Vol. 35, No. 1, January 1995 BIOCHEMISTRY *and* MOLECULAR BIOLOGY INTERNATIONAL
Pages 103–109

THE INFLUENCE OF CATIONS AND IONIC STRENGTH ON ACTIN POLYMERIZATION IN THE PRESENCE/ABSENCE OF α-ACTININ.

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Received October 5, 1994

SUMMARY: The presence of α-actinin has little influence on polymerizing actin with regards to the lag phase, rate and amplitude. Rising KCl or $MgCl_2$ concentrations parallel an increase in polymerization rate and a decrease in lag phase for both protein species. The observed overall smaller actin fluorescence amplitudes in the presence of α-actinin is due to a higher ratio of free G-actin to F-actin at *steady state*.

1. INTRODUCTION

α-Actinin belongs to the spectrin superfamily and is an actin binding and cross-linking protein found in both muscle and non-muscle cells [1]. It is a rod-like dimer of approx 30-40 nm length composed of two identical subunits of ~100 kDa molecular mass each with a globular head region [2]. The head piece of 27 kDa in size contains the actin binding domain [3]. It has further been demonstrated by various researchers that α-actinin is located in the cytoplasmic surface of membranes [4,5] and recent *in vitro* observation using total internal reflection fluorescence microscopy combined with photobleaching (TIRFRAP) and the film balance method, confirmed [6] that α-actinin inserts into negatively charged lipid films and binds to actin filaments at the same time.

In the present study we have examined (a) the influence of monovalent and divalent cations at different ionic strength on the lag phase and rate of actin polymerizing in the presence/absence of α-actinin, and (b) have correlated changes in fluorescence amplitude with results from DNase-I inhibition assays to determine the G/F-actin ratio at *steady state*.

1039–9712/95/010103–07$05.00/0

2. MATERIALS AND METHODS

2.1. Buffers.

F-buffer = 2 mM Tris-HCl, pH 8.0, 0.2 mM ATP, 0.2 mM DTT, 0.005% NaN_3 at 20° C and 0; 50; 100 mM KCl or 100 mM NaCl or 0; 1; 1.5; 2 mM $MgCl_2$ or 0; 0.2 mM $CaCl_2$ were used in polymerization assays.

2.2. Proteins.

α-Actinin was prepared from smooth muscle by the method of Craig et al. [7] with the modification that fresh turkey gizzard was cut into small cubes before the glycerol extraction step [8].

Actin was prepared by the method of Pardee and Spudich [9] from acetone powder obtained from rabbit back muscles followed by a gel filtration step as described by MacLean-Fletcher and Pollard [10] but using Sephacryl S-300 instead of Sephadex G-150. The G-actin peak fractions at ~1-2 mg/ml were sterile filtered and stored in G-buffer = 2 mM Tris/HCl, pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM DTT, 0.5 mM ATP at 4 °C. Actin was labelled with 7-chloro-4-nitrobenzeno-2-oxa-1,3-diazole (NBD) for polymerization assays following the protocol of Detmers et al. [11].

2.3. Polymerization assays .

Polymerization assays were carried out in a SPEX Fluorolog 1680 0.22m double spectrophotometer following the increase in fluorescence of a mixture of 85% actin plus 15% NBD-actin. Polymerization was started in the absence/presence of α-actinin by adding various buffers (see section 2.1.). Fluorescence emission and excitation was measured at 530 nm and 480 nm, respectively. Simultaneous recording of changes in fluorescence were documented on a SPEX computer DM 3000 (version 2.41) and later transferred to an Apple Macintosh LC computer and analyzed on a commercially available programme (IGOR Pro).

The lag phase of the polymerization trace was determined by the polynominal equation:

$$K_o + K_1(x - K_2) + K_3 (x - K_4)^2 \qquad (1).$$

The best fit to the lag from 0→300 sec was used to calculate the elapsed time at the lowest point of fluorescence signal, which was taken as relative measure when comparing actin and actin/α-actinin at different buffer conditions.

The rate of polymerization monitored by the change of fluorescence signal for these reactions was calculated by using the following equation:

$$A = K_1 - K_o * e^{-k * t} \qquad (2)$$

where, A is the fluorescence amplitude, K_1 is the endpoint of the fluorescence amplitude, K_o is the starting point of the fluorescent amplitude and, k is the polymerization rate constant determined from (t) ≥300 sec.

