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Conversion of ATP-actin to ADP-actin reverses the affinity of monomeric actin for Ca^{2+} vs Mg^{2+}

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Monomeric ATP-actin binds Ca^{2+} 3–4-times more strongly than Mg^{2+} at pH 8. On conversion of G-ATP-actin to G-ADP-actin, the relative affinity of actin for the divalent cations is reversed, so that Mg^{2+} is bound 6-times more strongly than Ca^{2+}. The dissociation rate constant of Ca^{2+} from Ca-ADP-actin is 50-fold higher than that for Ca^{2+} from Ca-ATP-actin, suggesting that this reversal of divalent cation affinities is due primarily to a higher equilibrium dissociation constant for Ca-ADP-actin. These results demonstrate an interaction between the actin-bound nucleotide and divalent cation or their binding sites.

Monomeric actin; Actin-bound divalent cation; Actin-bound nucleotide

1. INTRODUCTION

A characteristic of monomeric actin (G-actin) which has generated considerable interest since its discovery [1] is the ability of actin to bind one ATP molecule per monomeric unit with high affinity ($K_d = 10^{-10}$M) [2]. Although the actin-bound ATP is tightly bound, it will exchange with other nucleotides in solution. Direct measurements by Neidl and Engel [3] of the exchange of G-actin-bound 1:N^6 ethenoadenosine 5′-triphosphate with ATP and ADP indicate that ATP is bound to G-actin 175-fold more strongly than is ADP.

Another interesting characteristic of monomeric actin is its tight-binding site for 1 mol divalent cation per mol protein [4]. The tightly-bound cation is usually Ca^{2+} when standard, well-accepted procedures are used for the in vitro purification of monomeric actin, but Mg^{2+} is probably the tightly-bound divalent cation in vivo. We have recently reported [5] that Ca^{2+} is bound much more tightly to ATP-actin ($K_d = 1.9 \times 10^{-9}$ M) than previously thought [6] and that Mg^{2+} binds to ATP-actin about 5-times weaker than Ca^{2+} does.

Early studies of the removal of the actin-bound nucleotide and actin-bound cation by prolonged dialysis suggested that the two tight-binding sites on the protein are in close proximity [7], but more recent work has indicated that the two sites are distinct and are separated by 10 Å to as much as 30 Å [8–12]. However, though the two binding sites may be some distance apart, we have shown that exchange of the actin-bound Ca^{2+} for Mg^{2+} accelerates the release of actin-bound ATP [13], suggesting that there is an interaction between the nucleotide and divalent cation or their binding sites.

To prepare ADP-actin for polymerization kinetics studies we used this increased dissociation

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Abbreviations: Quin2, 2[2-bis-(carboxymethyl)amino-5-methyl(phenoxyl)methyl]-6-methoxy-8-bis-[carboxymethyl]amino quinoline; 8-OHQ, 8-hydroxyquinoline-5-sulfonic acid

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rate of ATP from Mg-actin [13] to shorten the preparation time of ADP-actin using the hexokinase method described by Pollard [14]. However, while attempting to exchange the actin-bound Mg^{2+} for Ca^{2+} on ADP-actin, we found it difficult to prepare homogeneous Ca-ADP-actin even at high [Ca]/[Mg] ratios. We therefore monitored the bound divalent cation during the conversion of ATP-actin to ADP-actin and found that the affinities of Ca^{2+} and Mg^{2+} for ADP-actin are significantly altered from those for ATP-actin.

2. MATERIALS AND METHODS

2.1. *Actin preparation*

Actin was extracted from rabbit skeletal muscle acetone powder [15] and purified by published procedures [16] except that the column eluent for the final column chromatography step was 0.2 mM ATP, 0.02 mM $CaCl_2$, 0.01% NaN_3 and 5 mM Hepes, pH 7.0. This monomeric actin contained ATP as bound nucleotide and Ca^{2+} as bound divalent cation (Ca-ATP-actin). The actin-bound ATP was converted to ADP by incubation with 0.3 mM glucose and 20 U/ml hexokinase [13,14] in the presence of 0.04 mM $MgCl_2$ (all final concentrations). For experiments requiring ^{45}Ca-labelled actin, purified Ca-ATP-actin in column eluent was labelled by incubation with $^{45}CaCl_2$ (final spec. act. = 5 μCi/μmol).

