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PREPARATION, PURIFICATION AND PROPERTIES OF A CROSSLINKED TRIMER OF G-ACTIN

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SUMMARY: Phenylenebismaleimide has been used to form crosslinks between actin monomers [Knight, P. and Offer, G. (1978) Biochem. J. 175, 1023-1032]. We have purified a trimer of actin monomers as well as a dimer and a mixture of higher molecular weight oligomers. The trimer is much more effective than the dimer in enhancing the rate of polymerization while higher oligomers do not appear to be any more effective than the trimer. A lag in the polymerization process, as measured fluorescence enhancement of trace pyrene-actin, still occurs in the presence of trimers serving as the nuclei, suggesting that the mechanism for polymerization is more complex than nucleation followed by elongation.

The minimal mechanism for the polymerization of actin is considered to be an unfavorable nucleation step followed by a highly cooperative elongation process (1). The nucleation step is necessary to explain 1) a lag in the polymerization reaction, 2) a "critical concentration" of actin below which no polymer is formed under polymerizing conditions and 3) the fact that addition of F-actin fragments markedly enhance the rate of polymerization. Investigation of the concentration dependence of the polymerization reaction has implied that the nucleation step involves the formation of an oligomer of 3-4 G-actin molecules (1-3).

One approach to this problem is to use covalently cross-linked oligomers of G-actin as a defined species in the polymerization. Knight and Offer (4) have utilized p-N,N'-phenylenebismaleimide to cross-link actin monomers and have shown that dimers and higher molecular weight oligomers can be formed. Grumet and Lin (5) showed that the cross-linked preparation could enhance polymerization of G-actin. Mockrin and Korn have isolated and partially characterized the cross-linked dimer (6) by passing the crude cross-linked

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material over a Sephadex G-200 column although the material may still contain a small fraction of monomeric actin (6).

In the present paper, we show that it is possible to prepare a purified cross-linked trimer using the same cross-linking reaction. The trimer is much more effective than dimer in enhancing the rate of polymerization. We also find that oligomers higher than trimer are not more effective than the trimer in enhancing the polymerization rate.

MATERIALS AND METHODS

Materials - Phenylenebismaleimide (Aldrich) was recrystallized from dimethylformamide by the addition of 20% (v/v) ethanol. All other reagents were of analytical grade. G-Actin was purified from rabbit skeletal muscle according to the method of Spudich and Watt (7), with the gel filtration (Sephadex G-150) modification of MacLean-Fletcher and Pollard (8). For the preparation of cross-linked actin oligomers (as described below) the final G-150 step was omitted.

Preparation of Cross-Linked Oligomers of G-Actin - G-actin (7 mg/ml), purified as above but omitting the Sephadex G-150 step, in 2 mM Tris/Cl, 0.2 mM Ca^{2+}, 0.2 mM ATP, 1.5 mM NaN_3, pH 8 [G-Buffer] was mixed with an equal volume of 5 mM Na borate, pH 9.3, containing 0.2 M KCl, 0.4 mM ATP and 2 mM Mg^{2+} (6). A 2.4 molar excess of phenylenebismaleimide was added, the mixture incubated at 25° for 10 minutes and then quenched by the addition of a 320 molar excess of β-mercaptoethanol (relative to the initial concentration of phenylenebismaleimide) according to the procedure of Knight and Offer (4). Unless noted, all subsequent steps were carried out at 4°. The cross-linked actin was centrifuged at 100,000 x g for 2 hrs, the pellet suspended in G-buffer and dialyzed against this buffer for 3 days. Residual F-actin was removed by centrifugation at 100,000 x g for 1-2 hrs. The supernatant was diluted to 1 mg/ml, made 1 mM in Mg^{2+} and incubated for 5 min at 25° and then immediately centrifuged at 4° at 100,000 x g for 2 hrs. The pellet was suspended in G-buffer to give a protein concentration of about 1 mg/ml and dialyzed for 3 days against this buffer to depolymerize the actin. After centrifugation to remove residual F-actin, the material (in 10 cc) was applied to a Sephadex G-150 column (2.5 x 45 cm). Two peaks were obtained from this column. The leading peak contained dimer, trimer and higher oligomers while the trailing peak was essentially all monomer (6).

The fractions from the leading peak were pooled, diluted to 1 mg/ml and the material repolymerized at 1 mM Mg^{2+} for 5 min as above. After resuspending the pellet, dialyzing for 3 days against G-buffer and centrifuging to remove residual F-actin, the cross-linked actin (3-4 cc at 2-3 mg/ml) was applied to the Sephadex G-150 column. Fractions of 2.4 cc were collected and samples from each fraction were analyzed by Na dodecyl sulfate gel electrophoresis (7.5% cross-linked gels). Judicious pooling of fractions of this and the previous column yielded reasonably pure dimer or trimer as well as material which contained trimer, tetramer and some higher oligomers (Fig. 1). If necessary, trimer fractions were rechromatographed on Sephadex G-150. Figure 1 shows that isolated dimer and trimer each show 2 major bands in SDS gels. This observation was also made by Mockrin and Korn for the dimer (6) who attributed it to different extents of unfolding as a consequence of intramolecular cross-links. Their conclusion is strengthened by the observation (not shown) that the monomeric G-actin from the phenylenebismaleimide-treated material also shows two bands in SDS gels.

