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Chemical Modification of Actin. Acceleration of Polymerization and Reduction of Network Formation by Reaction with *N*-Ethylmaleimide, (Iodoacetamido)tetramethylrhodamine, or 7-Chloro-4-nitro-2,1,3-benzoxadiazole†

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ABSTRACT: We examined the properties of rabbit skeletal muscle actin labeled at Cys-373 with *N*-ethylmaleimide or with (iodoacetamido)tetramethylrhodamine, and of *N*-ethylmaleimide–actin further modified with 7-chloro-4-nitro-2,1,3-benzoxadiazole (which primarily labels Lys-372). All three derivatives polymerize more rapidly than unlabeled actin. As measured by fluorescence photobleaching recovery and low-shear viscometry, all three also show a lower extent of network formation relative to native actin. *N*-Ethylmaleimide has a much smaller effect on the rate of polymerization and on network formation than do the other two derivatives. We suggest that chemical modification of actin with these compounds may stabilize nuclei, accounting for the acceleration of polymerization. Stabilization of nuclei also reduces the average filament length at equilibrium, thereby reducing the extent of network formation. We note a parallel between these results and the effects that cytochalasin and capping proteins have on the polymerization of actin.

The polymerization of actin involves several processes, including formation of oligomers (nuclei) from monomeric actin, elongation of the nuclei into long filaments, and end to end reactions between filaments (Oosawa & Asakura, 1975; Pollard & Craig, 1982). In addition, filaments can interact to form networks in solution (Kasai et al., 1960; Maruyama et al., 1974; Tait & Frieden, 1982b). These processes may be important in the regulation of cell motility and the structure of the cytoskeleton and can all be affected by many recently discovered proteins (Craig & Pollard, 1982; Korn, 1982; Schliwa, 1981).

Chemical modification of actin is one approach to an understanding of how these events occur. Some modifications slow or prevent polymerization. For example, Bender et al. (1976) showed that modification of actin at Tyr-53 with 5-diazonium-1*H*-tetrazole completely prevented polymerization. Actin nitrated at Tyr-69 polymerized more slowly than native actin, and attachment of a dansyl group at this residue completely prevented polymerization (Chantler & Gratzer, 1975). Carbethoxylation of several histidine residues also prevented polymerization (Hegyi et al., 1974). Until now, there have been no reports of chemically modified actins which polymerize more rapidly than native actin, nor of derivatives which have altered properties in reactions such as annealing or network formation.

There have been many studies of derivatives of actin labeled at Cys-373. All studies so far have concluded that modification of this residue does not alter the rate or extent of polymerization or other properties of the actin (e.g., Lusty & Fasold, 1969; Stone et al., 1970; Lin, 1978; Ikkai et al., 1979; Thomas et al., 1979; Wang & Taylor, 1980; Taylor et al., 1981). However, there are a few discrepancies in these results. Although Stone et al. (1970) found that modification of actin with a maleimide-based spin-label did not impair its ability to polymerize, they also said that "occasionally spin-labeled samples showed an enhanced rate of viscosity development". Wang & Taylor (1981) found that actin labeled with *N*-ethylmaleimide (MalNEt),[1] eosin–maleimide, or 5-(iodo-

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[1] Abbreviations: FPR, fluorescence photobleaching recovery; IATR, (iodoacetamido)tetramethylrhodamine; MalNEt, *N*-ethylmaleimide; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole; rhodamine–actin, actin labeled at Cys-373 with IATR; Tris, tris(hydroxymethyl)aminomethane.

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acetamido)fluorescein had a reduced low-shear viscosity compared to that of unlabeled actin, although they suggested that this effect could be due to "aging" of the actin during labeling. In earlier studies, we showed that trace quantities of rhodamine–actin did not affect the rate or extent of polymerization of unlabeled actin or its ability to form networks, but we did not rule out the possibility that rhodamine–actin might differ from unlabeled actin (Tait & Frieden, 1982a,b). Using fluorescence photobleaching recovery (FPR), which is a new technique for measuring network formation (Tait & Frieden, 1982b), we found that rhodamine–actin forms networks less well than unlabeled actin and that these effects also occurred for other labeled actin derivatives as well.

