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The Experimental Basis of Some Recent Hypotheses on the Mechanism of the Polymerization of Actin: A Reappraisal

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We have studied the effect of sonication on the fluorescence of *N*-(1-pyrenyl)iodoacetamide-labeled F-actin as well as of native actin–pyrenyl-actin mixed oligomers in which the subunits were covalently attached to each other by phenylenebismaleimide. In both cases the fluorescence of the solution was largely decreased by sonication. We have found that this effect is due (a) to a 20–30% decrease of the specific fluorescence of the polymers; (b) to the release of monomers (or other nonfluorescing species) from the polymers. These results question the validity of the novel mechanism for the polymerization of actin recently proposed (D. Pantaloni *et al.* (1984) *J. Biol. Chem.* **259,** 6274–6283). In these studies, in fact, the implicit assumption was made that the quenching of the fluorescence of the solution under sonication was due exclusively to the conversion of F-actin into G-actin.

Under suitable ionic conditions, sonication is known to promote the formation of actin oligomers from both G-actin and F-actin (1, 2). Under sonication the critical concentration of actin remains approximately the same as in the case of the ordinary G–F tranformation of actin (1). During sonic vibration actin oligomers exchange the bound nucleotide, ADP, with free ATP in the solution and, as a result of this exchange, inorganic phosphate is continuously liberated (3, 4). The continuous hydrolysis of ATP was explained by the loosening of the rigid F-actin structure caused by the vibration, which allows the exchange of the nucleotide to take place at a high rate (3). Due to the increase of the number of the filament ends, sonication enhances the ability of F-actin to act as a primer for the polymerization of G-actin (5). The new filament ends appear to be in an "activated" state since their priming activity decays rapidly, even under conditions in which either reannealing or depolymerization are excluded (2).

Recently the polymerization of actin under sonication was reinvestigated by Pantaloni *et al.* (6) and by Carlier *et al.* (7) by following the decrease of the fluorescence of *N*-(1-pyrenyl)iodoacetamide-labeled F-actin. Because the polymerization of labeled G-actin is accompanied by a large increase in fluorescence, the experimental data were interpreted with the implicit assumption that, under sonication, the fluorescence quenching is exclusively due to F-actin into G-actin conversion (i.e., to the increase of the monomer concentration). On this assumption it was claimed (a) that the critical concentration of actin depends on the number concentration of filaments (6), and (b) that the length redistribution of filaments after sonication is due to an unidimensional random walk mechanism and not to an end to end reannealing of filaments (7).

Because of the novelty of the proposal we have carefully checked its experimental design. We have now found that, under

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sonic vibration, the quenching of the fluorescence of *N*-(1-pyrenyl)iodoacetamide-labeled F-actin is mostly due to rearrangement and loosening of the polymers and not only to the increase in concentration of the monomer as assumed by Pantaloni *et al.* (6) and Carlier *et al.* (7).

EXPERIMENTAL

G-actin from rabbit muscle was prepared as in Spudich and Watt (8) and further gel filtered through Sephadex G-200 (9). Actin was kept at a concentration of 5 mg/ml in 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM 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 1 cm) being taken to be 0.62 (10). Alternatively, the Coomassie blue method was used (11). Molar concentration of G-actin was calculated on the basis of a M_r of 42,000 (12). Viscosity was measured with Ostwald viscosimeters (water flow time 60 s at 20°C).

Fluorescence measurements were performed with a Perkin-Elmer MPF3 spectrofluorometer equipped with a Linseis recorder. The exciting wavelength was 347 nm, the emission wavelength was 387 nm. A cutoff filter for the removal of the scattered light (wavelength below 390 nm) was inserted in front of the photomultiplier. The filter quenched about 20% of the light emitted at 387 nm.