2.4. DNase -I inhibition assays.

The amount of free G-actin for various polymerization conditions in the presence/absence of α-actinin was determined by the DNase-I inhibition assay. Calf thymus DNA (80 μg/ml) was dissolved in 0.1 M Tris-HCl, pH 7.5, 4 mM $MgSO_4$

and 1.8 mM $CaCl_2$ by gentle stirring at 4° C for 24 - 48 h. The insoluble DNA was removed by centrifugation at 30.000 x g, and the supernatant was stored at -20° C. Measurements of samples were carried out in a spectrophotometer at 260 nm for 30 sec, and the results were plotted on a Mannesmann Tally printer. The linear increase in absorbance was directly proportional to the free DNase-I, and the amount of free G-actin was determined from a standard chart (0→100% inhibition of DNase-I) [12].

3. RESULTS

The use of NBD fluorescence of labelled G-actin allows the determination of polymerizing actin [13]. In a typical polymerization experiment 3 μM G-actin (15% NBD-actin and 85 % unlabelled actin) and actin/α-actinin at a molar ratio of 10:1 were mixed at various ionic conditions. Fig. 1 shows typical traces at 50 mM KCl for both species, and the inset gives the polynominal fit for the lag of actin/α-actinin at (t) 0→300 sec.

The lag phases for actin and α-actinin/actin in Fig. 2A under the various ionic conditions are similar when considering the high standard deviation (SD ±25%). However, an impressive decrease in lag is observed for both species with an

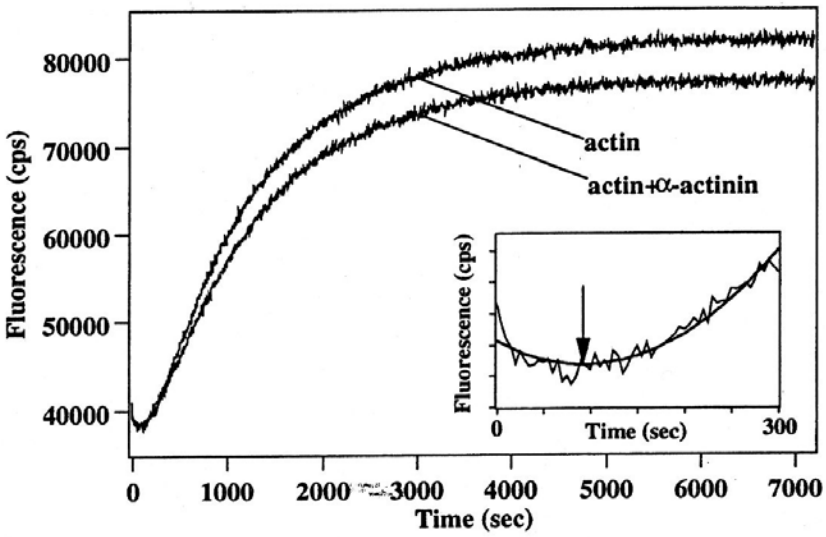

Figure 1:
Fluorescent traces of 3 μM actin polymerizing in the absence/presence of α-actinin (total of 15% NBD-actin and 85% unlabelled actin) at a molar ratio of 10:1. Inset: Determination of the lowest point of fluorescence (=lag) by the polynomial fit for actin in the presence of α-actinin. Buffer: *50 mM KCl*, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$, 1 mM EGTA, 2 mM Tris-HCl, pH 7.5, 0.2 mM ATP, 0.2 mM DTT, 0.005% NaN_3 at 20 °C.