We show here that chemical modification of actin at Cys-373 with MalNEt or (iodoacetamido)tetramethylrhodamine (IATR) accelerates the rate of polymerization and partially inhibits network formation. Modification of MalNEt–actin with 7-chloro-4-nitro-2,1,3-benzoxadiazole (NBD-Cl), a reagent which primarily labels Lys-372 (Detmers et al., 1981), causes further increases in both effects. We suggest that a possible explanation for both these observations is stabilization of nuclei by chemical modification. This would result in an increased number of nuclei during polymerization, leading to faster polymerization. It would also shorten polymers, even at equilibrium. Since shorter polymers are less likely to be cross-linked to other polymers, a reduction in the average filament length would reduce the extent of network formation.

Materials and Methods

Materials. Labeling reagents were from the following sources: MalNEt, Sigma; IATR, Research Organics (Cleveland, OH); NBD-Cl, Pierce (Rockford, IL). They were used as supplied. The buffer used for all experiments ("depolymerizing buffer") contained 2 mM Tris-HCl (pH 8.0 at 25 °C), 0.2 mM $CaCl_2$, 0.2 mM ATP, and 1.5 mM NaN_3.

Preparation of Actin. Actin was prepared from rabbit skeletal muscle according to Spudich & Watt (1971), except that 0.8 M KCl was used instead of 0.6 M KCl prior to ultracentrifugation, and 2-mercaptoethanol was omitted from buffers. The actin was further purified by gel filtration on Sephadex G-150 or Sephacryl S-300 and was greater than 99% pure by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. G-Actin samples were centrifuged before use. Since the rate of polymerization and the apparent low-shear viscosity varied from one preparation to the next, we limited direct comparisons of these parameters for labeled and unlabeled actins to material which had all been made from the same actin preparation.

Labeling of Actin. Actin was usually labeled in the polymerized state (1 mg/mL, 2 mM $MgSO_4$, 100 mM KCl). The labeled F-actin was then collected by centrifugation and depolymerized by dialysis against depolymerizing buffer. The resulting labeled G-actin was centrifuged prior to use or storage. As a control, unlabeled actin was put through exactly the same procedure except that the modifying reagent was not added; this actin will be referred to as sham-reacted actin. The recovery of actin from the labeling reactions was usually 70–90%. Concentrations of rhodamine–actin and NBD–MalNEt–actin were determined by the Bradford (1976) assay, with unlabeled actin as the standard.

MalNEt–actin was prepared by incubation of F-actin with 2 mol of MalNEt (dissolved in ethanol) per mol of actin monomer for 1 h at room temperature. It is well established that MalNEt modifies Cys-373 preferentially and quantitatively in both G- and F-actins (Elzinga & Collins, 1975; Bender et al., 1976; Detmers et al., 1981).

NBD–MalNEt–actin was prepared from MalNEt–actin according to Detmers et al. (1981). MalNEt–actin (1 mg/mL) was incubated with 0.4 mM NBD-Cl at 15 °C for 5 h in the standard buffer plus 2 mM $MgSO_4$ and 100 mM KCl. Detmer et al. (1981) showed that under these conditions about 60% of the NBD was attached to Lys-372, with the remainder distributed over several other lysine residues. The extent of labeling was determined spectrophotometrically, by using $E_{480} = 2.6 \times 10^4\ M^{-1}\ cm^{-1}$ which Aboderin et al. (1973) determined for NBD–lysine. Most preparations contained about 0.5 mol of NBD per mol of actin. The sham-reacted actin used as a control for experiments with NBD–MalNEt–actin underwent two cycles of polymerization and depolymerization to simulate the effect of first preparing MalNEt–actin as the starting material for labeling with NBD-Cl.