Sonic vibration was performed at the lowest possible setting with a Kontes (22.5 kHz, maximal power 25W) applied directly in the optical cell placed in the thermostatted jacket of the fluorometer and fluorescence was recorded simultaneously. *N*-(1-Pyrenyl)-iodoacetamide was purchased from Molecular Probes (USA). *N*-(1-Pyrenyl)iodoacetamide-labeled actin was prepared according to Kouyama and Mihashi (13). Crosslinking of F-actin (75% native actin, 25% pyrenyl-labeled actin) with a twofold molar excess of *p-N,N'*-phenylenebismaleimide was performed according to Knight and Offer (14). Crosslinked F-actin was dialyzed 4 days at 2°C against the dialysis buffer. (After dialysis a sample of the oligomeric preparation, diluted to a concentration of 0.75 mg/ml, displayed a η_{sp} of 0.25, which increased to 0.76 after polymerization in the presence of 2 mM $MgCl_2$. Fluorescence intensity, in the absence of $MgCl_2$, was 67% of that found after full polymerization in the presence of 2 mM $MgCl_2$) (oligomers, crude preparation).

The crosslinked actin was sedimented for 15 min at 95,000 rpm in the TL100 Beckman centrifuge. The sedimented crosslinked oligomers were dissolved in the dialysis buffer, dialyzed for 24 h at 2°C against the same buffer, and employed in most of the experiments described below (oligomers, step 1 of the purification).

In some experiments a further purified oligomer preparation was employed. Oligomers (step 1 of the purification) (2 mg/ml), dissolved in the dialysis buffer were sonified for 60 s. The solution was then centrifuged for 20 min at 95,000 rpm in the TL100 centrifuge. The pellets were dissolved in the dialysis buffer supplemented with 2 mM $MgCl_2$ and then dialyzed for 24 h at 2°C against the dialysis buffer without $MgCl_2$ (oligomers, step 2 of the purification).

At all the stages of the purification the fluorescence per unit of protein weight of the oligomers polymerized in 2 mM $MgCl_2$ was approximately the same, independent of the relative amount of monomeric actin. This shows that pyrenyl-actin was not discriminated, with respect to native actin, in the incorporation into the oligomers.

The oligomer preparations were further characterized by SDS-polyacrylamide gel electrophoresis[2] (Table I) and by electron microscopy (Fig. 1).

RESULTS

Effect of Sonication on Pyrenyl-Labeled Actin Oligomers Crosslinked with p-N,N'-Phenylenebismaleimide

To test whether sonication alters the fluorescence of pyrenyl-actin filaments, we have prepared mixed (native actin-*N*-(1-pyrenyl)iodoacetamide-labeled actin) oligomers crosslinked with *p-N,N'*,-phenylenebismaleimide (see Experimental). When these oligomers (step 1 of the purification), dissolved in the dialysis buffer, are submitted to sonication, the fluorescence intensity of the solution decreases to 67% and rises to 74% 1 min after the end of the sonication. After a second sonication, the fluorescence intensity of the solution decreases to 51% and rises to 58% 2 min after the end of the sonication (Fig. 2). Centrifugation for 15 min at 95,000 rpm of the F-actin reaction mixtures, both before and after sonication, yields a supernatant solution which displays the same specific fluorescence of a G-actin solution. Furthermore, the specific fluorescence of the sedimented oligomers is always higher before sonication than after sonication (Table II).

The addition of a polymerizing agent (1 mM $MgCl_2$) to the sonicated incubation mixture restores the initial fluorescence

[2] Abbreviation used: SDS, sodium dodecyl sulfate.

TABLE I

COMPARISON OF THE RELATIVE MASS FRACTION OF OLIGOMERS IN CROSSLINKED ACTIN AT DIFFERENT STAGES OF PURIFICATION

	Crude preparation	Step I of purification	Step II of purification	Data of Knight and Offer
Monomer	67.0	52.7	26.1	29.8
Dimer	23.0	29.6	36.5	26.8
Trimer	8.5	13.4	28.0	18.9
Tetramer	1.2	3.0	7.7	11.5
Pentamer	0.25	1.3	1.7	6.27
Hexamer	—	—	—	3.4
Heptamer	—	—	—	1.7

Note. The oligomeric preparations (100 μg of protein) were submitted to 7% polyacrylamide-SDS gel electrophoresis (17). The gels were stained with Coomassie brilliant blue R-250. Densitometric scanning was performed with a Seroskop Elvi 160.

intensity. Furthermore, in the presence of $MgCl_2$, the effect of sonication on the fluorescence of the oligomers is fully reversible as happens with uncrosslinked F-pyrenyl-actin (Fig. 2).