2.2. *Actin-bound cation analysis*

2 ml samples were polymerized by the addition of 0.1 M KCl and phalloidin equimolar with the actin. After allowing 90 min at 25°C for polymerization to occur, the samples were centrifuged at 110000 × g for 90 min. The resulting pellets were rinsed 3 × with 0.1 M KCl, and each sample was then brought to its original volume with H_2O and allowed to soften overnight at 0°C. Then, after a brief sonication to completely disperse the pellet, the protein concentrations were determined by UV absorption, actin-bound Ca^{2+} by liquid scintillation counting, and actin-bound Mg^{2+} by atomic absorption spectrophotometry.

2.3. *Actin-bound nucleotide analysis*

Actin and its bound nucleotide were separated from the reaction mixture by rapid (7–10 min) chromatography of 2.0 ml samples on a 1 cm × 20 cm Sephadex G-50 column using 0.02 mM $CaCl_2$, 0.01% NaN_3 and 5.0 mM Hepes, pH 7.0 as eluent. The peak fraction was collected and both the protein concentration and actin/nucleotide ratio were determined by UV absorption. A 0.75 ml aliquot of this fraction was removed and mixed with 0.15 ml of 30% $HClO_4$. After centrifugation to remove the precipitated protein, a 0.5 ml aliquot from the supernatant was neutralized with 0.125 ml saturated $KHCO_3$, and the resulting precipitate was removed by centrifugation. An aliquot of the supernatant was analyzed for nucleotide by HPLC using a Whatman Partisil 10/25 SAX column and 0.5 M KH_2PO_4, pH 2.8, as eluent.

2.4. *Actin-bound cation exchange kinetics*

Fluorescence measurements were made with an SLM SPF 500 C spectrofluorometer equipped with a Hi-Tech rapid kinetics accessory. The fluorescent Ca^{2+} specific chelator Quin2 was used to deter-

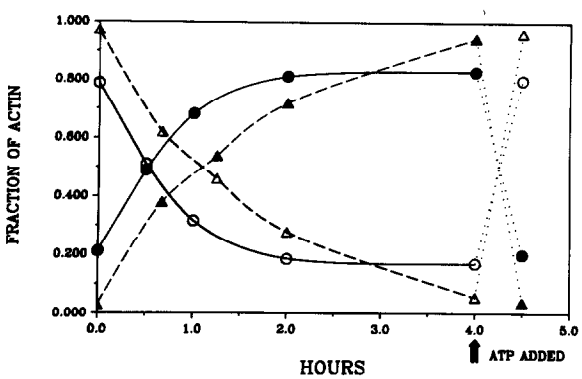

Fig.1. Time course of actin-bound cation exchange and actin-bound nucleotide exchange at pH 8. Monomeric Ca-ATP-actin (15–20 μM) was incubated with 0.04 mM $MgCl_2$ and $^{45}CaCl_2$ for 10 min at pH 8 before addition of 0.3 mM glucose and 20 U/ml hexokinase (final concentrations) at time $t = 0$. Samples were removed at the times indicated and analyzed for cations (circles) and nucleotides (triangles). After 4 h, the nucleotide and cation bound to actin were reversed by the addition of 0.3 mM ATP (final concentration) to the reaction mixture and final samples removed for analysis half an hour later. Total [Ca^{2+}], 0.039 mM; total [Mg^{2+}], 0.04 mM. ATP-actin, (△); ADP-actin, (▲); Ca-actin, (○); Mg-actin, (●).

mine rates of Ca^{2+} release from actin and the fluorescent Mg^{2+}-indicator 8-OHQ was used to measure Mg^{2+} dissociation from actin.

3. RESULTS AND DISCUSSION

Fig.1. shows the results of experiments in which the tightly-bound divalent cation of actin was monitored during conversion of ATP-actin to ADP-actin. Monomeric Ca-ATP-actin (in 0.04 mM total [Ca^{2+}]) was incubated with 0.04 mM $MgCl_2$ (final concentration) for 10 min – a period long enough for equilibration of divalent cation [17]. Initial samples were removed for assay of bound nucleotide and divalent cation. Then at time $t = 0$, 0.3 mM glucose and 20 U/ml hexokinase (final concentrations) were added to initiate conversion of ATP-actin to ADP-actin and samples were taken at intervals for analysis of bound nucleotide and divalent cation. After 4 h, when the conversion of actin-bound ATP to ADP was about 95% complete, reversibility was verified by adding excess ATP (to 0.3 mM) to re-convert the ADP-actin to ATP-actin, incubating for 30 min, and analyzing final samples for bound nucleotide and divalent cation. From the measured total and actin-bound cation concentrations and

