions and their use to determine the binding of nucleotide to cation-free G-actin. Evidence is presented that distinctive absorbance changes can be attributed to the initial removal of bound cation by EDTA and that quite different absorbance changes result from subsequent loss of bound nucleotide. A third set of absorbance changes apparently are related to slow denaturation of actin after nucleotide removal.

Materials

Actin was extracted from an acetone-dried powder of rabbit muscle (Feuer *et al.*, 1948) at 0° (Drabikowski and Gergely, 1962) and purified by repeated polymerization and ultracentrifugation (Mommaerts, 1951). ATP- and ADP-G-actin and actin containing bound calcium were prepared as previously described (West, 1970a). The calcium or magnesium content of the actin was determined by atomic absorbance measurements and was found to be essentially equimolar to the actin (West, 1970a). ITP-G-actin was prepared by addition of 1.0 mM ITP to ADP-G-actin solutions prior to clarification at 100,000*g* for 10 min. UTP-G-actin was prepared by repeatedly homogenizing pellets of [^{14}C]ADP-actin in 0.5 mM UTP and then repolymerizing until at least 90% of the ^{14}C label was displaced. Just prior to studies, C-actin preparations were treated with Dowex 1-Cl-X2 (West, 1970a) to remove free nucleotide and the desired concentration of nucleotide was readded. All solutions were prepared in 1 mM Tris-HCl (pH 8.0).

There did not appear to be contamination of ADP-G-actin solutions with either ATP-G-actin or myokinase since the viscosity of ADP-G-actin solutions did not appreciably increase during a 3-hr interval after addition of 0.1 M KCl, 1 mM $MgCl_2$, and 1 mM ADP at 0° (West, 1970a). ATP-G-actin solutions appeared tropomyosin free since their viscosity was similar to that of previously reported tropomyosin free ATP-G-actin solutions (Drabikowski and Gergely, 1962). AMP-deaminase appeared to be absent since there was no absorbance change attributable to AMP deamination over a 24-hr period at 25° following addition to ADP-G-actin of 0.1 mM

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AMP (Lowey and Luck, 1969). ITP–G-actin solutions appeared to be largely free of residual adenine nucleotide since the 249:259 mμ ratios of perchloric acid extracts of ITP–G-actin showed nearly the same value as the added ITP. UTP–G-actin solutions did not appear to contain appreciable residual adenine nucleotide since less than 5% of the [^{14}C]-ADP label remained and since paper chromatography of neutralized perchloric acid extracts of the UTP-actin treated with an isobutyric acid–ammonium hydroxide–water (66:1:33, v/v) solvent system did not reveal adenine nucleotide.

[^{14}C]ATP and -ADP were obtained from Nuclear-Chicago. [^{3}H]ATP and -ADP were ordered from Schwarz BioResearch, Inc. ATP, ITP, and UTP were obtained from P-L Biochemicals. ADP free of contaminating ATP was purchased from Calbiochem.

Methods

Separation of actin from nucleotides and cations was carried out on a column of Sephadex G-25. The Sephadex was allowed to swell in distilled water at 70° for 2 hr and the supernatant and fines were aspirated after settling. The Sephadex was three times resuspended and permitted to settle in 1 mM Tris·HCl (pH 8.0) and the supernatant and fines were again aspirated. The 1.6-cm i.d. column was then filled at 0.5° and allowed to settle to a height of 37 cm. The Sephadex was washed over a prolonged period with 1 mM EDTA in 1 mM Tris·HCl (pH 8.0) until the ultraviolet absorbance of the effluent was the same as the eluent. Before all studies, the column was preequilibrated with the eluent solution until the ultraviolet absorbance or the counts of the effluent were the same as the eluent. All studies were performed at 0.5° since actin denatured rapidly at higher temperatures.

Difference spectra were recorded with a Durrum PGS spectrophotometer. Matched cuvets with a path length of 1 cm, 0.1 cm, or split compartment cuvets with a chamber path length of 0.44 cm were employed. The cells were maintained at 0.5 ± 0.4° in a thermostatted cell compartment. Protein and buffer were placed in separate compartments of the sample and reference cuvets; a base line was determined prior to EDTA addition. EDTA ordinarily was added to the sample actin and reference buffer in a volume of 0.5% of the actin volume to give a final EDTA concentration of 1 mM and the lambda pipet was also used to add an equal voume of buffer to the reference actin. Studies were also run with EDTA concentrations of 5 or 10 mM without apparent change in the results. Scans were run from 320 to 240 mμ either at 100 or 20 mμ per min. Since actin solutions have no absorbance at 315 mμ, changes in absorbance at 315 mμ were taken as an indication of moisture condensation or other cause of light-scattering or instrumental variations. Ordinarily, changes at this wavelength were very small and apparently instrumental and were corrected for in the computations at other wavelengths, but in the few instances where appreciable changes occurred at 315 mμ the curves were discarded. The absorbance changes at 256 mμ or other wavelengths were calculated as a per cent of the final absorbance change and plotted on semilogarithmic paper as 100 minus the per cent. When plotted in this manner, the rate of absorbance change at 256 mμ in ADP–G-actin solutions was linear but in ATP–G-actin preparations an initial curvature often was noted for the first 5–10 min, especially at concentrations above 1.2 mg/ml.