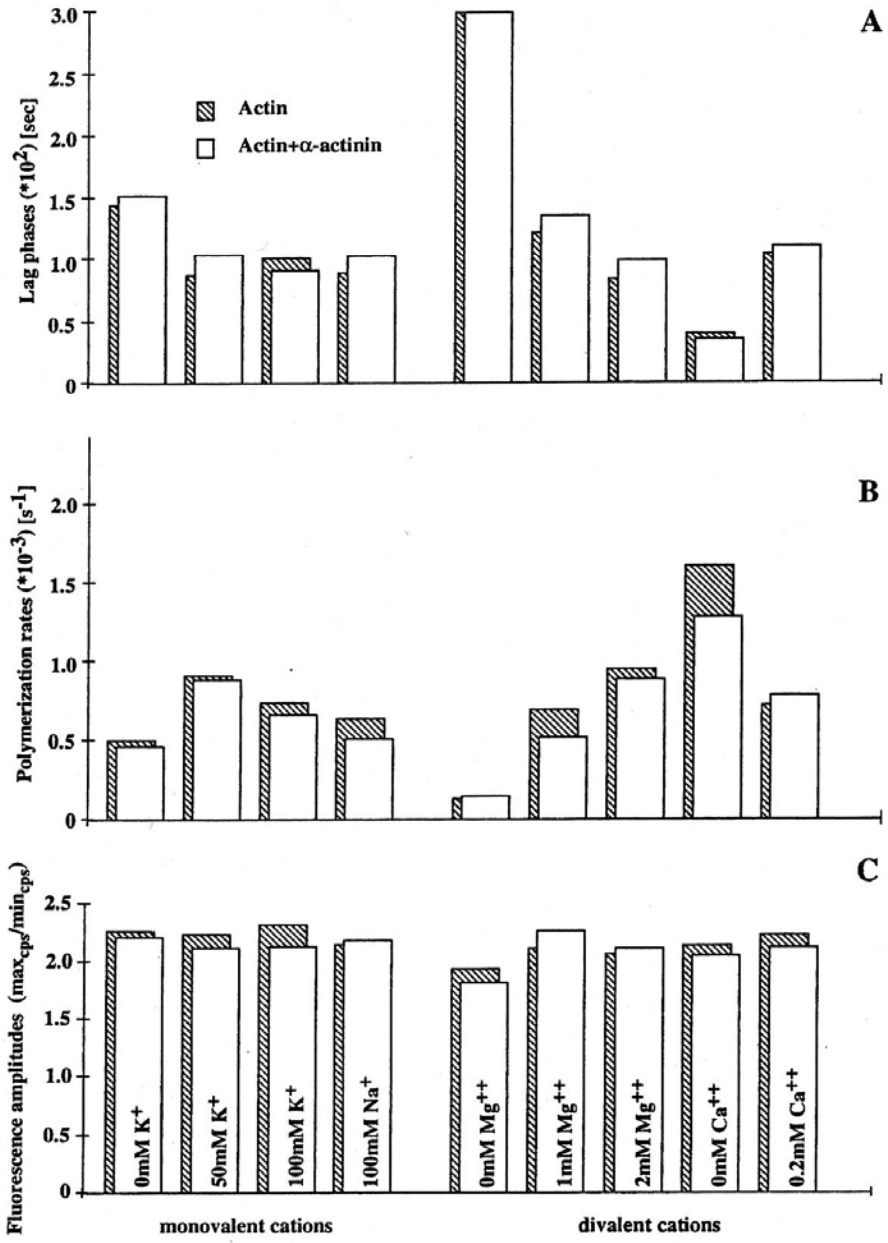

Figure 2:
Plot of fluorescent lag phases (**A**), polymerization rates measured by NBD fluorescence (**B**) and fluorescent amplitudes (**C**) *versus* different monovalent, divalent cations at various ionic strength. Standard deviation: (a) ±25 %, (b) ±20 % and (c) ±3 % (with n=3 measurements each). Experimental conditions: 3 μM actin (crossed bars) and actin/α-actinin (blank bars) at a molar ratio of 10:1. *Normal buffer:* 100 mM KCl, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$, 2 mM Tris-HCl, pH 7.5, 0.2 mM ATP, 0.2 mM DTT, 0.005% NaN_3 at 20° C; where indicated, these cations were used with other ions of the *normal buffer* kept constant. (Note: k, the polymerization rate constant was determined (t) ≥300 sec).

increase in KCl (0→100 mM) and $MgCl_2$ (0→2 mM) concentrations. The polymerization rates from (t ≥300 sec) for actin in the presence of α-actinin at various monovalent cation concentrations show a slight decrease and at different divalent cation concentrations a more pronounced change compared to pure actin (Fig. 2B). An increase in polymerization rate is observed with rising $MgCl_2$ concentrations. However, since the standard deviations are fairly high (SD ±20%), the data do not allow further interpretation. The fluorescence amplitudes for actin in the presence of α-actinin in Fig. 2C show a similar trend under these buffer conditions with the exception that divalent cations have no special influence, and the data show a smaller standard deviation (SD ±3%).