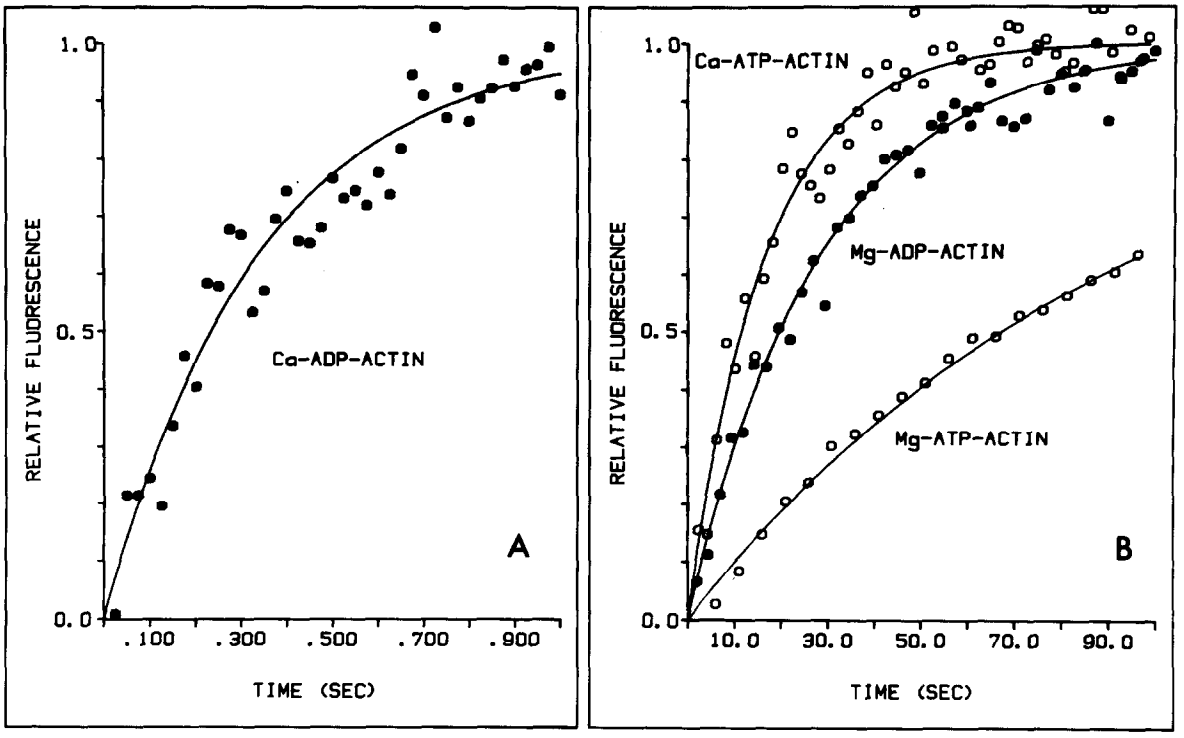

Fig.2. Dissociation of bound cation from ATP- and ADP-actin; representative time course data with experimental curve-fits. Ca^{2+} release from Ca-actin was detected with Quin2 (excitation wavelength = 340 nm; emission wavelength = 500 nm) and Mg^{2+} release from Mg-actin was detected with 8-OHQ (excitation wavelength = 327 nm; emission wavelength = 506 nm). Experiments were performed using actin as in fig.1, adjusted as noted, and diluted with an equal volume of fluorescent indicator solution containing 0.1 mM Quin2 or 8-OHQ in 5 mM Hepes, pH 8.0, 25°C, in a stopped flow apparatus. Panel A: Ca-ADP-actin, actin solution as in fig.1 at time $t = 4$ h. Panel B: Ca-ATP-actin, actin solution as in fig.1 at time $t = 50$ min. Quin2 indicator solution also contained 0.1 mM $MgCl_2$. Mg-ADP-actin, actin solution as in fig.1 at time $t = 4$ h also contained 0.5 mM EGTA. 8-OHQ indicator solution also contained $CaCl_2$ at concentrations of 0.3 to 2.4 mM. Mg-ATP-actin, actin solution as in fig.1 at time $t = 0$ except glucose concentration was 0.175 mM. 8-OHQ indicator solution also contained 0.6 mM $CaCl_2$.