Since ADP–G-actin is unstable even at 0°, some absorbance changes at 256 mμ occurred due to the spontaneous denaturation of ADP–G-actin in the reference cuvet. The rate of this reference cell absorbance change was determined by measuring the decrease in absorbance at 256 mμ after the absorbance change in the sample cell had been completed, thereby using the sample cell as a reference. Essentially the same rate of reference cell absorbance change was found when bovine serum albumin was used as a reference. This absorbance decrease was found to follow first-order kinetics and, hence, could be extrapolated to zero time. In computing the binding constant of cation-free G-actin for nucleotides other than ATP, the absorbance changes were corrected for change in absorbance of the reference cell utilizing such a plot. In the case of ATP, the absorbance of the reference cell was essentially stable at 0° and no correction was necessary. The binding constant of cation-free actin for nucleotide was determined from the difference spectral data as described in results. ATP binding to cation-free actin was also determined by the rate of denaturation method (West, 1970a) as outlined in Results.

Actin concentrations were determined by measuring the absorbance of the nucleotide after extraction in 0.6 M perchloric acid, using an extinction coefficient of 14.7×10^3/mole at 257 mμ for ATP, 14.9×10^3/mole at 257 mμ for ADP (Pabst, 1956), or of 10.9×10^3/mole at 250 mμ for ITP and 10.0×10^3/mole at 262 mμ for UTP at pH 1. This was checked with the concentration determined from the protein absorbance at 280 mμ using an $E^{1\%}_{1cm}$ value of 11.08 (West, 1970a). Agreement within 15% was generally obtained. Results which did not agree to within 30% were discarded. Viscosity measurements were performed in Ostwald viscometers with outflow times of about 25 sec at 25 ± 0.1°, 50–60 min after salt addition.

Results

Chromatographic Studies. When [^{45}Ca]ATP–G-actin (5 mg/ml) was applied to a 32-cm column of Sephadex G-25 preequilibrated with 1 mM EDTA and 0.1 mM Ca-free ATP in 1 mM Tris·HCl (pH 8.0) and eluted at 0° with the same solution, the ^{45}Ca was eluted at the same position as ^{45}Ca chromatographed in the absence of added protein. The eluted G-actin polymerized after addition of 0.1 M KCl with 1 mM $MgCl_2$ but not after 0.1 M KCl alone. G-actin eluted with 1 mM EDTA without nucleotide was not polymerizable. When [^{14}C]ATP–G-actin (5 mg/ml) was eluted at 0° from a similar Sephadex column with 1 mM EDTA and 0.2 mM [^{14}C]ATP in 1 mM Tris·HCl (pH 8), a nucleotide peak was found at the same site as the protein at a concentration almost as great as the protein concentration. Nucleotide was not eluted at the same site as the protein when the actin was denatured by exposure to EDTA for 200 min at 0° prior to addition to the column.

Time Course of Absorbance Changes Compared to Rate of Loss of Actin-Bound Nucleotide and Cation. Absorbance changes occur at many wavelengths following EDTA addition to G-actin, including a prominent increase in absorbance at 256 mμ which has been related to a hypochromic change of actin-bound nucleotide (West *et al.*, 1967). To determine if the 256-mμ absorbance change occurred at the same rate as the loss of bound nucleotide viscosity, bound nucleotide content and absorbance changes were measured in aliquots of the same solution after addition of EDTA at 0°. To measure viscosity and bound nucleotide, the solutions were polymerized at various intervals after addition of EDTA, by addition of 0.1 M KCl, 1 mM $MgCl_2$, and 5 μM ATP. At low free ATP concentrations the actin preparations lost polymerizability at a somewhat slower rate than the rate of absorbance change

