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Kinetics of Nucleotide and Metal Ion Interaction with G-Actin†

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ABSTRACT: The kinetics of interaction of Ca^{2+} ions and nucleotides with G-actin have been investigated by making use of the enhancement of 1,N^6-ethenoadenosine 5′-triphosphate (ϵATP) fluorescence on binding to actin, the enhancement of 2-[[2-[bis(carboxymethyl)amino]-5-methylphenoxy]methyl]-6-methoxy-8-[bis(carboxymethyl)amino]quinoline (Quin-2) fluorescence on binding to Ca^{2+}, and the sensitivity of the fluorescence of an *N*-(iodoacetyl)-*N*′-(5-sulfo-1-naphthyl)ethylenediamine (1,5-AEDANS) group on Cys-374 to metal ion binding. It is concluded that metal ion dissociation is the rate-limiting step in nucleotide dissociation (0.016 s^{-1} for Ca^{2+} at pH 7.2 and 21 °C) and that earlier conclusions that metal ion release is relatively fast and subsequent nucleotide release slow are incorrect. Results presented here and obtained by others on the metal ion concentration dependence of the effective rate of nucleotide exchange can be interpreted in the light of this conclusion in terms of a limiting rate which corresponds to that of metal ion release and an "apparent" dissociation constant for Ca^{2+} which is without direct physical significance. This apparent dissociation constant is more than 2 orders of magnitude greater than the real dissociation constant of Ca^{2+} from the Ca–actin–ATP complex, which was estimated to be 2×10^{-9} M from a titration with Quin-2. Confirmation that the rate of Ca^{2+} release is rate limiting both in nucleotide dissociation reactions and in replacement of Ca^{2+} by Mg^{2+} was obtained with 1,5-AEDANS–actin, since both the replacement of Ca^{2+} by Mg^{2+} and the removal of Ca^{2+} to give the actin–ATP complex occurred at the same (slow) rate. Metal ion exchange kinetics (Mg^{2+}/Ca^{2+}) can be explained by use of the simplest possible kinetic model of interaction of both metal ions and with appropriate rate constants for the forward and reverse rate constants of metal ion binding. In particular, the rate of dissociation of Mg^{2+} is about 1 order of magnitude lower than that for Ca^{2+}. The data obtained, together with thermodynamic considerations, lead to the conclusion that the tightly bound Ca^{2+} cannot be bound as a complex with ATP. Kinetic evidence suggesting that a metal ion–nucleotide complex is recognized in the binding reaction at relatively high metal ion concentration thus requires that this is a further metal ion, possibly one of the "weakly" bound ones.

The kinetics and thermodynamics of nucleotide and metal ion interactions with G-actin have been the subjects of numerous investigations over the past 2–3 decades [e.g., Asakura (1961), Barany et al. (1962), Kuehl and Gergely (1969), Waechter and Engel (1975, 1977), Neidl and Engel (1979), Frieden et al. (1980), and Carlier et al. (1986)]. Several years ago, a consensus of opinion appeared to have been reached which may be summarized briefly as follows. ATP can bind to G-actin either in the presence or in the absence of divalent metal ions (normally Ca^{2+} or Mg^{2+}), but its affinity is much higher in their presence. Calcium binding to the so-called high-affinity site was considered to be a relatively rapid equilibrium reaction, but the rate constant for nucleotide release, which only occurs after dissociation of metal, was thought to be very low (ca. 0.02–0.05 s^{-1}) and to be rate limiting in nucleotide exchange or dissociation processes. The latter points, in particular the apparently relatively weak binding of Ca^{2+} even to the high-affinity site (ca. 10^5–10^6 M^{-1}) and the low rate constant for the release of nucleotide, appeared to be strongly supported by experiments on the rate of nucleotide exchange as a function of free Ca^{2+} (Kuehl & Gergely, 1969; Waechter & Engel, 1975). However, very recent evidence has been interpreted to indicate that Ca^{2+} binding is much stronger than previously thought and that its rate of dissociation from its complex with G-actin–ATP is much slower than that implied by earlier investigations (Gershman et al., 1986), although this latter point was not considered to be confirmed in most recent work (Carlier et al., 1986).

In this paper, evidence is presented which leads to a rationalization of the older and newer results. In the proposed scheme, which is consistent with all the data presented here and with relevant published data, Ca^{2+} release is slow, and subsequent release of ATP from its metal-free complex with G-actin is much faster.

Materials and Methods

Protein Preparations. Actin from rabbit skeletal muscle was obtained as described earlier (Drabikowski & Nowak,

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1973). Final ATP–G-actin solutions in 0.2 mM ATP, 0.2 mM $CaCl_2$, and 2 mM Hepes[1] buffer, pH 7.6, were kept at 0 °C for no longer than 1 week.

To obtain ϵATP–G-actin, the ATP–G-actin was polymerized with 0.1 M KCl; F-actin was collected by ultracentrifugation and then depolymerized by homogenization in a Teflon/glass homogenizer in a solution of 0.2 mM ϵATP, 0.2 mM $CaCl_2$, and 2 mM Hepes buffer, pH 7.6, followed by a 24-h dialysis against the same buffer solution.

Actin was labeled with 1,5-IAEDANS as described by Carlier et al. (1986).

Fluorescence Measurements. The binding of ϵATP to actin was monitored on the basis of the large ratio of relative molar fluorescence intensities of the ϵATP–G-actin complex and free ϵATP (Miki et al., 1974). The kinetics of displacement of bound ϵATP by added ATP and dissociation of ϵATP in the presence of EDTA in excess over $CaCl_2$ were examined in a fluorescence spectrofluorometer (SLM 8000) equipped with a thermostated cell holder which was maintained at 20.0 ± 0.5 °C. Excitation and emission wavelengths were 350 and 410 nm, respectively. Stopped-flow measurements of the dissociation of the ϵATP–G-actin complex in the presence of EDTA and its reassociation upon addition of $CaCl_2$ in excess over EDTA were carried out with a Durrum D-132 three-syringe rapid-mixing apparatus equipped with a D-137 dual detector unit. The mixing dead time was 2.0 ms. Fluorescence was excited at 340 nm, and the emitted light was monitored at 90° with a filter with a transmission cut-off at 360 nm. The fluorescence signal was divided by the signal obtained from the straight-through beam to improve the signal to noise ratio. The resulting signal was registered on a Nicolet 4094 digital oscilloscope. In the reassociation experiments usually three to six traces obtained from consecutive runs of the same reaction were averaged. For evaluation, traces on chart paper were obtained from the transient recorder with an X-Y recorder. They were analyzed by fitting to a single exponential with a Nord 500/100 computer.