The effect of the addition of 1 mM $MgCl_2$ to the sonicated oligomers was studied in detail. It was found that the fluorescence increase was taking place as a first-order process with a rate constant which was essentially independent of the oligomer concentration (Table III). This shows that the recovery of the fluorescence is not due to reaggregation of monomers and reannealing of oligomers but mainly to rearrangement of the crosslinked oligomers.

The Time Course of the Fluorescence Recovery after Sonication Cannot Be Reconciled with the Assumption That the Elongation Reaction Is the Main Phenomenon Taking Place

The main assumption made by Pantaloni *et al.* (6) was that the increase of fluorescence of the pyrenyl-labeled actin after the stop of sonication was due to the conversion of G-actin into F-actin, i.e., to the elongation reaction. The rate of the elongation reaction is given by

$$\text{filament concentration} \times (k_+ \times \text{G-actin concentration} - k_-)$$

thus, at any time, the rate of the fluorescence recovery should obey the same law. This, however, is not the case, as is shown in the experiment described in Fig. 2 of (6). In this experiment the change in the weight number concentration of actin filaments was measured (Fig. 2b) as well as the time course of the recovery of fluorescence (Fig. 2a) after sonication of three solutions containing, respectively, 3.75 μM (A), 7.5 μM (B), and 15 μM (C) pyrenyl-labeled actin. Inspection of Fig. 2b shows that the concentration of filaments decreases very rapidly (at least for curves B and C) but the phenomenon is not accompanied by the decrease of the rate of the fluorescence recovery, as is expected for an elongation reaction. The same data, on the contrary, are described, at least approximately, by two exponentials. The faster of these two exponentials is almost independent of the initial actin concentration (apparent first-order rate constants: 0.015 s^{-1}, 0.014 s^{-1} and 0.01 s^{-1} at 15, 7.5, and 3.75 μM actin, respectively). These rate constants are of the same order of magnitude of those found in the previous section for the fluorescence recovery after sonication.

Pyrenyl-Labeled Actin Filaments Display a Lower Specific Fluorescence Immediately after Sonication

In principle it should be possible to determine the specific fluorescence of pyrene-