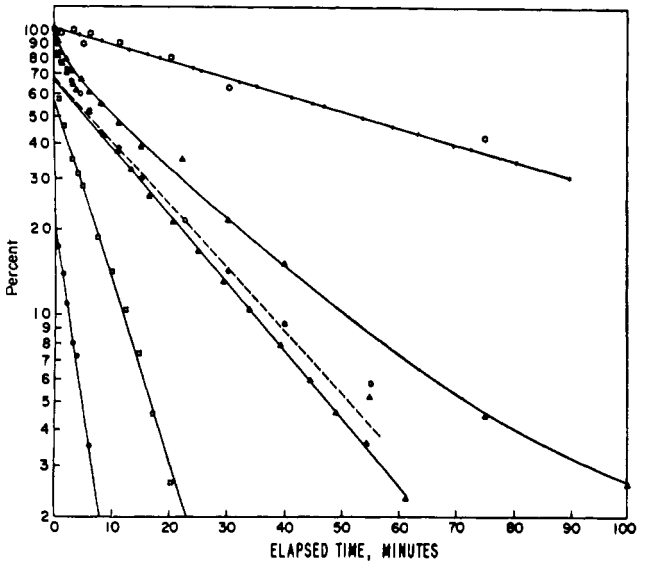

FIGURE 1: Rate of absorbance change at 256 mμ after addition of 1 mM EDTA at 0.4° to ATP-G-actin compared to the rate of decrease in polymerizability and the rate of loss of actin bound nucleotide. Absorbance change was recorded as a per cent of the final absorbance change at 256 mμ and plotted after subtraction from 100%. (▲) Absorbance change at 256 mμ following addition of 1 mM EDTA at zero time to 29 μM ATP-G-actin in 1 mM Tris·HCl (pH 8.0) containing ATP (0.21 μM). (●) Absorbance difference between the above absorbance values during the first 10 min and the solid line fitted to the remaining absorbance values. (○) Change in actin bound nucleotide of the same actin preparation. Aliquots of the actin were polymerized at the indicated times by addition of 0.1 M KCl, 1 mM $MgCl_2$, and 5 μM ATP. Once polymerization was complete and viscosity was measured, the preparations were mixed for 8 min with Dowex 1. The Dowex 1 was removed by centrifugation and the preparation was deproteinized by addition of 0.6 M perchloric acid. The bound nucleotide concentration was determined from the absorbance at 257 mμ and expressed as a per cent of the control. (△) Change in viscosity of aliquots of the same actin preparation. The actin was polymerized as described above and, after 60 min, the viscosity measured in Ostwald viscometers at 25°. Viscosity is expressed as a per cent of controls polymerized in the absence of EDTA. (•) Absorbance change at 256 mμ following addition of 1 mM EDTA at zero time to 29 μM ATP-G-actin in 1 mM Tris·HCl (pH 8.0) containing ATP (50 μM). (○) Change in viscosity of aliquots of the same actin preparation treated and calculated as in △. (□) Absorbance change at 256 mμ following addition of 1 mM EDTA at zero time to 20 μM ATP-G-actin in 1 mM Tris·HCl (pH 8.0) containing 0.21 μM ATP.

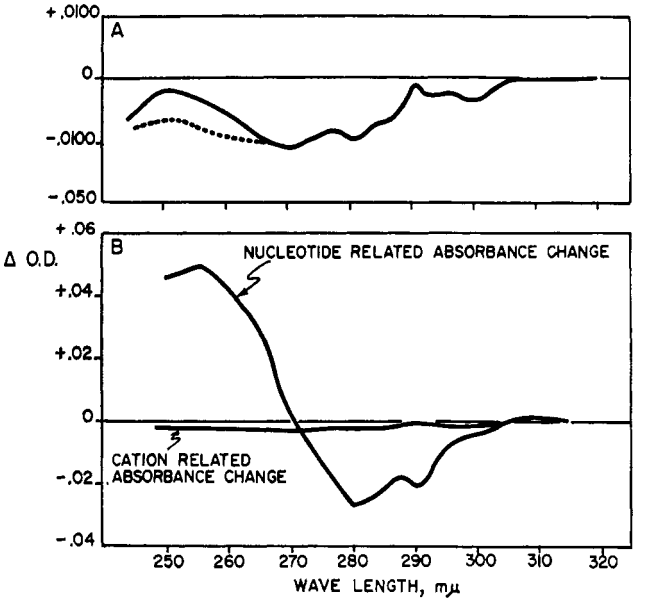

FIGURE 2: Absorbance change attributable to cation removal from ADP-G-actin compared to the total absorbance change. (A) The absorbance changes illustrated were those observed following addition of 1 mM EDTA to ADP-G-actin (40.0 μM) in the presence of 0.2 mM ADP at 0°. The solid line is the average of 15 studies of absorbance changes extrapolated back to the time of EDTA addition. In the dashed curve a correction has been applied at 256 mμ and adjacent wavelengths to subtract the absorbance change attributable to nucleotide dissociation from the G-actin under these conditions. Binding constant of cation-free nucleotide for actin of 2.5×10^6 M^{-1} and an absorbance change at 256 mμ/μmole of bound nucleotide dissociated of 0.0048 ODU were employed in this calculation. This later value was computed from the average total absorbance change observed following EDTA addition to ADP-G-actin. The effect of this correction was to make the absorbance change at 256 mμ slightly more negative. The magnitude of this curve per mole of actin was determined from the average absorbance change at 271 mμ in relatively dilute actin preparations since it appears that no absorbance change occurs at this wavelength after 1 min. At high protein concentrations, the size of the curve per mole was appreciably less in ATP-G-actin in contrast to ADP-G-actin because some aggregated ATP-G-actin was not immediately susceptible to the action of EDTA. (B) Comparison of the cation related absorbance change in ADP-G-actin to the subsequent absorbance change following addition of 1 mM EDTA to ADP-G-actin (10 μM) in the presence of 5 μM ADP at 0°. The subsequent absorbance changes were computed by subtracting the initial absorbance change illustrated in part A from the final absorbance change.

(Figure 1), although the difference in rates was less apparent at high free ATP concentrations (Figure 1). However, the bound nucleotide content, obtained by measuring the 259-mμ absorbance of perchloric acid extracts following an 8-min treatment of the F-actin solution by Dowex 1, showed a rate of decrease that was quite similar to the rate of the absorbance change at 256 mμ (Figure 1).