Rhodamine–actin was prepared as described (Tait & Frieden, 1982a), and the extent of labeling was determined by using $E_{554} = 2.3 \times 10^4\ M^{-1}\ cm^{-1}$. Most preparations contained about 0.5 mol of rhodamine per mol of actin. Prior labeling of actin with MalNEt prevents labeling with IATR, suggesting that IATR labels Cys-373 (Tait & Frieden, 1982a). Takashi (1979) has shown directly that a similar iodoacetamide-based fluorescent label (*N*-[[(iodoacetyl)amino]-ethyl]-5-naphthylamine-1-sulfonic acid) labels Cys-373, and Sleigh & Burley (1973) showed that an iodoacetamide-based spin-label also labels Cys-373.

Fluorometry. Static fluorescence intensity during polymerization was measured with a Spex Fluorolog fluorometer equipped with a 150-W xenon arc lamp and operated in the ratio mode to compensate for fluctuations in illumination intensity. Measurements were made at 20 °C in a 3-mL quartz cuvette; samples were stirred with a rotating Teflon stirrer for 30 s at the beginning of polymerization. For rhodamine–actin, the excitation wavelength was 555 nm and the emission wavelength 570 nm; for NBD–MalNEt–actin, the corresponding values were 480 and 520 nm. There is no contribution of light scattering to the apparent fluorescence change under the conditions we used. Fluorescence was recorded continuously during polymerization, and no detectable bleaching occurred. The half-time for polymerization is defined as the time required for the fluorescence enhancement to reach half the value achieved upon completion of polymerization. In the figure legends, the concentration of fluorescent actin is given in terms of the total concentration of labeled actin added; the actual concentration of labeled actin molecules is about half the indicated value because rhodamine–actin and NBD–MalNEt–actin contain about 0.5 mol of fluorophore per mol of actin.

FPR Measurements. FPR measurements were done as described (Tait & Frieden, 1982b). Under the conditions we used, only the diffusion of polymeric actin is observed; the diffusion of monomer makes no contribution to the measured recoveries after bleaching. The excitation wavelength was 515 nm for rhodamine and 476 nm for NBD. Samples were first mixed in a test tube by repeated pipetting and then placed in flat glass capillaries (50-μL volume) at room temperature (22–24 °C). The parameters t_d, mobile fraction, and percent recovery (see next paragraph) were calculated as described (Tait & Frieden, 1982b).

The FPR measurements give several pieces of information. First, the fluorescence enhancement of rhodamine–actin or NBD–MalNEt–actin indicates how much polymer is present in the sample. Second, the rate of fluorescence recovery after bleaching is a measure of the apparent translational diffusion coefficient of polymeric actin, D [$D = w^2/(4t_d)$, where w is

the Gaussian beam radius (approximately 1.8 μm in this study) and t_d is the "characteristic" recovery time (approximately equal to the half-time of fluorescence recovery under most conditions) (Axelrod et al., 1976)]. Third, the fraction of the bleached fluorescence which does not recover indicates what fraction of the F-actin is immobilized (unable to diffuse), and hence what fraction of the F-actin is cross-linked into a network (Tait & Frieden, 1982b). The extent of immobilization is quantified by the mobile fraction, which is obtained from a curve-fitting procedure which extrapolates the observed recovery to infinite time. When curve fitting is inaccurate due to slight recoveries, the extent of immobilization can be estimated from the percent recovery, which is the fraction of the bleached fluorescence which has returned by the end of the measured recovery period (usually ~40 s long) (Tait & Frieden, 1982b).

Viscometry. Falling-ball viscometry was carried out as described by MacLean-Fletcher & Pollard (1980). We used 0.2-mL glass pipets with internal diameters of approximately 1.5 mm and stainless-steel balls with diameters of 0.79 mm (Micro-Ball Co., Peterborough, NH). For measurements, the pipets were held at 60° to the horizontal. Under these conditions, the balls fall at a rate of 10.7 ± 0.29 cm/s (mean ± SD, n = 10) in water at 22 °C. Samples were drawn up in the pipets immediately after addition of $MgSO_4$, so that they were subjected to shear only at the beginning of polymerization. For a given sample, each measurement was made on a fresh region of solution not previously exposed to the ball, since the passage of the ball reduced subsequent readings in the same location. The apparent viscosity of the samples is given as the reciprocal of the velocity of the falling ball, which is directly proportional to the viscosity for a Newtonian fluid. For a non-Newtonian fluid, the viscosity cannot be determined because the rate of shear around the ball is unknown (MacLean-Fletcher & Pollard, 1980).