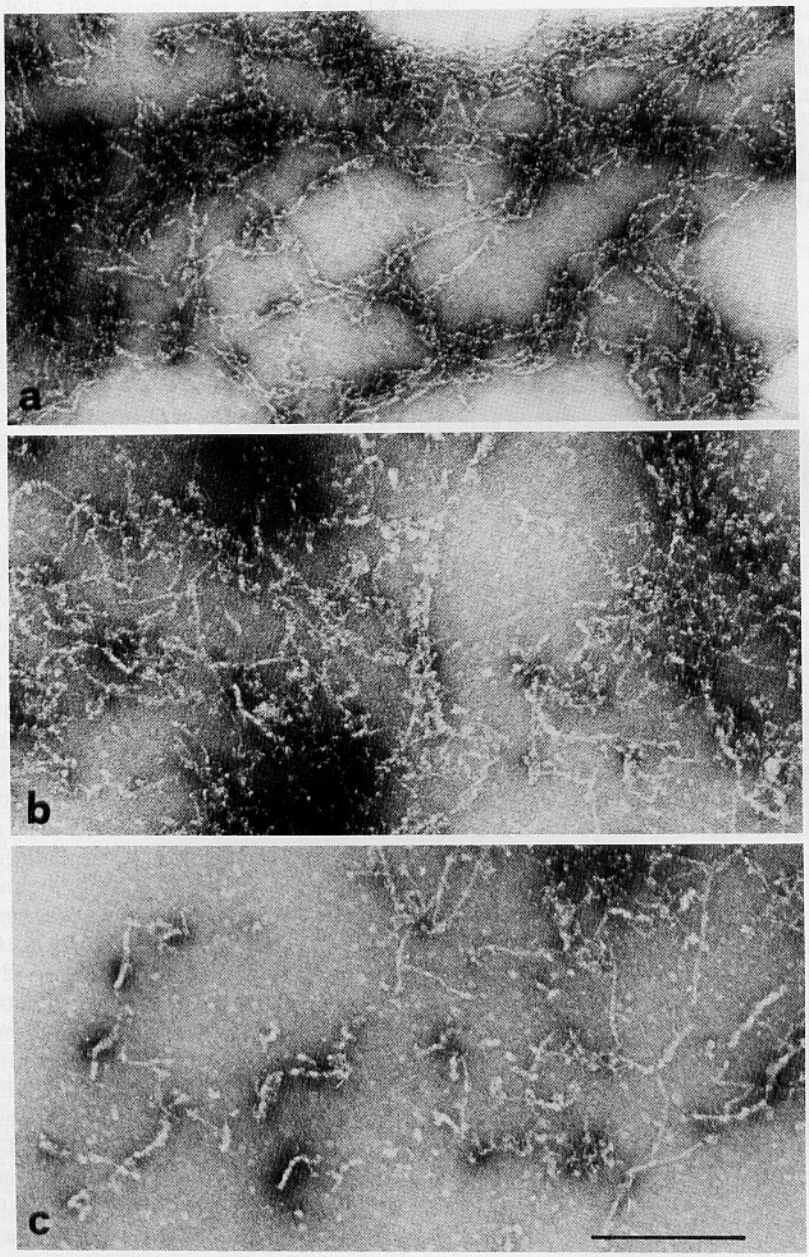

FIG. 1. Electron micrographs of the actin oligomers stained with uranyl acetate. Oligomers (100 μg of protein/ml) dissolved in the dialysis buffer were applied on a carbon-coated 400 mesh grid, washed with the dialysis buffer, and stained with 5 drops of 1% (w/v) uranyl acetate, pH 4.25. The actin solutions were transferred by a Pasteur pipet to avoid mechanical damage to the filaments. Electron microscopy was performed on a Hitachi H-800 electron microscope. Actin oligomers (a); actin oligomers sonicated twice for 60 s each time (b) and (c). The bar (shown in (c)) represents 250 nm.

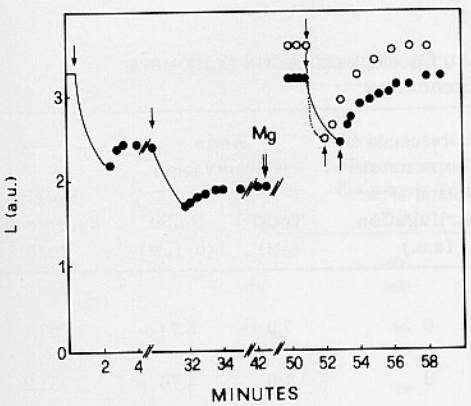

FIG. 2. Effect of sonication on the fluorescence intensity of the crosslinked (native actin-*N*-(1-pyrenyl)iodoacetamide-labeled actin) oligomers. The reaction mixtures contained crosslinked (native actin-*N*-(1-pyrenyl)iodoacetamide-labeled actin) oligomers (●) (7 μM as the monomer), dissolved in the dialysis buffer, pH 8.2, 23°C. The mixture was sonicated twice for 102 s each time, at the first and at the 29th min of the experiment (solid arrow). At the 42nd min of the experiment 1 mM $MgCl_2$ was added to the mixture (open arrow), which was then sonicated for 120 s at the 51st min of the experiment (filled arrow). Fluorescence emission was followed throughout the experiment. The effect of sonication (70 s) on the fluorescence emission of native F-actin (○) (7 μM as the monomer) in 1 mM $MgCl_2$ is also presented for comparison.