Measurement of Actin Polymerization - Actin polymerization was followed continuously by fluorometric methods using trace amounts of pyrene-labeled actin (excitation at 365 nm, emission at 386 nm). As described elsewhere (9), this technique is a valid measure of incorporation of monomer into polymer and results in a fluorescence enhancement of about 25-fold. The fluorescence enhancement was measured on a Spex Fluorolog fluorometer using an emission/reference mode. Solutions were stirred for only 20 sec following addition of Mg^{2+} to initiate polymerization. When used, cross-linked actin was added prior to Mg^{2+} addition. In the absence of Mg^{2+}, these nuclei did not induce polymerization.

RESULTS

Figure 2 shows the time course of polymerization of G-actin in the absence and presence of cross-linked dimer or trimer. While the dimer accelerates the rate of polymerization, the trimer is much more effective. As measured by the time required to reach 50% of the maximum under these conditions (0.5 mg/ml G-actin, 1.5 mM Mg^{2+}), 2.4×10^{-9} M dimer (.2 μg/ml) enhances the rate 1.4-fold while the same molar concentration of trimer enhances it 5.5-fold. Thus, trimer is at least 4-fold more effective than dimer. Of interest is that a mixture of trimer, tetramer, pentamer (and possible higher molecular weight species) does not appear to be as effective as trimer (data not shown).

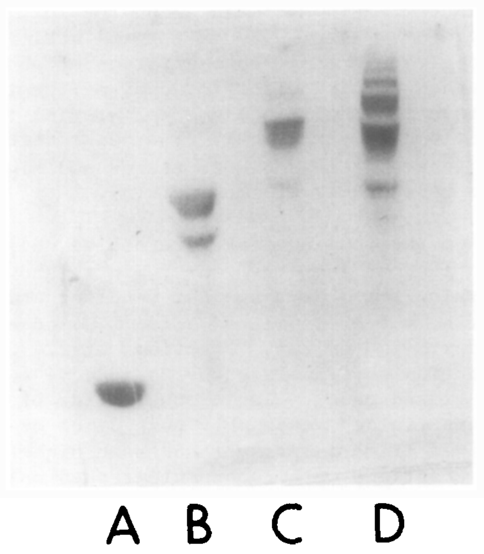
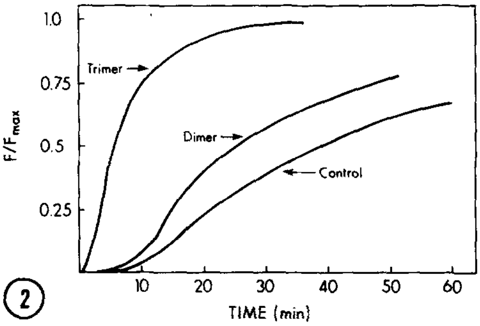


Fig. 1. SDS gels of cross-linked oligomers of G-actin. Lane A: G-actin; Lane B, dimer; Lane C, trimer; Lane D, trimer and higher molecular weight oligomers.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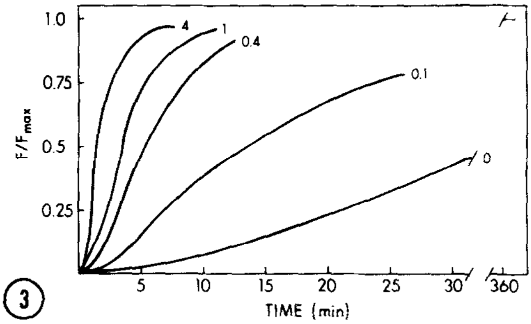
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Fig. 2. The rate of polymerization of 0.5 mg/ml G-actin using the pyrene actin fluorescence change. Conditions: pH 8, 2 mM Tris/Cl, 20° in "G-buffer" plus 1.5 mM Mg^{2+}. The curve labeled dimer contains .2 μg/ml dimer (2.4×10^{-9} M) while that labeled trimer contains an equimolar amount of trimer (.3 μg/ml).

Fig. 3 Rate of polymerization of 0.5 mg/ml G-actin induced by 1.5 mM Mg^{2+} as a function of trimer concentration. Conditions as given in legend to Fig. 2. Trimer concentrations are noted for each curve in μg/ml.

Fig. 3 shows the dependence of the rate of polymerization on trimer concentration. At the highest trimer concentration shown, 4 ug/ml (3.2×10^{-8} M), a lag in the polymerization reaction is still observed.

DISCUSSION

This paper presents a method of isolation of cross-linked trimer of G-actin. The cross-linked dimer has been previously prepared by a similar method (6). Unfortunately, the yield of trimer is much lower than that of dimer so that trimer preparation is somewhat more difficult.

With covalently prepared dimer and trimer it is possible to compare their effectiveness in enhancing the polymerization rate. While trimer is much more effective than dimer, it is of interest that higher oligomers (data not shown) do not appear to be any more effective than the trimer in enhancing polymerization. These results imply that the trimer has all the characteristics of any higher molecular weight nucleus, i.e., the trimer contains both the fast and slow growing ends. On the other hand, the dimer may only grow from one end. In this regard, differences in ATP exchange in the cross-linked dimer have been observed (6). Since larger cross-linked oligomers are no more effective than trimer, we may conclude that the trimer is the effective nucleus under these conditions of polymerization.

Another feature of the trimer-enhanced polymerization is that even when there is considerable enhancement of the rate, a lag still exists in the polymerization reaction. This result is surprising since it would be expected under these conditions that the concentration of added trimer would be considerably higher than that generated from monomer and thus that one should simply observe the elongation process which should not show a lag. One interesting explanation for this result is that during the polymerization reaction there is considerable breakage of short filaments which may in turn serve as nuclei, i.e., that there is an autocatalytic process with respect to nuclei formation. Another possibility is that the nuclei undergo an Mg^{2+}-dependent conformational change required for polymerization as we have previously observed in G-actin (10).

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