Results

Effect of Chemical Modification on the Rate of Polymerization. For most experiments, we measured the rate of polymerization using either NBD–MalNEt–actin [with a fluorescence enhancement of roughly 2-fold on polymerization (Detmers et al., 1981)] or rhodamine–actin [with a fluorescence enhancement of roughly 1.5-fold on polymerization (Tait & Frieden, 1982a)]. These previous studies have shown that fluorescence measurements are likely to be valid as a quantitative assay of incorporation of monomer into polymer. As we will show under Discussion, the rate constants for addition of labeled monomers to polymers are unlikely to differ from the values for unlabeled actin. This means that trace quantities of rhodamine–actin and NBD–MalNEt–actin can be used to measure the polymerization of actin, even though these derivatives polymerize more rapidly in pure form.

Figure 1 shows the acceleration of polymerization due to modification of actin with MalNEt. Samples of sham-reacted actin and MalNEt–actin were prepared at 0.5 mg/mL (with 0.025 mg/mL NBD–MalNEt–actin as a tracer) and polymerized with 1.5 mM $MgSO_4$. The MalNEt–actin polymerizes faster than its sham-reacted control (half-time = 18.3 min for MalNEt–actin, 23.0 min for sham-reacted actin), but both reach essentially the same extent of fluorescence enhancement (they are within 1% of each other at 84 min). The difference in rate is therefore not due to differences in the amount of polymerizable actin in the two samples. A pelleting assay also showed that the unlabeled actin preparation contained as much or more sedimentable actin as the preparation of MalNEt–actin. Although the acceleration of polymerization by MalNEt

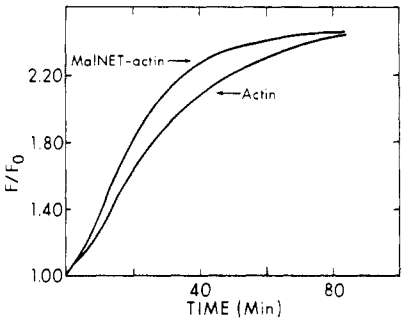

FIGURE 1: Polymerization of MalNEt–actin and sham-reacted actin. MalNEt–actin and sham-reacted actin were polymerized at 0.5 mg/mL (containing 0.025 mg/mL NBD–MalNEt–actin as tracer) with 1.5 mM $MgSO_4$ at 20 °C in a buffer containing 0.2 mM $CaCl_2$, 0.2 mM ATP, 1.5 mM NaN_3, and 2 mM Tris-HCl, pH 8. At zero time, Mg^{2+} was added, and the cuvette's contents were stirred for 30 s. Data are presented as the ratio of fluorescence intensity to the fluorescence intensity prior to addition of Mg^{2+}.

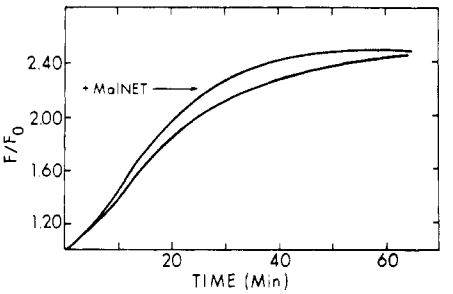

FIGURE 2: Polymerization of MalNEt–actin and actin directly after the modification reaction. Samples of G-actin (0.5 mg/mL total, 0.025 mg/mL NBD–MalNEt–actin as tracer) were prepared in cuvettes under the same conditions as in Figure 1. Samples then received either 2 mol of MalNEt/mol of actin or an equal volume of the solvent for MalNEt, ethanol. After a 1-h incubation at 20 °C, samples were polymerized with 1.5 mM $MgSO_4$.

is relatively modest, it is easily demonstrated due to the precision of the fluorescence measurement and has been repeatedly observed for a number of different preparations of MalNEt–actin.