labeled actin oligomers and polymers, before and after sonication, since, once they have been sedimented by centrifugation at 95,000 rpm, the fluorescence of the supernatant solution is found to be at the baseline (i.e., to display the fluorescence of pyrenyl-labeled G-actin). Unfortunately, under the conditions employed previously (6, 7) (0.2 mM ATP, 1 mM $MgCl_2$) extensive fluorescence changes occur during the time needed to sediment the polymers. After many trials, conditions were found (38 μM ATP, 0.5 mM $MgCl_2$) under which fluorescence remains stable for about 30 min after sonication and rises to the original level after the addition of 0.2 mM ATP. When analysis of the polymers obtained under these conditions was performed, it was found that the ratio between the fluorescence and the weight of the polymer after sonication was about 25% lower than before sonication (Table IV).

DISCUSSION

p-*N*,*N*′-Phenylenebismaleimide is reported to crosslink F-actin by reacting with cysteine 373 of one monomer and with an unidentified lysyl residue of a second monomer (14). We have used a polymer formed by a mixture of native actin (75%) and of pyrenyl-labeled actin (25%). After the addition of phenylenebismaleimide, cysteine 373 of the native actin reacts rapidly so that position 373 is now modified in all the actin population. A slower crosslinking reaction then follows, which involves a lysyl residue of a neighboring subunit. When the process involves pyrenyl-labeled actin, the crosslinking comes to a stop, so that, very likely, pyrenyl-labeled actin is usually the terminal monomer of each oligomer. The large amount of monomeric actin found in our crude preparation, in comparison to that found by Knight and Offer (14), may be due to imperfect optimization of our reaction conditions; the lower average mass of the oligomers produced is, however, very likely due to the presence of pyrenyl-labeled actin, which interferes with the crosslinking process.

A faithful picture of the state of aggregation of the oligomers at low ionic strength is provided by electron microscopic observation (Fig. 1a) which shows that oligomers form long, winding filaments. After sonication, in some fields, filaments are still quite long (Fig. 1b) while, in other fields, filaments are much shorter (Fig. 1c) but, even the shortest filaments are composed by many tens of monomers.

Sonication produces a large decrease of the fluorescence of the oligomers. This phenomenon is due (a) to a 20–30% decrease of the specific fluorescence of the polymers; (b) to the release of monomers (or other nonfluorescing species) from the polymers.

These phenomena were confirmed with oligomer preparations of different composition as well as with uncrosslinked F-

TABLE II

SPECIFIC FLUORESCENCE OF PYRENYL-LABELED CROSSLINKED ACTIN OLIGOMERS BEFORE AND AFTER SONICATION

	ΔFluorescence of the sample before centrifugation (*a*) (a.u.)	ΔFluorescence of the supernatant solution after centrifugation (a.u.)	Actin concentration		Specific fluorescence (*a*/*b*)
			Total (μM)	Pellet (*b*) (μM)	
A Centrifugation before sonication	328	0	7.0	5.74	57.1
Centrifugation after the first sonication	244	0	7.0	4.76	51.2
Centrifugation after the second sonication	192	0	7.0	4.34	44.2
B Centrifugation before sonication	305	0	7.0	6.33	48.2
Centrifugation after sonication	116	0	7.0	3.22	36.0

Note. (A) From the incubation mixture of the experiment described in Fig. 2, samples were taken before sonication, after the first sonication (20th min), after the second sonication (40th min), and were centrifuged for 15 min at 95,000 rpm. The fluorescence emission and the protein content of the supernatant solutions were then determined. (B) The experiment was performed as in (A) except that oligomers (step 2 of the purification) were employed and sonication was applied for 60 s. ΔFluorescence = fluorescence of the sample − fluorescence of the original actin solution.

pyrenyl-actin. The effect of sonication is not surprising since in the course of the polymerization of actin a systematic deviation also was observed between the time course of the increase of the fluorescence of pyrenyl-labeled actin and the increase of the weight of the polymer determined directly by centrifugation (15).