When ATP (0.2–0.8 mM) was present in the G-actin solutions prior to EDTA addition, the initial absorbance changes were not similar to absorbance changes observed subsequently (Figure 2B). These initial changes appeared to be complete within about 90 sec at 0° whereas absorbance changes related to nucleotide loss occurred over a period of several days. The initial absorbance changes following EDTA addition to actin solutions containing 0.2 mM free nucleotide were similar for ADP or ATP except that there was a negative peak at 300 mμ in ADP-G-actin preparations (Figure 2). Pretreatment of the G-actin with 20% v/v Dowex 50 for 4 min had no effect on these initial absorbance changes, but they could be reversed by addition of 0.7 mM $CaCl_2$ and did not occur when the EDTA contained 0.7 mM $CaCl_2$ or when the G-actin was denatured by heating at 85° for 4 hr prior to EDTA addition. At low protein and low nucleotide concentrations, the initial absorbance change after EDTA addition represented as much as 50% of the total absorbance change, in contrast to the small size and different wavelength distribution of the initial absorbance change following EDTA addition in the presence of 0.2 mM nucleotide. When ATP-G-actin concentrations in excess of 2 mg/ml were employed even with low added nucleotide, the initial absorbance changes were small and resembled the initial change found in the presence of 0.2 mM nucleotide. The rate of absorbance change at 256 mμ or elsewhere for these preparations at 0° followed first-order kinetics after about 10 min, but in ATP-G-actin though not ADP-G-actin, there was an early faster change (Figure 1). When the corresponding values

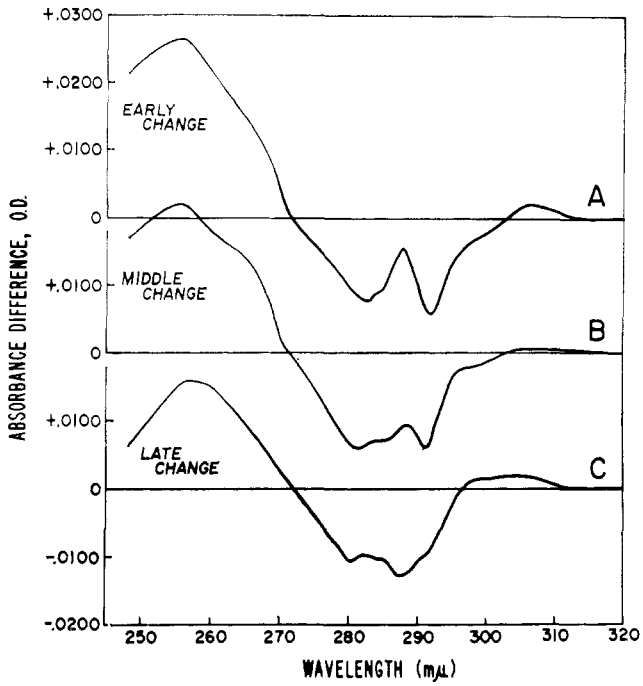

FIGURE 3: The early absorbance changes in ADP–G-actin compared to the later changes following EDTA addition at 0°. EDTA (1 mM) was added at zero time to ADP–G-actin (22.5 μM) in 1 mM Tris·HCl (pH 8.0) containing 10.3 μM ADP and scans from 320 to 240 mμ were run at various intervals. Path length was 1 cm. (A) Absorbance difference between the 1- and 8-min scans. (B) Absorbance difference between the 8- and the 20-min scans. (C) Absorbance difference between the 20- and the 80-min scans.

of the line fitted to the later absorbance changes were subtracted from the changes in the first 10 min, the difference followed first-order kinetics (Figure 1). To evaluate the possibility that these results were due to aggregation of ATP–G-actin inhibiting bound nucleotide dissociation at high protein concentrations, the rate of exchange of actin-bound ATP with added [^{14}C]ATP was studied at high and at low protein concentrations, employing the procedure described by Keuhl and Gergely (1969). ^{14}C-labeled ATP was found to exchange for bound ATP almost as rapidly at high protein concentrations as at low actin concentrations.

To investigate the possibility of a slow conformational change of actin following nucleotide loss, absorbance changes in the 310- to 270-mμ region immediately after EDTA addition were compared to those observed at later intervals. The absorbance change in the first few minutes following EDTA addition with the initial absorbance change due to cation removal eliminated was appreciably different from changes observed later (Figure 3). In ADP–G-actin solutions a prominent trough at 286 mμ in the early curves was later replaced by a peak at almost the same wavelength (Figure 3) while negative peaks at 292 and 297 mμ decreased in size. In contrast the curves changed little between 280 and 240 mμ. In ATP–G-actin the absorbance changes were similar except that a negative peak at 283 mμ in early curves gradually shifted to higher wavelengths.

Determination of Binding Constant of Nucleotide for G-Actin. The binding of nucleotide to cation-free G-actin was determined by two methods. The first, a modification of a technique developed by Asakura (1961), has been described by West (1970a). In this method the initial rates of G-actin inactivation were measured in the presence of various concentrations of added nucleotide (Figure 4A) and the binding constant was computed from these rates according to eq 6 of our previous communication (West 1970a) (Figure 4B). The rates of G-actin inactivation were determined by measuring the viscosity of polymerized aliquots of the G-actin solution taken at intervals following EDTA addition. The results of three such studies indicated a binding constant of 2.6×10^5 M^{-1} (Table I).

