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EVALUATION OF THE ACTIN FILAMENT LENGTH FROM THE TIME COURSE OF THE DEPOLYMERIZATION PROCESS

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Summary : When pyrenyl-labelled actin, at intermediate stages of polymerization, is diluted in the polymerization buffer, the decrease of fluorescence takes place stepwise through pseudo zero order reactions of decreasing rate. It is shown that the analysis of the kinetic course of the reaction allows the evaluation of the length of the actin filaments.

Formally the release of monomer (c_1) from actin filaments take place as a first order process :

$$c_{n+1} \underset{k_-}{\overset{k_+}{\rightleftharpoons}} c_1 + c_n$$

It happens, however, that the old filament (c_{n+1}) generates a new filament (c_n) so that the total filament concentration does not change. For this reason the depolymerization, which follows F-actin dilution, is expected to occur as a pseudo zero order process, which will proceed at a constant rate until the shortest filament class is depolymerized. A slower pseudo zero order reaction will then take place, corresponding to the depolymerization of the second shortest filament class. The process will go on, step by step until the total depolymerization of actin filaments. If the system is not exceedingly polydisperse, the kinetic course of actin depolymerization allows to determine the relative length distribution of F-actin filaments as well as the absolute mass of each class of filaments. If the dissociation rate constants of the two filament ends are known, also the absolute length of the filaments and the concentration of each class of filaments can be determined.

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MATERIALS AND METHODS

G-actin from rabbit muscle was prepared as in Spudich and Watt (1) and further gel filtered through Sephadex G-150 (2). Actin was kept at the concentration of 5 mg/ml in 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5mM 2-mercaptoethanol, 2 mM NaN_3, and 2 mM tris-HCl buffer, pH 8.2 (dialysis buffer). Actin concentration was measured from the absorbance at 290 nm, the absorbance of 1 mg of pure actin/ml (light path 1cm) being taken to be 0.62 (3). Alternatively, the Coomassie blue method was used (4). Molar concentration of G-actin was calculated on the basis of a molecular weight of 42,000 (5).

N-(1-pyrenyl)iodoacetamide labelled actin was prepared as previously described (6). Fluorescence measurements were performed with a Perkin Elmer MPF3 spectrofluorimeter equipped with a Linseis recorder. A cut off filter for the removal of the scattered light (wavelengths below 390 nm) was inserted in front of the photomultiplier. Excitation was at 347 nm with a 2 nm slit width. Emission was detected at 387 nm with a 6 nm slit width using the "10" sensitivity range. With this combination, bleaching of fully polymerized pyrene-actin was $<1\%$ per h, so continuous measurements could be made with ease.

RESULTS

16.8 μM pyrenyl-G-actin, which had been polymerized for 45 min at 25°C in 30 mM KCl, was made to depolymerize by 100 fold dilution in the same salt solution. As it is shown in the upper part of fig. 1, the decrease of the fluorescence, which monitors depolymerization, took place stepwise through subsequent, apparently linear, tracts. Since, after dilution, the concentration of actin is much lower than the critical concentration, which was found to be equal to 3.0 μM, depolymerization can be treated as an irreversible process, the rate of each subsequent tract of the curve being:

$$v_0 = k_- c_{f0}$$

$$v_1 = k_-(c_{f0}-c_{f1}) \qquad c_{f1} = (v_0-v_1)/k_-$$

$$v_2 = k_-(c_{f0}-c_{f1}-c_{f2}) \qquad c_{f2} = (v_1-v_2)/k_-$$

$$\cdots\cdots\cdots\cdots \qquad \cdots\cdots\cdots\cdots$$

$$v_n = k_-(c_{f0}-c_{f1}-c_{f2}\cdots-c_{fn}) \qquad c_{fn} = (v_{n-1}-v_n)/k_-$$

where $k_- = k_-$(barbed end)$+ k_-$(pointed end); c_{f0} is the total filament concentration; c_{f1}, c_{f2}, ..., c_{fn} are the concentrations

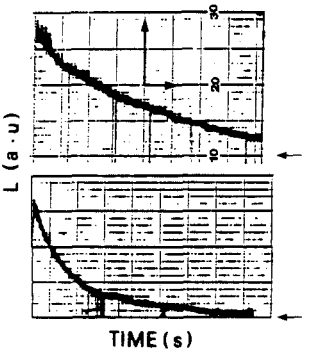


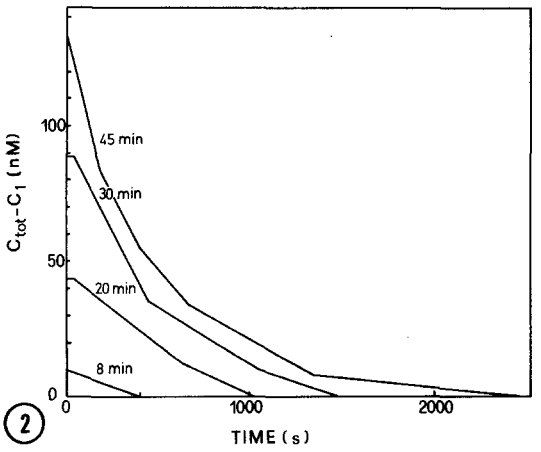
Fig. 1) The effect of fragmentation of F-actin on the fluorescence decay after dilution - Upper part of the figure : 16.8 μM pyrenyl-G-actin was polymerized at 25°C in the dialysis buffer supplemented with 30 mM KCl. After 45 min 0.02 ml of the mixture were transferred by means of a Smi digital adjusted micropipettor to the fluorimeter cuvette containing 1.98 ml of the dialysis buffer plus 30 mM KCl and the decay of the fluorescence was followed as it was described in the methods section.
Lower part of the figure : the filaments of the same polymerized actin sample were fragmented by repeated aspiration (8 times) through the needle of a 0.05 ml Terumo microsyringe; 0.02 ml of the fragmented actin sample were then transferred to the spectrofluorimeter cuvette containing 1.98 ml of the dialysis buffer plus 30 mM KCl and the decay of the fluorescence was followed. Vertical arrow : 10 arbitrary fluorescence units; horizontal arrow : 180 s; lateral arrows indicate the position of the base line.