Fluorescence measurements on 1,5-AEDANS–actin were performed with excitation and emission wavelengths of 340 and 470 nm, respectively.

Determination of the Rate of Displacement of ϵATP by ATP. ϵATP–G-actin solution was diluted in the fluorescence cell to a final concentration of ca. 1 μM directly before the measurement. The reaction was started by addition of ATP at a final concentration of 1 mM, and the decrease in the fluorescence intensity was followed. The concentration of free ϵATP introduced with actin was in the range of 2–4 μM, i.e., 500–250 times lower than that of the added ATP. Under these conditions the reassociation of ϵATP with actin could be neglected.

Determination of the Rate of Reassociation of the ϵATP–G-Actin Complex. The determinations were performed with the three-syringe rapid-mixing apparatus described in one of the preceding sections. ϵATP–G-actin was diluted to 3.2–7.6 μM in a solution of 0.2 mM $CaCl_2$ and 4 mM Hepes buffer, pH 8.3, in one of the syringes directly before the measurements. Dissociation of the complex was initiated by mixing with an equal volume of 2 mM EDTA solution in 4 mM Hepes buffer, pH 8.3, from the second syringe. The mixture (1.6–3.8 μM actin, 0.1 mM $CaCl_2$, 1 mM EDTA, 4 mM Hepes, pH 8.3) was allowed to react in an incubation tube for 7 s. After this time, when about 60% of the initial ϵATP–G-actin complex was dissociated, it was mixed with an equal volume of a solution from the third syringe containing 2.3 mM $CaCl_2$, 4 mM Hepes, pH 7.6, and various concentrations of ϵATP, and the fluorescence signal was recorded. It was experimentally established that with the combination of buffers used the final pH during the reassociation reaction was 7.6. The dissociation step was performed at pH 8.3 to increase its rate relative to the rate of actin denaturation and thus allow for significant accumulation of nucleotide-free but still active actin [see Waechter and Engel (1977)]. The measurements were performed at room temperature (21 ± 1 °C).

In each experiment the time course of the dissociation of the initial ϵATP–G-actin complex upon mixing with EDTA was recorded on a dual-mixing system with the third syringe disconnected. From the fluorescence intensity traces the concentration of ϵATP released from the complex at the time of mixing with the $CaCl_2$ solution in the reassociation experiment was calculated by assuming that the total fluorescence change in EDTA corresponded to ϵATP equimolar to actin. The initial concentration of free ϵATP at the time of mixing with $CaCl_2$ was then obtained as the sum of free nucleotide introduced with actin, the nucleotide released from its complex with actin, and that added together with $CaCl_2$.

Determination of the Ca^{2+} Binding Constant. The affinity of Ca^{2+} for the G-actin–ATP complex was determined in the presence of 200 μM ATP by competition with Quin-2, essentially as decribed by Gershman et al. (1986). To remove excess Ca^{2+}, G-actin was treated with Dowex 50 shortly before use as described by Strzelecka-Golaszewska and Drabikowski (1967).

Determination of the Exchange of Actin-Bound Ca^{2+} with Free $^{45}Ca^{2+}$ and with $^{45}Ca^{2+}$ Bound to Dowex 50. G-Actin solution (21 μM) freed of excess Ca^{2+} by Dowex-50 treatment was divided into two portions. To one portion $^{45}CaCl_2$ was added at the final concentration of 0.1 mM. After incubation for 20, 40, 60, 120, and 180 s, 1-mL aliquots of the solution were withdrawn and stirred with equal portions of Dowex 50 for 2 min, after which the resin was removed by centrifugation. In parallel, 1-mL aliquots of the other portion of the actin solution were stirred for various time intervals with portions of Dowex 50 preloaded with $^{45}Ca^{2+}$ by a 2-min stirring with 1-mL aliquots of a 0.1 mM solution of $^{45}CaCl_2$, followed by centrifugation and removal of the supernatants (it was checked that the supernatants contained no radioactivity). The Dowex treatment of the actin solutions was terminated by removal of the resin by centrifugation. No detectable radioactivity was released to the solution when Dowex 50 loaded with $^{45}Ca^{2+}$ was stirred with the buffer solution without actin.

Other Procedures. Actin concentration was determined spectrophotometrically at 290 nm with an absorption coefficient of 0.63 $mg \cdot mL^{-1} \cdot cm^{-1}$ (Houk & Ue, 1974). Molar actin concentration was calculated with the value of 42 000 for the molecular weight of G-actin (Elzinga et al., 1973). The concentration of 1,5-AEDANS–actin was determined as described by Frieden et al. (1980). The concentration of ϵATP was determined at 265 nm with an absorption coefficient of 5700 $M^{-1} \cdot cm^{-1}$ (Secrist et al., 1972).

Reagents. ϵATP was prepared by the method of Secrist et al. (1972) and separated from unreacted ATP by chromatography on a QAE-Sephadex G-50 column. ATP (disodium salt) was purchased from Pharma-Waldhof (Düsseldorf). ADP (Pharma, Waldhof) was purified before use on a column

[1] Abbreviations: ϵATP, 1,N^6-ethenoadenosine 5′-triphosphate; 1,5-AEDANS, *N*-(iodoacetyl)-*N*′-(5-sulfo-1-naphthyl)ethylenediamine; *EGTA*, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Quin-2, 2-[[2-[bis(carboxymethyl)amino]-5-methylphenoxy]methyl]-6-methoxy-8-[bis(carboxymethyl)amino]quinoline; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

of QAE-Sephadex A-25 with a gradient of triethylammonium bicarbonate (pH 7.5). The ca. 1% ATP impurity present in all commercial samples tested was removed by this procedure, as shown by HPLC on a C_{18} reversed column (Shandon) with an acetonitrile gradient in 50 mM triethylammonium acetate (pH 7.5). In this system, ADP is eluted before ATP because of ion pair effects. Quin-2 was from Calbiochem and 1,5-IEADANS from Aldrich. Hepes was a product of Carl Roth (Karlsruhe).

Results and Discussion

Kinetics of Ca^{2+} Interaction with G-Actin–Nucleotide Complexes. Earlier analyses of Ca^{2+} and nucleotide interaction with G-actin were based on Scheme I.