A second method for estimating the binding constant of nucleotide to cation-free actin depended on a measurement of the percentage of bound nucleotide which dissociated immediately following EDTA addition, as indicated by the absorbance change at 256 mμ. This measurment required the extrapolation of the linear part of the 256-mμ absorbance change back to zero time to determine the initial absorbance change at 256 mμ, a procedure based on the assumption that bound and free nucelotide reach rapid equilibrium following EDTA addition (West, 1970a). The absorbance change at 256 mμ attributable to cation removal (Figure 2) was subtracted from these results and the concentration of dissociated actin, G^0, calculated. The amount of actin dissociated from nucleotide before EDTA addition, G^1, was computed from previously determined binding constants of G-actin for ATP (Asakura, 1961) or ADP (West, 1970a). The binding constant of cation-free G-actin for nucleotide could be computed from the equation $K_b = [GN - (G^0 + G^1)]/(G^0 + G^1)(N + G^0 + G^1)$, where GN is the initial actin concentration computed from the nucleotide concentration measured in a perchloric acid extract of the Dowex-1-pretreated G-actin solution, and N is the concentration of added nucleotide. For 12 ATP–G-actin solutions with a protein concentration range of 6–30 μM and added nucleotide from 0.25 to 50 μM, the K_b was $3.4 \times 10^5 \pm 0.3 \times 10^5$ M^{-1} (Table I). Those studies employing only low protein concentrations in which there was an essentially linear 256-mμ absorbance change yielded the same binding constant. There was often an initial curvature during the first 5–10 min in preparations containing ATP–G-actin above 1 mg/ml. If this curvature is due to slow arrival at equilibrium following EDTA addition because of gradual dissociation of oligomers of actin to G-actin, the extrapolation of the linear part of the plot to zero time in the binding constant computation could cause error. The extent of the error was estimated by extrapolating the linear plot to the apparent mean life of the oligomeric actin instead of to zero time. Since on a semilogarithmic plot a straight line resulted when the extrapolation of the linear portion of the absorbance change was subtracted from these early points, a half-life of this process could be estimated and a mean life computed from the relationship T mean $= T_{1/2}/\ln 2$. This calculation should provide an estimate of the lower limit of the binding constant since it is based on the extreme assumption that none of the nucleotide dissociated from actin immediately following EDTA addition at zero time. For the same 12 ATP–G-actin solutions mentioned above, the binding constant when computed to the mean life of the first process, instead of to zero time, was 2.0×10^5 M^{-1} (Table I). For 13 preparations of ADP–G-actin with a similar range of protein and nucleotide concentrations, the K_b was 2.5×10^5 M^{-1} (Table I).

The binding constant of ITP to cation-free G-actin was estimated by measuring the absorbance change at 249 mμ following EDTA addition to determine the additional concentration of nucleotide-free G-actin after EDTA addition. A value of 4×10^6 M^{-1} was used for the binding constant of Mg–G-actin for ITP (Seidel *et al.*, 1967). The binding constant of cation-free G-actin for ITP appeared to be similar to that

TABLE I: Binding Constants of Cation-Free G-Actin for Nucleotide.

Character of Nucleotide	Method	Temp (°C)[a]	No. of Expt	Binding Constant K_b (M^{-1})	95% Confidence Limits of K_b (M^{-1})
ATP	Rate of G-actin inactivation	0.4	3	2.6×10^5	$1.0\text{–}8.3 \times 10^{5}$[b]
ATP	Initial dissociated nucleotide	0.6	12	3.4×10^5	$2.8\text{–}4.0 \times 10^{5}$[c]
ATP	Dissociated nucleotide at mean life of oligomeric actin	0.6	12	2.0×10^5	$1.5\text{–}2.6 \times 10^{5}$[c]
ADP	Initial dissociated nucleotide	0.6	13	2.5×10^5	$2.2\text{–}2.7 \times 10^{5}$[c]
ITP	Initial dissociated nucleotide	0.6	3	2.2×10^5	$2.1\text{–}2.4 \times 10^{5}$[c]
UTP	Initial dissociated nucleotide	0.6	6	2.7×10^4	$1.6\text{–}3.8 \times 10^{4}$[c]

[a] Temperature was regulated to within ±0.4°. [b] Confidence limits were calculated based on the asymptotic relation between the log likelihood function and the χ^2 distribution (West, 1970a). [c] Confidence limits were computed as the means plus or minus two standard errors.

for ATP (Table I). The binding of UTP to cation-free G-actin was similarly estimated from the absorbance change at 263 mμ following EDTA addition using a value for the binding constant of UTP to Mg–G-actin equal to that for ADP (Iyengar and Weber, 1964). Cation-free G-actin appeared to bind UTP with an affinity constant of about 3×10^4 M^{-1} (Table I).