the published values for the equilibrium dissociation constants for Ca^{2+} and Mg^{2+} complexed to ATP and ADP [18], the free concentrations of Mg^{2+} ($[Mg^{2+}]_f$) and Ca^{2+} ($[Ca^{2+}]_f$) were calculated. As a fortuitous result of the Mg^{2+} and Ca^{2+} binding characteristics to ATP, ADP and actin, the ratio $[Ca^{2+}]_f/[Mg^{2+}]_f$ changed little during the experiment (from 1.3 at $t = 0$ to 1.5 at 4 h). Fig.1 shows that ATP-actin was converted almost entirely to ADP-actin at the end of 4 h. The tightly-bound divalent cation, initially 80% Ca^{2+} and 20% Mg^{2+} at time $t = 0$, was exchanged during the same time period as the conversion of ATP-actin to ADP-actin, so that at the end of 4 h the actin-bound divalent cation was 20% Ca^{2+} and 80% Mg^{2+}. This indicates a reversal in the relative affinities of Ca^{2+} and Mg^{2+} for actin from a ratio of approx. 3 for ATP-actin to a ratio of approx. 0.17 for ADP-actin. Measurements after the addition of excess ATP at 4 h showed that, upon regeneration of ATP-actin, divalent cation binding reverts to the original ratio seen for ATP-actin. Thus, ATP-actin and ADP-actin have a reversed order of affinities for Ca^{2+} and Mg^{2+}.

In fig.2 are representative time course records which show the release of Ca^{2+} and Mg^{2+} from both ATP- and ADP-actin using the fluorescent indicators Quin2 and 8-OHQ to monitor cation release as described in the figure legend and elsewhere [19]. Note the hundred-fold difference in time scales between fig.2A and B. It may be seen from these records that although the dissociation rate for Mg^{2+} from ADP-actin is somewhat faster than that for ATP-actin, the dissociation rate of Ca^{2+} from ADP-actin is markedly faster than that from ATP-actin. Values for the rate constants (k_-) for dissociation of Ca^{2+} and Mg^{2+} from ATP-actin and ADP-actin were calculated from experimental curve-fits to the data records for a number of different experiments, and the average results and estimated errors are shown in table 1. The estimates for the rate constants for dissociation of Mg^{2+} (0.013 s^{-1}) and Ca^{2+} (0.06 s^{-1}) from ATP-actin agreed well with the values at pH 8 that we have recently reported [19]. The rate constant for dissociation of Ca^{2+} from ADP-actin (2.8 s^{-1}) is about 50-fold higher than that for Ca^{2+} from ATP-actin, while k_- for the dissociation of Mg^{2+} from ADP-actin (0.037 s^{-1}) is only about 3-fold higher than that for Mg^{2+} from ATP-actin. Thus, the major effect of nucleotide substitution is evidently a large decrease in the affinity of actin for Ca^{2+}.

Table 1

Summary of affinities and rate constants of Ca^{2+} and Mg^{2+} to actin

	ATP-actin	ADP-actin
Ratio of relative affinities Ca/Mg	3.5	0.17
k_- Ca (s^{-1})	0.06 ± 0.01	2.8 ± 0.4
k_- Mg (s^{-1})	0.013 ± 0.003	0.037 ± 0.005

Ratios of relative affinities from experiments described in fig.1. Dissociation rate constants from several experiments of the type described in fig.2. Errors estimated from the curve-fit procedure. k_- for Mg^{2+} dissociation from ADP-actin depended slightly (inversely) on the Ca^{2+} concentration in the 8-OHQ indicator solution; the value presented is from a linear least squares extrapolation to infinite $[Ca^{2+}]$

Using the dissociation rate constants from table 1 and equilibrium dissociation constants for Ca-ATP-actin (8 nM) and Mg-ATP-actin (28 nM) at pH 8 that we have reported [19], and if the association (forward) rate constants for Ca^{2+} and Mg^{2+} to actin are not significantly affected by the nature of the bound nucleotide, we estimate the equilibrium dissociation constants to be 375 nM for Ca-ADP-actin and 80 nM for Mg-ADP-actin. The ratio of affinities of Ca^{2+} and Mg^{2+} for ADP-actin that may be calculated from these values is 0.21, in good agreement with the ratio of affinities (0.17) measured directly in the equilibrium binding experiments of fig.1.

The reversal in the affinities of actin for Ca^{2+} and Mg^{2+} upon replacement of bound ATP with ADP results mainly from a 50-fold increase in the dissociation rate constant for Ca^{2+}. The relatively low dissociation rate constant for Mg^{2+} from either ATP- or ADP-actin may contribute to the stability of monomeric actin by retarding the formation of cation free actin, which is known to denature readily. The mechanism for these changes in affinity, and particularly for the large change in the Ca^{2+} dissociation rate constant, is not understood at present; however, the occurrence of these changes strongly implies an interaction between the actin-bound nucleotide and divalent cation or their binding sites.

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