Scheme I

$$A\text{–}N\text{–}M^{2+} \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} A\text{–}N + M^{2+} \quad (1)$$

$$A\text{–}N \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} A + N \quad (2)$$

Under the assumption that the first step is a rapid equilibrium and that nucleotide release from A–N is much slower (Kuehl & Gergely, 1969), the overall rate constant for exchange of the actin-bound nucleotide with free nucleotide in solution is given by (Waechter & Engel, 1975)

$$1/k_{exp} = K_1[M^{2+}]/k_{-2} + 1/k_{-2} \quad (3)$$

where $K_1 = k_1/k_{-1}$. This relationship was used to obtain values for k_{-2}, the rate constant for nucleotide release from A–N, and K_1, the association constant of metal ion to A–N, by plotting the experimental rate constant of nucleotide exchange against free-metal ion concentration. Typical values for k_{-2} for ϵATP and K_1 for Ca^{2+} were 5.5×10^{-2} s^{-1} and 7×10^5 M^{-1}, respectively (Waechter & Engel, 1975). This analysis is obviously incorrect if the first step in Scheme I is not in rapid equilibrium, as is indicated by the results recently reported by Gershman et al. (1986). In agreement with this work, by monitoring Ca^{2+} release with the fluorescent calcium chelator Quin-2 we have found that the rate of this reaction is indeed much slower than previously assumed. As reported by Gershman et al. (1986), unexpectedly high concentrations of Quin-2 are needed to compete with G-actin–ATP for Ca^{2+} binding. Competition titrations of the type reported by these authors led to a value of 4.6×10^8 M^{-1} for the affinity constant of Ca^{2+} for G-actin at pH 7.2, which is in excellent agreement with their value. This high affinity leads to difficulties in the determination of the rate of Ca^{2+} release from G-actin by competition with Quin-2 alone, since it is not possible to use such a large excess of Quin-2 that the kinetic equations simplify to the extent that only the rate of Ca^{2+} release contributes to the observed transient. The experimental situation was simplified greatly by mixing G-actin simultaneously with Quin-2 and Mg^{2+} in large excess over Ca^{2+}. The rate constant for ATP–actin at pH 7.2 was 1.57×10^{-2} s^{-1}, in good agreement with the value of 1.3×10^{-2} s^{-1} reported by Gershman et al. (1986). The corresponding value for ϵATP–G-actin was 2.51×10^{-2} s^{-1}.

Exchange of Actin-Bound Ca^{2+} with Free $^{45}Ca^{2+}$ and with $^{45}Ca^{2+}$ Bound to Dowex 50. The earlier consensus of opinion that the rate of Ca^{2+} release from G-actin is fast was based on measurements of the kinetics of exchange of G-actin-bound Ca^{2+} with radioactive $^{45}Ca^{2+}$ and the strong cation exchanger Dowex 50 to stop the exchange reaction (Barany et al., 1962). Since the results of these experiments are in direct contradiction to those obtained by the spectroscopic methods described here, we tested the possibility that exchange of Ca^{2+} between Dowex 50 and G-actin can occur. The results of the experiments are shown in Figure 1. In agreement with earlier work, when the exchange reaction was stopped by addition of Dowex 50 followed by a 2-min incubation, the exchange was complete after ca. 1 min (total time including incubation was 3 min), although there is an indication of a slow phase, albeit with small amplitude. The second curve in Figure 1 shows the results of incubating Ca–actin with Dowex 50 previously loaded with $^{45}Ca^{2+}$. It is clear that exchange occurs between the Dowex 50 bound and actin-bound Ca^{2+} with a half-life of approximately 2 min, so that in the actual exchange experiment we can expect that ca. 50% of Ca^{2+} which was still bound to actin exchanged *after* the reaction was considered to be stopped by the addition of Dowex 50. Using the manual mixing and sampling technique, it was not possible to obtain enough data at short times after mixing to test this interpretation quantitatively, i.e., to test whether the experimental results agreed with those expected using the rate of Ca^{2+} release determined by spectroscopic methods together with the rate measured for the Dowex/actin Ca^{2+} exchange reaction, but it seems that the latter reaction is fast enough to lead to the erroneous conclusion that the rate of Ca^{2+} exchange is so fast that it is essentially complete after ca. 20 s.

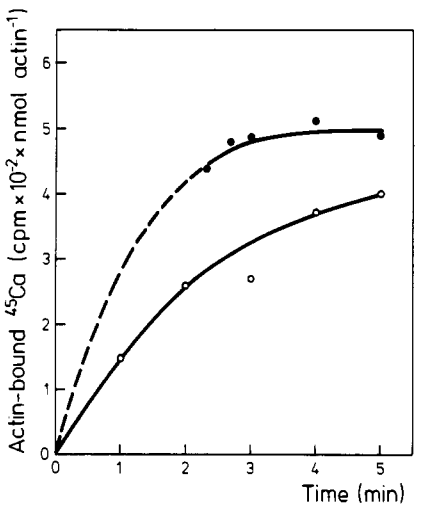


FIGURE 1: Exchange of actin-bound Ca^{2+} with free $^{45}Ca^{2+}$ and with $^{45}Ca^{2+}$ bound to Dowex 50. Experimental protocol and conditions are as described under Materials and Methods. The specific radioactivity of the supernatants is plotted against the combined time of the incubation and Dowex treatment (●) or against the time of Dowex–^{45}Ca treatment in the control experiment (○).

Kinetics of Metal Ion Release and Exchange Using AEDANS–Actin. The slow enhancement of fluorescence of 1,5-AEDANS specifically attached at Cys-374 in G-actin on replacement of Ca^{2+} by Mg^{2+} in solution under conditions where polymerization does not take place has previously been interpreted in terms of a slow isomerization of the Mg^{2+}–G-actin complex following rapid dissociation of Ca^{2+} and binding of Mg^{2+} (Frieden et al., 1980; Carlier et al., 1986). The evidence obtained with Quin-2 appears to contradict this conclusion. We therefore decided to reinvestigate the kinetics of the fluorescence changes in 1,5-IEADANS–actin on metal ion exchange or dissociation.