Assuming the size of the fluorescence amplitude is a measure of the amount of polymerized actin [13], then by using the highly specific DNase-I inhibition assay, the free amount of G-actin (%) could be determined at equilibrium. By deducting the free G-actin at (t) = 7200 sec from the free G-actin at (t) = 0 sec, the polymerized portion of actin (=F-actin) at *steady state* can be determined. The polymerization was started with 3 μM G-actin in the presence/absence α-actinin at a molar ratio of 10 : 1 (= 100% free G-actin at (t) = 0 sec), and measurements were carried out 2 hours thereafter. Results from these experiments - under all buffer conditions tested - showed for actin in the presence of α-actinin an average of 21% (n = 6 measurements) free G-actin (= 79% actin in polymerized form) in contrast to 15% (n = 6 measurements) free G-actin (= 85% actin in polymerized form) for pure actin. These findings are in good agreement with the overall observed reduction in the fluorescence amplitudes size at *steady state* for actin/α-actinin compared to pure actin (cf. Fig. 1).

4. DISCUSSION

Actin polymerization has been proposed as a four step mechanism: 1.) activation = salt-binding and conformational changes in monomers; 2.) nucleation = formation of oligomers; 3.) elongation = growth of polymers; and 4.) annealing = joining of filaments. All these steps are reversible and can be regulated by adding monovalent and/or divalent salts [for review, 14]. The assay form used here does not allow the evaluation of all individual reactions but is a direct kinetic study of the effects of monovalent/divalent cations on actin nucleation and elongation phases.

The nucleation is regarded as the rate-limiting step in spontaneous polymerization of actin monomers because the reaction is unfavourable with regards to its dissociation constants. The formation of nuclei is responsible for the

lag phase at the outset of polymerization. Thus, the nucleation activity can be easily changed by the addition of a nucleating protein to actin, e.g. talin [15]. In the presence of this nucleating protein actin polymerization occurs immediately, whereas in the absence of a nucleating protein (e.g. α-actinin) actin polymerization is preceded by a lag phase. When testing the nucleation activity, it is necessary to ensure that nucleation is not circumvented by spontaneous nucleation. Under the various experimental conditions the initial concentration of monomeric actin was sufficient so that during the time of the assay the spontaneous nucleation could be neglected. Assuming the time course of nucleation is expressed by the length of the lag phase, we observed a steady decrease with increasing monovalent salt for pure actin and actin in the presence of α-actinin (10:1). A similar behaviour was observed when adding magnesium chloride from 0→2 mM. However, the presence of 0.2 mM calcium chloride showed a higher value compared to no divalent salt present in both species. The influence of α-actinin on actin nucleation can be regarded as negligible since the values are within the standard deviation (SD ±25%). The significant difference in lag phase between Ca^{2+} and $Mg^{2+}/K^{+}/Na^{+}$ chloride of the two species can either be due to the binding of the added $CaCl_2$ to actin or to a conformational change of actin following its binding [16].

The elongation refers to the association and dissociation of actin molecules at the ends of filaments. At each end the rate of change of length is $\Delta l/\Delta t = k_{+1} * A_1 - k_{-1}$ where, k_{+1} is the association, k_{-1} is the dissociation rate constant and, A_1 is the critical concentration. The time course of spontaneous polymerization of monomers is sigmoidal, and at saturation the length of filaments are constant according to the relation $k_{+1} * (A_1) = k_{-1}$. The rate of actin polymerization has been investigated extensively by a number of laboratories (for review see [14]), using a fluorescent label covalently attached to actin. The NBD label has turned out useful because it changes the fluorescence intensity linearly with the concentration of polymerized actin (=F-actin) [13]. By determining the free amount of G-actin (%) by the DNase-I inhibition assay at (t) = 7200 sec and deducting it from the amount of G-actin (%) at (t) = 0 sec, the polymerized portion of F-actin was determined. Comparing these results with the observed changes in fluorescence amplitude of polymerized actin in the presence/absence of α-actinin, both experimental approaches agree reasonably well. These findings therefore support the notion that the cross-linking of α-actinin and actin probably leads to tighter actin network formation which prevents the binding of further actin monomers to the actin filaments.

Further, assuming equation (2) characterizes the elongation behaviour of actin, the presence of α-actinin influences the rates negligibly when using monovalent salt at different ionic strength. However, divalent salts show a more

pronounced effect. Increasing the magnesium chloride concentration (0→2 mM) parallels an increase in rates for both species whilst an increase in calcium chloride (0→0.2 mM) shows an adverse effect.

We thank Ms. H. Kirpal for protein preparations and Dr. M. Bärmann for stimulating discussions. This work was presented as a poster at the Biochemical Society Meeting in Canterbury (UK) and published in Biochem Soc. Trans. (1994) 22:57S in abstract form. Financial support was received from NATO (grant No. CRG 940666 to WHG).

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