TABLE III

RATE CONSTANTS OF THE RECOVERY OF THE FLUORESCENCE OF THE SONICATED OLIGOMERS AFTER THE ADDITION OF 1 mM $MgCl_2$

Crosslinked actin oligomers (μM as the monomers)	Fluorescence (% of the initial value)		Fluorescence recovery (s^{-1})
	After 3 min of sonication	After the addition of 1 mM $MgCl_2$ (final value)	
0.8	61	84	0.016
2.0	66	89	0.016
4.0	66	99	0.017

Note. Crosslinked (native actin– *N*-(l-pyrenyl)iodoacetamide-labeled actin) oligomers, at the concentrations indicated in the table, were dissolved in the dialysis buffer, pH 8.2, 23°C. The mixtures were sonicated twice for 90 s each time with a 60-s interval between the pulses. At the end of the sonication 1 mM $MgCl_2$ was added and the recovery of the fluorescence was followed as described under Experimental.

TABLE IV

SPECIFIC FLUORESCENCE OF PYRENYL-LABELED F-ACTIN BEFORE AND AFTER SONICATION

	Time of the experiment (min)	ΔFluorescence of the sample (*a*) (a.u.)	ΔFluorescence of the supernatant solution (a.u.)	Actin concentration			
				Total (μM)	Supernatant (μM)	Pellet (*b*) (μM)	Specific fluorescence (*a*/*b*)
Centrifugation before sonication	0th	260	0	13.7	1.8	11.9	21.8
	8th	260					
First centrifugation after sonication	11th	80	0	13.7	9.0	4.7	16.4
	19th	75					
Second centrifugation after sonication	24th	78	0	13.7	9.1	4.6	16.9
	32nd	78					

Note. *N*-(1-pyrenyl)iodoacetamide-labeled actin (72 μM) was polymerized with 2.56 mM $MgCl_2$. The polymerized sample was diluted with 2 mM Tris-HCl buffer, pH 8.0, to yield a final concentration of 13.7 μM actin, 38 μM ATP, 38 μM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1 mM 2-mercaptoethanol, and 0.38 mM NaN_3. Three hours after the dilution, when the new steady state had been attained, as judged from the fluorescence of the solution, the sample was submitted to sonication for 60 s; fluorescence emission was recorded simultaneously. Samples were also taken before and after sonication and centrifuged for 8 min at 95,000 rpm. Protein contents of both supernatant solutions and of the pellets (dissolved in the original volume of 2 mM Tris-HCl buffer, pH 8.0) were determined. Fluorescence was recorded exactly at the start and at the end of each centrifugation. The fluorescence of the supernatant solutions after centrifugation was also determined. ΔFluorescence = fluorescence of the polymerized samples − fluorescence of the original G-actin solutions.

At low ionic strength the effect of sonication is reversed only to a minor extent when sonication stops. This is an important observation. It shows that the filaments formed by aggregation of oligomers, which are stable for weeks, do not reanneal once they have been fragmented. Evidently, fragmentation is accompanied by the rearrangement of the filament structure in such a way to prevent reannealing. The fluorescence recovery of the oligomers, after sonication, depends on the addition of 1 mM $MgCl_2$. The process, being concentration independent, is due neither to elongation nor to reannealing (at least to a significant extent). It must be due, on the contrary, to a rearrangement of the filament structure accompanied by a fluorescence recovery.

Because of these observations we believe that the fluorescence signal cannot be safely used to monitor the process of polymerization and depolymerization in the course of sonication as was done previously (6, 7).

A further cause of perplexity about the reliability of the interpretation of the data obtained in the course of sonication comes from the work of Carlier *et al.* (16), which indicates that the pyrenyl probe attached to actin fluoresces twice as intensely in ADP-F-actin as in ATP-F-actin. To our knowledge this large difference of the fluorescence was not taken into account when the effect of sonication on the fluorescence of F-actin was studied. This represents a serious drawback since, during sonication, the rate of the nucleotide exchange on the polymer is increased and the relative proportion of ADP-F-actin and ATP-F-actin must necesssarily be different than in the polymer at rest.

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