On addition of 0.4 mM EGTA to AEDANS–actin in the presence of 1 mM ATP at pH 7.95 and 22 °C, there was a relatively slow increase of approximately 8% in the fluorescence of the dansyl group. The magnitude and rate (0.11 s^{-1}) were not effected when the experiment was repeated in the presence of 20 μM Mg^{2+} (Figure 2a). Thus, either the fluorescence of metal-free 1,5-AEDANS-labeled actin–ATP and actin–

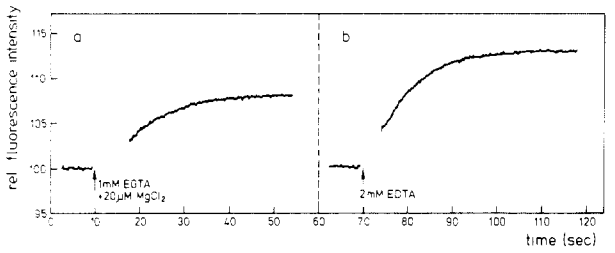


FIGURE 2: Kinetics of replacement of Ca^{2+} by Mg^{2+} (a) and of removal of Ca^{2+} (b) using the fluorescence signal of 1,5-AEDANS–actin. The rate constant is 0.11 s^{-1} in both cases. Conditions: 5 mM Tris·HCl pH 7.95, 3 μM 1,5-AEDANS–actin, 1 mM ATP, T = 21 °C, λ_{ex} = 340 nm, and λ_{em} = 470 nm.

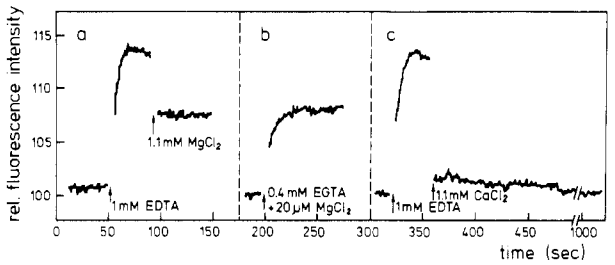


FIGURE 3: Changes of fluorescence intensities of 1,5-AEDANS–actin on interaction with Ca^{2+} and Mg^{2+}. Each experiment (a–c) starts with Ca^{2+}–1,5-AEDANS–actin. Conditions as in Figure 2.

ATP–Mg is identical, and the Ca^{2+} release is rate limiting, or actin–ATP–Mg is produced in both experiments due to the presence of Mg^{2+} impurities in the solutions. This question was not answered by attempts to remove Mg^{2+} impurities with Dowex-50 and Chelex resins, since the same result was obtained with such solutions. However, in view of the high affinity of G-actin–ATP for Mg^{2+} (Carlier et al., 1986), it is not likely that these measures would have led to a sufficiently large reduction in the contaminating Mg^{2+} concentration to allow metal-free G-actin–ATP to be produced by removal of Ca^{2+} by EGTA.

A decision between the two interpretations can be made on the basis of Figure 2b. Here, 2 mM EDTA was used to remove both Ca^{2+} and Mg^{2+} (the relatively high pH of 7.95 was needed to ensure complete complexation of Mg^{2+}), and it can be seen that the fluorescence change is larger (ca. 14%), but the rate is identical (0.11 s^{-1}) with that in the experiment of Figure 2a. The change cannot be due to actin denaturation, since this is largely prevented on this time scale by the presence of 1 mM ATP. Moreover, denaturation is accompanied by a loss of fluorescence rather than an increase (Frieden et al., 1980) and can in fact be seen as a separate slower process after the initial enhancement, as described later. Thus, it appears that the fluorescence intensity of metal-free 1,5-AEDANS-labeled actin–ATP is higher than that of actin–ATP–Mg, which is in turn higher than that of actin–ATP–Ca. This experiment leads to the conclusion that Ca^{2+} release itself is the process which occurs at 0.11 s^{-1} under these conditions. The interpretation is confirmed and extended by the experiments shown in Figure 3. In Figure 3a, metal ions were removed from actin–ATP by treatment with 1 mM EDTA, after which 1.1 mM Mg^{2+} was added. Within the mixing time (<5 s), it can be seen that the typical Mg^{2+} level is established (for a comparison, see Figure 3b in which the Mg^{2+} complex is produced directly). Thus, there is no sign of a step in the binding of Mg^{2+} which is as slow as the rate of replacement of Ca^{2+} by Mg^{2+}. In a similar experiment in Figure 3c, metal ion free actin–ATP was treated with 0.1 mM free Ca^{2+}, which results in rapid establishment of a level only slightly above the level registered before addition of EDTA. The remaining slow

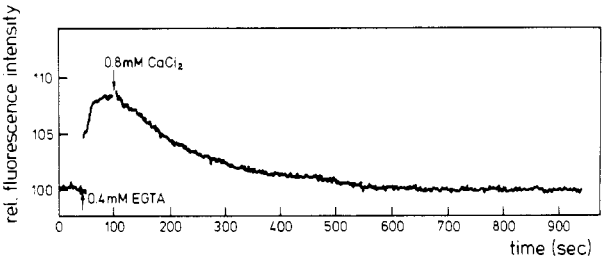


FIGURE 4: Kinetics of displacement of Mg^{2+} by Ca^{2+}. On removal of Ca^{2+} by addition of EGTA, Mg^{2+}–1,5-AEDANS–actin is formed from traces of Mg^{2+} in the solutions used. Addition of excess Ca^{2+} leads to slow displacement of Mg^{2+} and return to the original fluorescence intensity at a rate of 6.4×10^{-3} s^{-1}. Tris (free base) was added simultaneously with Ca^{2+} at the same concentration as the already present EGTA. This was shown to prevent a pH change. The rate of Ca^{2+} release was 0.18 s^{-1} under these conditions (as for Figure 2 except that the pH was 8.1).

return to the original level could be due to a small amount of Mg^{2+} bound to the actin, although this cannot be taken as proven. Supporting evidence comes from the experiment shown in Figure 4, in which Mg^{2+} is replaced by Ca^{2+}. A pH of 8.1 was used, since this led to a faster and more easily measurable rate of Mg^{2+} release. The measured rate constant was 6.4×10^{-3} s^{-1} under these conditions, compared with a value of 0.18 s^{-1} at this pH for Ca^{2+} release.

The conclusions reached here differ from those of Frieden et al. (1980) and Carlier et al. (1986) with respect to the rate of Ca^{2+} release from its complex with ATP–G-actin and the kinetics of Mg^{2+} association with ATP–G-actin. The interpretations of Carlier et al. (1986) were based largely on the kinetics of the fluorescence increase on replacement of Ca^{2+} by Mg^{2+} at varying ratios and absolute concentrations of the two metal ions. At fixed Ca^{2+} concentrations, the observed rate constant of the fluorescence change increased in an approximately hyperbolic manner (not starting at rate = 0) with increasing Mg^{2+} concentration. The authors state that this behavior cannot be explained with a model in which simple one-step binding of both metal ions occurs, with the dissociation of Ca^{2+} from G-actin being the rate-limiting step of the fluorescence change. Further, they state that in terms of this model the change in observed rate constant with increasing Mg^{2+} concentration would be in the opposite direction to that which they observed. For these reasons, in agreement with Frieden et al. (1980), a two-step binding mechanism of Mg^{2+}, i.e., rapid binding followed by a slow isomerization step, was postulated. These conclusions appear to us to be incorrect, independent of the evidence presented above showing that Ca^{2+} release is rate limiting in replacement of Ca^{2+} by Mg^{2+}. Our reasoning is presented briefly here.