Discussion

The data in this communication suggest that actin retains a significant capacity to bind nucleotide in the absence of cation and that ultraviolet absorbance changes can be detected which correspond to each of several phases of change in the actin molecule following addition of EDTA. That cation is removed by EDTA is indicated by the fact that ^{45}Ca initially bound to actin chromatographed after EDTA addition at the same speed as ^{45}Ca which is not protein bound. Maruyama and Gergely (1961) also observed the rapid removal of actin-bound cation following addition of EDTA despite retention of polymerizability. Strohman and Samorodin (1962), using chromatographic techniques, found that [^{14}C]EDTA was not bound to G-actin, but their chromatographic data suggest little nucleotide binding to actin in the absence of bound cation. In contrast we found that at least 70% of the actin contained bound nucleotide following chromatography in the presence of 1 mM EDTA and 20 μM [^{14}C]ATP which indicates a binding constant of 7×10^4 M^{-1} (Mudd and Mann, 1963).

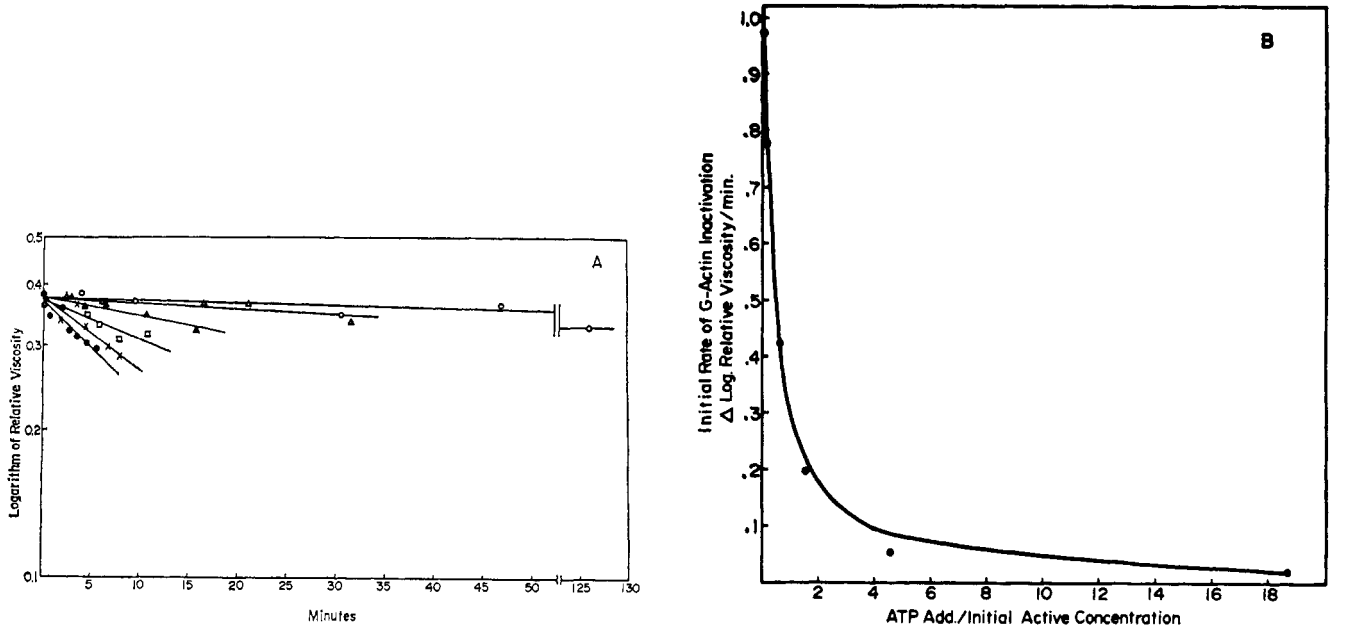

FIGURE 4: Effect of added nucleotide on rate of denaturation of ATP–G-actin in the presence of EDTA at 0°. (A) Aliquots were polymerized at the times after EDTA addition indicated on the abscissa by addition of 0.1 M KCl, 1 mM $MgCl_2$, and 0.5 mM ATP and the viscosity at 25° was determined 60 min later. Actin samples were maintained at 0° prior and for 15 min subsequent to salt addition. All solutions contained Dowex-1-pretreated ATP–G-actin which was diluted to 32 μM in 1 mM EDTA, 1 mM Tris·HCl (pH 8.0) at zero time. Solutions contained added ATP at concentrations (×, μM) of (●) 0, (×) 5, (□) 20, (▲) 50, (△) 146, and (○) 600. (B) Initial rates of cation free ATP–G-actin inactivation at 0° *vs.* the concentration of free ATP. Initial rates of inactivation were calculated from the slopes of the lines in Figure 4A expressed as logarithm of relative viscosity per minute. The abscissa indicates the ratio of the ATP and the actin concentrations. The line was the computed best fit to these points by the method of the least-mean squares and corresponds to a K_b of 3.3×10^5 M^{-1}.