of each single class of filaments of increasing length.
The average length of each class of filaments is given by :

$$f_1 = ak_-t_1$$
$$f_2 = ak_-(t_1+t_2)$$
$$\ldots\ldots\ldots\ldots$$
$$f_n = ak_-(t_1+t_2+ \ldots\ldots +t_n)$$

where a = 2.8 nm is the length per actin filament subunit (7) and t_1, t_2 .... and t_n are the time lengths of the subsequent linear tracts of the curve.

Fragmentation of actin filaments decreases the time of depolymerization (lower part of fig. 1) as it is expected from the increase of the filaments concentration as well as from the decrease of their average length. Furthermore, the decay of fluorescence occurs smoothly. This indicates that the system is much more polydisperse than before fragmentation.



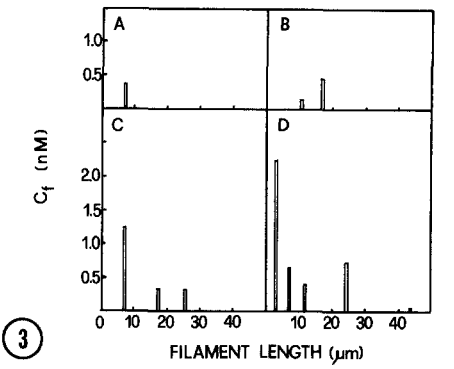


Fig. 2) Fluorescence decay, after dilution, as a function of the time of polymerization of actin - Experimental conditions were as described in the legend of the upper part of fig. 1. c_t-c_1 refers to the actual concentration of the sample in the fluorimeter cuvette after a 100 fold dilution. Polymerization times were as indicated in the figure.

Fig. 3) Filament length distribution of actin at different stages of polymerization in 30 mM KCl - Times of polymerization : 8 min (a); 20 min (b); 30 min (c) and 45 min (d). c_f refers to the filament length concentration in the polymerized sample before dilution.

The method we have proposed was applied to study the filament length distribution in the course of polymerization by 30 mM KCl. KCl was selected as the polymerizing agent because it does not allow spontaneous fragmentation of actin to occur (8-10). After 8, 20, 30, 45 and 60 min of polymerization, respectively, samples were taken and diluted 100 times in the polymerization buffer. As it is shown in fig. 2; the decrease of the fluorescence of the 8, 20, 30 and 45 min samples took place, respectively, in one, two, three and five linear tracts. The decay of the 60 min sample was smooth and did not present evident bends. For the analysis of the results (Table I and Fig. 3) a value of 6.7 s^{-1} for the $k_- = k_-$(barbed end) + k_-(pointed end), obtained under conditions (20 mM KCl) (11), very close to those of the present experiment, was selected.

TABLE I

FILAMENT LENGTH DISTRIBUTION OF ACTIN AT DIFFERENT STAGES OF POLYMERIZATION IN 30 mM KCl

Time of polymerization (min)	Reaction tract	v (pMs^{-1})	t (s)	c_f (pM)	f (μm)
8	I	24.7	405	3.7	7.6
20	I	41.0	585	1.5	10.5
	II	31.0	405	4.6	17.8
30	I	129.0	405	12.5	7.6
	II	45.0	585	3.3	17.8
	III	23.0	440	3.4	25.7
45	I	277.0	180	22.4	3.2
	II	127.0	225	6.5	7.6
	III	83.0	270	3.9	12.1
	IV	57.0	675	7.3	24.3
	V	8.0	1080	1.2	43.7

Data are taken from fig. 2. Filament concentration (c_f) is calculated by making use of a value of $k_- = 6.7\ s^{-1}$ (11)

DISCUSSION

Apparently, the method of election to determine the length of actin filaments is the direct electron microscopic observation. This method, however, suffers from some drawbacks : (a) the absolute concentration of F-actin filaments cannot be determined and the histograms of different stages of the reaction can be compared only indirectly (12); (b) artificial fragmentation of filaments during preparation of samples is likely to occur and (c) partial depolymerization certainly takes place between dilution of samples and staining on the grid. This last factor, provided total actin concentration is the same, is expected to introduce a larger error in the filament length determination of a short than of a long filament population.

The method we propose has the advantage of the extreme simplicity It allows to determine the absolute mass as well as the relative length of each class of filaments. If the dissociation constants of the two ends of the filaments are known, determination of the concentration and of the absolute length of the filaments is also possible.

Our study on actin polymerization in 30 mM KCl has revealed that, in the intermediate stages of the polymerization, filaments display a quite narrow range of lengths while, at the steady state, the system is much more polydisperse. This behaviour could be explained by the slow increase of the nuclei available for elongation. Calculations based on a value of $k_- = 6.7\ s^{-1}$ (11) have shown that filament length vary from 3 to 40 μm, a figure at least five times larger than those usually reported (12, 13). At this purpose we would like to remind that we previously described a lack of proportionality of the fluorescent signal to the polymerization process (6). Now we estimate that this source of error is low in comparison to the uncertainty of the determination of the dissociation constant but, in any case, a considerable progress can be achieved by an extensive use of our method.

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