Scheme II

$$\text{A–ATP–Ca} \underset{k_{+Ca}}{\overset{k_{-Ca}}{\leftrightarrow}} \text{A–ATP} \underset{k_{-Mg}}{\overset{k_{+Mg}}{\leftrightarrow}} \text{A–ATP–Mg} \qquad (4)$$

For the mechanism shown in Scheme II, it is possible to show that the observed rate constant for exchange of Ca^{2+} for Mg^{2+} (or Mg^{2+} for Ca^{2+}) is given by the following relationship (under the assumption that the effective association rates of the metal ions are much faster than their dissociation rates, which will be the case at the metal ion concentrations used):

$$k_{obsd} = \frac{k_{-Ca}}{1 + \dfrac{k_{+Ca}[Ca^{2+}]}{k_{+Mg}[Mg^{2+}]}} + \frac{k_{-Mg}}{1 + \dfrac{k_{+Mg}[Mg^{2+}]}{k_{+Ca}[Ca^{2+}]}} \qquad (5)$$

Thus if k_{-Ca} is much larger than k_{-Mg} (as indicated by results

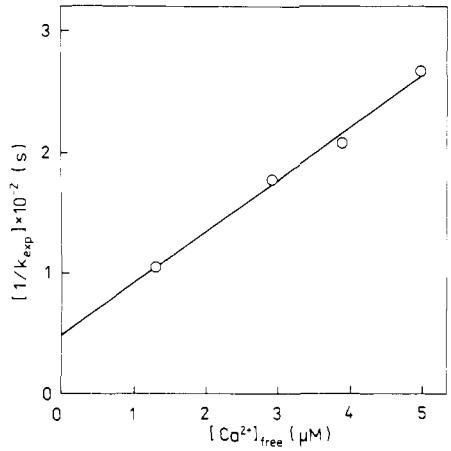


FIGURE 5: Dependence of the reciprocal of the rate constant of displacement of G-actin-bound ϵATP by ATP on the free Ca^{2+} concentration. Conditions: 0.67 μM ϵATP–G-actin, 4 mM Hepes, pH 7.6, 2 μM ϵATP, 1 mM ATP, and 20 °C. Total $[Ca^{2+}]$ was varied, and the free concentration was calculated from the Ca–ATP association constant given in the text.

described above), increasing $[Mg^{2+}]$ should cause the value of k_{obsd} to increase from a limiting value of k_{-Mg} as $[Mg^{2+}] \rightarrow 0$ in an approximately hyperbolic fashion (dominated by the second term of the equation) to reach k_{-Ca} as $[Mg^{2+}] \rightarrow \infty$. This is, qualitatively, the behavior observed by Carlier et al. (1980), and it appears that with adequate data and appropriate choices of the constants (with k_{-Ca} and k_{-Mg} independently determined as described above) the results could be rationalized in terms of the simplest scheme for the exchange reaction (Scheme II).

Kinetics of ATP Exchange with G-Actin-Bound ϵATP. Figure 5 shows, in agreement with Waechter and Engel (1975), a linear relationship between the reciprocal of the observed rate constant of displacement by ATP of ϵATP originally bound to G-actin. The concentrations of free Ca^{2+} at various total Ca^{2+} concentrations and constant ATP concentration used in our experiments were calculated from the association constant between ATP and Ca^{2+}. The latter value was redetermined under the conditions used in our experiments, making use of the quenching of ϵATP fluorescence on binding Mn^{2+} ions and the competing effect of Ca^{2+} ion, which cause no quenching. The association constant of ϵATP for Mn^{2+} was found to be 1.17×10^6 M^{-1} and that for Ca^{2+} to be 8.4×10^4 M^{-1} (pH 7.6, 5 mM Hepes, 20 °C). It was also shown that the altered structure of the nucleoside in ϵATP and ATP had no effect on the affinity for divalent metal ions. The value of 2.05×10^{-2} s^{-1} for the rate of nucleotide exchange at infinitely low Ca^{2+} concentration obtained from the ordinate intercept in Figure 5 is in reasonable agreement with earlier reported values of 5.5×10^{-2} s^{-1} (Waechter & Engel, 1975), 4.8×10^{-2} s^{-1} (Mannherz et al., 1980), and 9.4×10^{-3} s^{-1} (Strzelecka-Golaszewska et al., 1985) obtained under slightly different conditions. The reciprocal of the intercept on the abscissa, which was previously interpreted according to eq 3 to give the equilibrium association constant of Ca^{2+} binding to ϵATP–G-actin, is 8.8×10^5 M^{-1}.

In light of the evidence presented above on the rate of Ca^{2+} release from G-actin, these results must be interpreted in a different manner to that given previously. Considering the mechanism shown in Scheme I, two limiting situations could lead to the type of kinetic behavior seen. One of these is the widely accepted mechanism, i.e., that Ca^{2+} release is very rapid and nucleotide release is slow. Under these conditions, the differential equations describing the time course of dissociation

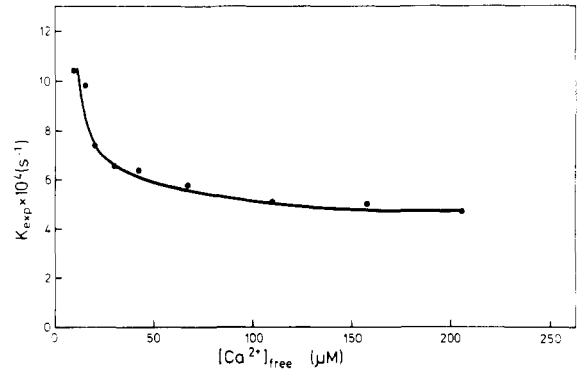


FIGURE 6: Dependence of the rate constant of displacement of ϵATP by ATP at high free $[Ca^{2+}]$. Conditions: 0.67 μM ϵATP–G-actin, 4 mM Hepes, pH 7.6, 100 μM ATP, and 21 °C.