This is a minimum value for the binding constant since it is assumed that all the protein in the Sephadex column is native actin whereas a portion of the actin denatured during chromatography. Measurements of the binding constant of cation-free actin for ATP utilizing absorbance changes at 256 mμ as a measure of bound nucleotide indicate a binding constant of 3.4×10^5 M^{-1}, a value in agreement with the result (2.6×10^5 M^{-1}) determined from the rate of actin inactivation in the presence of various nucleotide concentrations (Figure 4). The result appears to conflict with the conclusion that bound nucleotide is largely released following EDTA addition at the actin concentrations employed by Gergely *et al.* (1968). The presence of substantial bound nucleotide when EDTA-treated actin is polymerized by addition of 0.1 M KCl, 1 mM $MgCl_2$, and 5 μM ATP (Figure 1) does not appear attributable to rebinding of nucelotide as polymerization occurred, in view of the close correspondence of the rate of absorbance change at 256 mμ in unpolymerized preparations to the rate of decrease in actin bound nucleotide measured in polymerized preparations. In addition Seidel *et al.* (1967) found that the binding constants of G-actin and F-actin for various nucleotides were essentially the same. The use of absorbance changes at 256 mμ as a measure of loss of bound adenine nucleotide was previously suggested by West *et al.* (1967) since a new peak appeared at 249 mμ when ITP was substituted for actin-bound ADP. Similar hypochromic effects at 240–270 mμ have been observed with other proteins which bind nucleotide such as ribonuclease (Hummel *et al.*, 1961), bacterial nuclease (Cuatrecasas *et al.*, 1967), creatine kinase, hexokinase, and bovine serum albumin (J. West, unpublished data, 1969). However, a portion of the absorbance change at 256 mμ may represent effects other than nucleotide loss.

Taking the rate of absorbance change at 256 mμ as an indicator of the rate of loss of actin-bound nucleotide, it appears that, following EDTA addition to G-actin at concentrations lower than 1 mg/ml, nucleotide rapidly dissociates to arrive at a new equilibrium determined by the binding constant of the cation-free actin for nucleotide (Figure 1). The subsequent first-order absorbance decrease at 256 mμ (Figure 1) represents the loss of actin-bound nucleotide due to shifting of the equilibrium to the right as actin irreversibly loses capacity to bind nucleotide. Hence, it appears that denaturation after EDTA addition can be described by eq 1,

$$\begin{aligned}\text{CaATP–G-actin} + \text{EDTA} &\longrightarrow \text{CaEDTA} + \\ \text{ATP–G-actin} &\rightleftharpoons \text{ATP} + \text{G-actin} \longrightarrow \text{I} \quad (1)\end{aligned}$$

where I is the irreversibly denatured form of actin. Consequently the concentration of nucleotide dissociated by addition of EDTA and hence the binding constant of cation-free G-actin for ATP can be determined, as described in Methods, by extrapolating to zero time the line recording the first-order rate of absorbance decrease at 256 mμ. For G-actin preparations containing added ATP at concentrations ranging from 0.05 to 50 μM, the binding constant was found to be 3.4×10^5 M^{-1}, a value two orders of magnitude lower than the binding constant of G-actin containing bound cation for ATP (Asakura, 1961) and one-tenth the binding constant of Mg–G-actin for ADP (West, 1970a). Despite the curvature of the 256-mμ absorbance data during the 10 min following EDTA addition to ATP–G-actin at concentrations above 1 mg/ml (Figure 1) the intercept of the line fitted to the later points yields the same value for the binding constant. The linear rate of residual absorbance change obtained by subtracting the values of this line from the observed early points (Figure 1) is probably attributable to a slow disaggregation of the actin following EDTA addition with consequent retarded removal of bound cation by EDTA. As might be expected from the failure of MgADP–G-actin to polymerize at 0° (Hayashi and Rosenbluth, 1967), the early nonlinear absorbance change was generally not observed in ADP–G-actin solutions. Retarded exchange of actin bound [^{14}C]ATP was not found at actin concentrations up to 4 mg/ml, but similar studies could not be performed accurately using ^{45}Ca (Kuehl and Gergely, 1969) so it is possible that at high ATP–G-actin concentrations limited aggregation reduces exchangeability of cation but not nucleotide. Supporting this concept is the progressively smaller size, per mole of actin, of the cation-related absorbance change as ATP–actin concentration increases, a finding not observed with increasing concentrations of ADP–G-actin. Consequently the binding constant was recalculated assuming that dissociation of nucleotide from actin was retarded because of this initial process. The binding constant was found, when the extrapolation was carried out to the mean life of this process, to be 2.0×10^5 M^{-1}, which is still quite similar to the value (2.5×10^5 M^{-1}) found for ADP (Table I). According to eq 1, addition of nucleotide reduces the concentration of G-actin free of bound nucleotide and, hence, retards the rate of actin denaturation in the presence of EDTA. This effect is illustrated in Figure 4A and is the basis for the computation of the nucleotide binding constant by the actin inactivation method (West, 1970a). The result (Table I) seems to be in reasonable agreement with that obtained by the spectral technique.