can be solved explicitly, and the expression for the dependence of the rate of the single exponential process has the form given in eq 3. If, as is now apparent, Ca^{2+} release is slow, an interpretation based on this relationship is incorrect. However, identical kinetic behavior (i.e., simple exponential exchange kinetics and a linear dependence of the reciprocal rate constant on free Ca^{2+} concentration) can also be obtained in this case, but only under the condition that the rate of nucleotide release from its metal-free complex with actin is much faster than that of Ca^{2+}. The kinetic behavior is then described by

$$1/k_{exp} = k_1[M^{2+}]/k_{-2}k_{-1} + 1/k_{-1} \quad (6)$$

Thus, the intercept on the ordinate of a plot of $1/k_{exp}$ against $[M^{2+}]$ gives the reciprocal of the rate constant for Ca^{2+} release from the ternary complex ($k_{-1} = 2.05 \times 10^{-2}$ s^{-1}) rather than the rate of nucleotide release from the actin–nucleotide complex. This is in reasonable agreement with the value of 2.52×10^{-2} s^{-1} obtained by direct measurement of the rate of Ca^{2+} release from the G-actin–ϵATP–Ca complex with Quin-2 as described above.

The reciprocal of the intercept on the abscissa (8.8×10^5 M^{-1}) does not give the actual affinity of Ca^{2+} for the actin–nucleotide complex but the ratio k_1/k_{-2}, which is without direct physical significance. However, since the values of k_{-1} and of K_1 ($=4.6 \times 10^8$ M^{-1} from the titration of G-actin with Quin-2) are known, k_1, the rate constant for Ca^{2+} association with the G-actin–ϵATP complex, can be calculated to be 9.4×10^6 M^{-1} s^{-1}, and k_{-2} can be calculated to be 11 s^{-1} at pH 7.6.

Equation 6 is only approximately correct, since earlier studies on the ATP exchange kinetics have shown that there is a finite rate of ATP exchange even at "infinite" Ca^{2+} concentration (Kuehl & Gergely, 1969; Strzelecka-Golaszewska, 1973). In the range of Ca^{2+} concentrations which have normally been used, the approximation is apparently justified, since the plot of $1/k_{exp}$ against $[Ca^{2+}]$ is linear up to the highest concentration used. However, at higher concentrations the value of $1/k_{exp}$ tends toward a plateau. This is shown in Figure 6 in a plot of k_{exp} against $[Ca^{2+}]$, where it is apparent that the value of k_{exp} tends not toward zero but toward a definite limit of about 5×10^{-4} s^{-1}. This value represents the rate constant for ϵATP dissociation from the G-actin–ϵATP–Ca^{2+} complex *without* prior Ca^{2+} dissociation. The limiting value for the dissociation of ATP from G-actin at infinite Ca^{2+} concentration was previously reported to be about 3×10^{-4} s^{-1} at room temperature, pH 7.0 (Kuehl & Gergely, 1969), and 4×10^{-5} s^{-1} at 0 °C, pH 7.6 (Strzelecka-Golaszewska, 1973).

Metal Ion Exchange Kinetics Studied with ϵATP–Actin. Since the release of actin-bound cation is rate limiting in

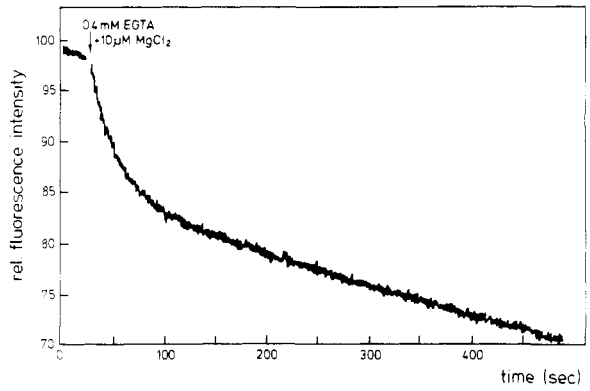


FIGURE 7: Displacement of Ca^{2+} by Mg^{2+} monitored by changes in the fluorescence of ϵATP–actin. Conditions: 1 μM ϵATP–actin, no added Ca^{2+}, <1 μM free ϵATP, 5 mM Hepes, pH 7.2, and T = 21 °C. At the time indicated by the arrow, 0.4 mM EGTA and 10 μM $MgCl_2$ were added, and the fluorescence intensity (λ_{ex} = 350 nm, λ_{em} = 410 nm) was registered. The rate constant for the faster phase on addition of EGTA/$MgCl_2$ is 0.037 s^{-1}.

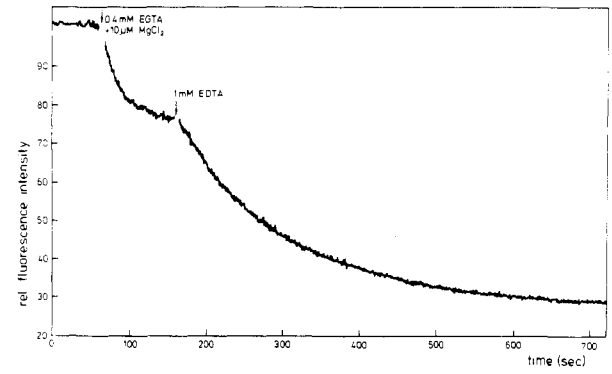


FIGURE 8: Changes in the fluorescence of ϵATP on replacement of Ca^{2+} by Mg^{2+} in ϵATP–actin, followed by removal of bound Mg^{2+}. The initial part of the curve was obtained as in Figure 7, after which 1 mM EDTA was added to study fluorescence changes on release of bound Mg^{2+}. Conditions as in Figure 7. Rate constant for Mg^{2+} release = 6.9×10^{-3} s^{-1}.

nucleotide dissociation, the difference in the rates of release of Ca^{2+} and Mg^{2+} from their respective complexes with G-actin–nucleotide species, observed above by IAEDANS fluorescence, should also be measurable with the quenching of nucleotide fluorescence which occurs on release of ϵATP in the presence of EDTA.

To measure the rate of Mg^{2+} release, Ca^{2+} must first be replaced by Mg^{2+}. As shown in Figure 7, this process appears to be associated with a loss of fluorescence. Under the conditions used (very low ϵATP concentration, ca. 1 μM, low Ca^{2+} concentration), there is slow decrease in fluorescence due to loss of bound nucleotide upon dilution of the protein to 1 μM before addition of Mg^{2+} ions. However, this can be easily distinguished from a faster phase corresponding to replacement of Ca^{2+} by Mg^{2+} when the former is removed by addition of EGTA. The rate constant of this phase is 3.7×10^{-2} s^{-1} at pH 7.2, which is identical with the value of the rate constant measured for removal of calcium (and nucleotide) by excess EDTA under the same conditions (not illustrated) and in reasonable agreement with the value obtained for Ca^{2+} release from its complex with ϵATP–G-actin by Quin-2 (2.52×10^{-2} s^{-1}). The quenching of fluorescence of bound ϵATP observed on replacing Ca^{2+} by Mg^{2+}, which was approximately 25% of the quenching seen on release of metal ion and nucleotide in the presence of EDTA, was followed by a slower phase which continues for tens of minutes and seems to correspond to denaturation-driven release of nucleotide at the low free Mg^{2+} and free nucleotide concentrations used in this experiment.

The quenching effect of bound Mg^{2+} (in comparison to Ca^{2+}) on the fluorescence intensity of actin-bound ϵATP is qualitatively similar to that observed on replacement of bound Ca^{2+} by Mn^{2+} (Loscalzo & Reed, 1976) and by Co^{2+} and Ni^{2+} (Miki & Wahl, 1985). The effects of the transition metals have been interpreted in terms of fluorescence energy transfer between the etheno group of the nucleotide and the metal ions and were taken as evidence for the proximity of the high-affinity metal binding and nucleotide binding sites. The similarity of the effects produced by the transition metal ions and by Mg^{2+} suggests that the quenching may be due to changes in structure of the nucleotide binding site causing an increased exposure to solvent rather than to direct proximity and interaction between the metal ion and the nucleotide in the ternary complex. This suggestion is supported by the observed effect of the bound cation on the local conformation around Cys-374, an amino acid relatively close in sequence to Lys-336 and Trp-356 which have been identified as the sites for covalent binding of a photoaffinity analogue of ATP to G-actin (Hegy et al., 1986).

Figure 8 shows an experiment in which the rate of Mg^{2+} release from its complex with ϵATP–G-actin is determined. In the first stage of the experiment, bound Ca^{2+} is replaced by Mg^{2+} (as in Figure 7). In the second stage, bound Mg^{2+} is removed by addition of EDTA. It can be seen that this occurs at a rate (6.9×10^{-3} s^{-1}) which is much slower than that for Ca^{2+} replacement by Mg^{2+}.

Earlier measurements of ATP exchange kinetics indicated faster dissociation of ATP from Mg–actin than from Ca–actin (Strzelecka-Golaszewska, 1973), which at first sight appears to be in contradiction to the slower dissociation of Mg^{2+} than Ca^{2+} from actin shown here, if cation release is really rate limiting for ATP dissociation. However, from eq 6 it can be shown that the ratio of observed ATP-exchange rate constants in the presence of Mg^{2+} and Ca^{2+}, respectively, is given by K_{Ca}/K_{Mg}, where K_{Ca} is the association equilibrium constant for Ca^{2+} to G-actin–ATP and K_{Mg} the corresponding constant for Mg^{2+}. Since it is well documented that Ca^{2+} has a higher affinity constant than Mg^{2+} for G-actin–ATP (Kasai & Oosawa, 1968; Strzelecka-Golaszewska & Drabikowski, 1968; Carlier et al., 1986), this means that the rate constant for ATP exchange seen in the presence of Mg^{2+} will be higher than that in the presence of Ca^{2+} by a factor equal to the ratio of these affinities. Thus, there is no contradiction between our present findings and those of Strzelecka-Golaszewska (1973).

Slower release of ATP from Mg–G-actin than from Ca–G-actin on incubation with EDTA (i.e., at very low concentrations of divalent metal ions) has been observed by Strzelecka-Golaszewska et al. (1974), although it was not explained at that time in terms of slower release of Mg^{2+}.

Kinetics of ϵATP Binding to G-Actin in the Presence of Ca^{2+}. Reassociation of ϵATP to actin in the presence of Ca^{2+} ions after transient removal of Ca^{2+} has been reinvestigated in experiments similar to those described by Waechter and Engel (1977) but at pH 7.6, for which the rate constant for ϵATP dissociation from actin, k_{-2}, has been calculated in one of the preceeding sections. The reassociation rates were measured under pseudo-first-order conditions established by using a high molar excess of free nucleotide (5–50 μM) over the transient nucleotide-free actin. The slope of a plot against free ϵATP concentration (results not shown) yields a value of 7.3×10^6 $M^{-1} \cdot s^{-1}$ for the apparent second-order rate constant of ϵATP reassociation, under the assumption that the total free nucleotide concentration (i.e., metal ion free nucleotide plus

Table I: Rate Constants for Interaction of ATP and Ca^{2+} and of ATP with G-Actin at 21 °C

method of determination	conditions	k_1 (s^{-1})	k_{-1} ($M^{-1}\cdot s^{-1}$)	k_2 (s^{-1})	k_{-2} ($M^{-1}\cdot s^{-1}$)
Quin-2 (fluorescence)	pH 7.2 ATP–actin	1.6×10^{-2}			
	ϵATP–actin	2.5×10^{-2}			
1,5-AEDANS–actin + EDTA or EGTA (fluorescence)	pH 7.95	1.1×10^{-1}			
	pH 8.10	1.8×10^{-1}			
ϵATP–actin + EDTA (fluorescence)	pH 8.3	1.5×10^{-1}			
ϵATP–actin + EGTA + $MgCl_2$ (fluorescence)	pH 7.2	3.7×10^{-2}			
ϵATP exchange kinetics according to eq 6	pH 7.6	2.1×10^{-2}			
calculation from K_1 with $k_1 = 2.1 \times 10^{-2}$ s^{-1}	pH 7.2–7.6		9.4×10^6		
calculation from k_1/k_2 ratio from eq 6	pH 7.6			11	
calculation from K_2 with $k_2 = 11$ s^{-1}					5.2×10^5 [a]
association of Ca–ϵATP with G-actin	pH 7.6				7.3×10^6 [b]

[a] This is an approximate value, since the values for K_2 and k_2 were obtained at different pH values (8.2 and 7.6, respectively). [b] This is not the value for k_2 but for the corresponding constant for the Ca–ϵATP complex.