Actin appears to lose polymerizability (Figure 1) at a slower rate that it loses bound nucleotide (Figure 1). The viscosity of the initial sample after EDTA addition is essentially the same as that before EDTA addition, in contrast to the appreciable decrease in bound nucleotide indicated by absorbance (Figure 1) and perchloric acid extract measurements (Figure 1), which suggest that nucleotide-free G-actin polymerizes as fully as G-actin containing bound nucleotide and hence that actin does not denature immediately following loss of bound nucleotide. Polymerization of nucleotide-free G-actin has been previously observed (Kasai *et al.*, 1965) and suggests that actin bound nucleotide has a role other than that of promoting formation of F-actin. The slower decline of viscosity compared to loss of actin bound nucleotide may be due to copolymerization of denatured actin with native actin or possibly a nonlinearity between viscosity and native actin concentration. Alternatively, a form of G-actin no longer capable of binding nucleotide may still polymerize. Since absorbance changes in the 270- to 300-mμ region are different later than in the early period following EDTA addition to G-actin (Figure 3, West *et al.*, 1968), it appears that delayed conformational changes subsequent to nucleotide removal occur which are presumably related to gradual actin denaturation.

Thus the binding constant of actin containing bound cation for ATP (Asakura, 1961) is appreciably greater than for ADP (West, 1970a) which, in turn, is greater than that of cation-free actin for ATP or ADP. Hence, it appears that, in the presence of cation, binding occurs primarily *via* the two terminal phosphates of ATP whereas binding does not appear to be mediated by the terminal phosphate when cation is absent. Despite the lower binding of ITP than ATP to Mg–G-actin (Iyengar and Weber, 1964), the data in this study indicate ITP binding in the absence of bound cation is essentially the same as that of ATP. Thus the different purine configuration of ITP may not entirely explain the reduced binding of

ITP compared to ATP when cation is present. In view of the markedly different ultraviolet absorbance of ITP–G-actin compared to ATP–G-actin (West, 1970b), which suggests altered actin conformation as well as an interaction of G-actin with the nucleotide purine ring, the decreased binding constant of Mg–G-actin for ITP may be due to steric problems secondary to altered position of the cation relative to the nucleotide. The reduced binding of UTP to G-actin, with or without bound cation, may be attributable to an even more markedly altered actin conformation.

At ATP concentrations of 0.2 mM only about 2% of the actin bound nucleotide would be released by EDTA addition assuming cation-free actin has a binding constant for ATP of about $3.4 \times 10^5\ M^{-1}$. Hence, nucleotide dissociation should not contribute substantially to the observed initial absorbance change when 0.2 mM ATP is present. Nevertheless, appreciable initial absorbance changes were observed at many wavelengths which were different in appearance from later absorbance changes attributable to loss of bound nucleotide (Figure 2). In particular, the initial absorbance changes at 271 mμ accounted for the entire absorbance change at that wavelength since there were no subsequent absorbance changes at 271 mμ (Figures 2 and 3). Since the chromatographic data suggest that bound cation is rapidly removed by EDTA, it appears that the initial absorbance change in the presence of high concentrations of ATP likely is a direct consequence of removal of bound cation. The initial absorbance changes appeared the same whether Ca or Mg was the bound cation. Since pretreatment of the actin with Dowex 50 did not alter the initial absorbance change, it seems unlikely that the effect is due to removal of cation nonspecifically bound to actin. The possibility that EDTA itself had a direct effect on actin absorbance does not seem likely since no pH changes occurred; EDTA itself does not bind to actin (Strohman and Samorodin, 1962), and EDTA two-thirds neutralized with $CaCl_2$ had no effect on G-actin absorbance. In addition EDTA had no effect on the absorbance of G-actin denatured by heating at 85° for 4 hr. The absorbance change induced by 1 mM EDTA was almost totally reversed by 0.7 mM $CaCl_2$ so that, in contrast to the conclusion of Bárány *et al.* (1962), it appears that removal of actin-bound cation is reversible. This initial change appeared to be similar in actin containing ATP or ADP except for the presence of a negative absorbance change at 300 mμ when EDTA was added to ADP–G-actin (Figure 2). A decrease in absorbance in this spectral region has been attributed to the removal of a negative charge adjacent to a tryptophyl chromophore (Ananthanarayanan and Bigelow, 1969). Thus, it appears that the conformation of ADP–G-actin is different from that of ATP–G-actin to the extent at least that a tryptophyl group is nearer a negative charge in ADP–actin, presumably due to a conformational difference between ATP– and ADP–actin. Actually, as discussed previously (West, 1970b), there are also differences between the absorbance of ADP– and ATP–G-actin at other wavelengths than 300 mμ. These changes largely are attributable to an effect of the bound nucleotide on actin although some differences in absorbance attributable to cation can be found also. When ITP or UTP is the bound nucleotide, the cation related absorbance changes are not similar to those observed with bound adenine nucleotide, perhaps because the conformation of ITP– or UTP–actin is appreciably different from that of ATP–actin (West, 1970a). The cation related absorbance changes of UTP–actin include an early absorbance peak at 300 mμ like ADP–G-actin, but unlike ATP– or ITP–G-actin. Since, in ADP–G-actin, this 300-mμ peak appears to result from conformational change due to interaction of a cation charge with the actin molecule instead of to the terminal phosphate of ATP (West, 1970b), this observation on UTP–actin suggests that cation does not bind to both terminal phosphates of UTP.

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