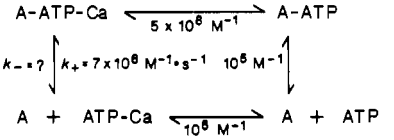
nucleotide–metal ion complex) is the relevant concentration term to use in calculating this constant. This value is in good agreement with that reported by Waechter and Engel (1977) for somewhat different conditions (6×10^6 M^{-1} at pH 8.2). The same value was obtained independent of whether the measurements were performed in the presence of 0.5 mM EDTA and 0.7 or 1.2 mM $CaCl_2$ or in the presence of 1 mM EDTA and 1.2 mM $CaCl_2$.

It is not immediately obvious from the results obtained which species associates with G-actin, i.e., whether it is the free nucleotide or the Ca–ϵATP complex. With the association constant determined as described in one of the preceding sections, the fraction of nucleotide as metal chelate is 94.1% and 98.3% at 0.2 and 0.7 mM Ca^{2+} in excess over EDTA, respectively. Thus, if free nucleotide were the species recognized, increasing the free Ca^{2+} concentration from 0.2 to 0.7 mM should result in a decrease of the observed pseudo-first-order rate constant for ϵATP association since this leads to a decrease in the free ϵATP concentration by a factor of ca. 3. As mentioned above, there was no detectable difference in the observed rates at these two values of $[Ca^{2+}]$. This is evidence in favor of Ca–ϵATP as the species recognized and bound by actin, at least at relatively high concentrations of the metal ion. However, as discussed at the end of this paper, this Ca^{2+} ion (or Mg^{2+} in the presence of $MgCl_2$) is probably not the tightly bound metal ion.

Kinetics of G-Actin Inactivation and the Affinity of ATP for Metal Ion Free G-Actin. Using the drop in fluorescence intensity of the IAEDANS group on cysteine-374 as a signal of actin denaturation (Frieden et al., 1980), we have studied the ATP concentration dependence of the rate of this process following the removal of the bound cation with EDTA, which results in a transient increase in fluorescence intensity as described above. The results (not shown) were used to calculate the association constant of ATP to metal-free actin (4.71×10^4 M^{-1} at 21 °C, pH 8.17) and k_{in}, the rate constant for denaturation of nucleotide and metal-free actin (6.45×10^{-2} s^{-1}). The presently obtained value for the inactivation rate constant, k_{in}, is almost the same as that obtained by Waechter and Engel (1977) under similar conditions but using a different procedure. The value for K_{ATP} (K_2 in Scheme I), the binding constant of ATP to metal-free G-actin, is in good agreement with the value of 2.6×10^5 M^{-1} at 0 °C and pH 8.0 obtained by West (1971) from EDTA–denaturation kinetics followed by measuring the loss of actin polymerizability. An order of magnitude higher value at 0 °C, pH 7.8, was obtained by Strzelecka-Golaszewska et al. (1985) from denaturation kinetics as a function of ATP concentration in the presence of Dowex 50.

The apparent equilibrium binding constant for ϵATP at infinite Ca^{2+} concentration, calculated from the effective rate constants obtained here for the dissociation and reassociation reaction under these conditions, is 7.3×10^6 M^{-1} $s^{-1}/5 \times 10^{-4}$ $s^{-1} = 3.6 \times 10^{11}$ M^{-1} at pH 7.6, 20 °C. Thus, it is clear that, as is generally believed, the affinity of nucleotide for metal-free G-actin is much lower than for actin with the high-affinity site saturated.

Scheme III



Conclusions

The results presented in this paper are consistent with the generally accepted mechanism (Scheme I) of nucleotide and metal ion interaction with G-actin, with the important modifications that the tightly bound metal ion exhibits a much higher affinity than determined much earlier, but in agreement with most recent estimates (Konno & Morales, 1985; Gershman et al., 1986; Carlier et al., 1986), that its rate of dissociation is very slow for Ca^{2+}, in agreement with Gershman et al. (1986) but not with Carlier et al. (1986), and that the rate of nucleotide release from metal ion free G-actin is relatively rapid, in contrast to Waechter and Engel (1975), who concluded that this was the rate-limiting step in the exchange of nucleotides. We find no evidence for a slow isomerization step in the binding of Mg^{2+} to the high-affinity sites. The rate constants obtained here for the interaction of Ca^{2+} and ϵATP/ATP with G-actin are listed in Table I.

As argued in an earlier section, it seems very likely that the rate constant for association of ϵATP with G-actin measured in the presence of Ca^{2+} is that for the association of the Ca–ϵATP complex, and not of free ϵATP. In Scheme III, the assumption has been made that the Ca^{2+} ion which binds with ATP is identical with the tightly bound Ca^{2+} ion in the final ATP–G-actin–Ca^{2+} complex.

If this scheme is correct, the value of k_-, the rate constant for release of the Ca–ATP complex from the ternary complex with actin, can be calculated, since the product of the equilibrium constant for Ca–ATP binding to actin and the equilibrium constant for binding of Ca^{2+} to ATP must be equal to the product of the known equilibrium constants for ATP and Ca^{2+} binding to actin in a stepwise fashion, i.e., $5 \times 10^8 \times 10^5 = 5 \times 10^{13} = 10^5 \times 7 \times 10^6/k_-$. From this relationship, assuming that the k_+ values for ATP and ϵATP are similar (our recent unpublished results show them to be almost indentical), k_- would be ca. 10^{-2} s^{-1}. This is in direct contradiction to the results obtained in this work and by others, from which it is apparent that the effective rate of ϵATP dissociation

is 1–2 orders of magnitude slower than this at high Ca^{2+} concentrations (Figure 6). Moreover, the observed dependence of nucleotide exchange rate on Ca^{2+} concentration would not be predicted on the basis of Scheme III if k_- were ca. 10^{-2} s^{-1}. We are thus forced to the conclusion that the Ca^{2+} ion which appears to be associated with ATP in the ATP binding reaction at relatively high Ca^{2+} concentrations is not that which is bound at the high-affinity site in G-actin. We therefore suggest that the tightly bound metal ion is spatially separated from the ATP binding site but that, at physiological concentrations of divalent metal ions, a further metal ion is bound with the ATP. This could be one of the "weakly bound" metal ions which are directly responsible for polymerization of actin. Perhaps the potential to hydrolyse ATP is only realized after binding of divalent metal ions (or monovalent ions if present at high concentrations) in analogy with all other ATPases and GTPases studied in enough detail to allow a decision on this point to be made. However, it is clear that the situation is more complex than this, since ATP is not actually hydrolyzed until actin monomers are already incorporated into the polymers (Pantaloni et al., 1985a,b).

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