Acceleration of polymerization also occurs when MalNEt–actin is polymerized directly after modification of G-actin (rather than after the usual purification by sedimentation and depolymerization following the modification reaction). Figure 2 shows the course of polymerization at 1.5 mM $MgSO_4$ and 0.5 mg/mL (using 0.025 mg/mL NBD–MalNEt–actin as the tracer) directly following a 1-h preincubation of the G-actin in the cuvette with MalNEt or with solvent alone. [MalNEt preferentially modifies Cys-373 in both G-actin and F-actin (Elzinga & Collins, 1975).] This result suggests that the rate acceleration is not due to any differences between actin and MalNEt–actin which might develop during the purification step after the reaction.

NBD–MalNEt–actin (0.44 mol of NBD/mol of G-actin) polymerizes much more rapidly than actin which has been sham reacted under conditions used to prepare NBD–MalNEt–actin (Figure 3). At 0.5 mg/mL and 1.5 mM $MgSO_4$, NBD–MalNEt–actin has a half-time of 5.3 min compared to 16.4 min for the sham-reacted actin control; both reach essentially the same final fluorescence level. Since the relative acceleration of polymerization is much greater for NBD–MalNEt–actin than for MalNEt–actin, we conclude that the addition of NBD to MalNEt–actin makes a large additional contribution to the rate acceleration. The rate of polymerization of NBD–MalNEt–actin was the same when it was measured with trace quantities of rhodamine–actin, proving

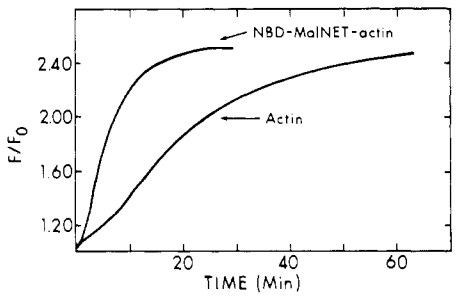

FIGURE 3: Polymerization of NBD–MalNEt–actin and sham-reacted actin at 0.5 mg/mL and 1.5 mM $MgSO_4$. Conditions are as in Figure 1. The NBD–MalNEt–actin contained 0.44 mol of NBD/mol of actin; the sham-reacted actin contained 0.025 mg/mL NBD–MalNEt–actin as tracer.

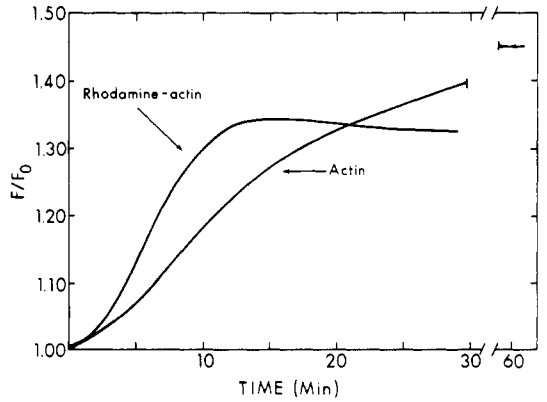

FIGURE 4: Polymerization of rhodamine–actin and sham-reacted actin at 0.5 mg/mL and 1.5 mM $MgSO_4$. Conditions are as in Figure 1. The rhodamine–actin contained 0.60 mol of rhodamine/mol of actin; the sham-reacted actin contained 0.025 mg/mL rhodamine–actin as a tracer.

that the differences in Figure 3 are not due to possible differences (e.g., photoinduced effects or inner filter effects) between rate measurements with trace and stoichiometric amounts of NBD–MalNEt–actin.

Rhodamine–actin (0.60 mol of rhodamine/mol of G-actin) also polymerizes more rapidly than its sham-reacted control at 0.5 mL/mL and 1.5 mM $MgSO_4$ (Figure 4). Rhodamine–actin also reaches a lower fluorescence level than its sham-reacted control. However, this difference was not due to a different critical concentration, at least as conventionally defined (see next paragraph). We should also note that the effect of modification on the rate of polymerization was more variable for rhodamine–actin than for NBD–MalNEt–actin or MalNEt–actin. These differences may have been due to variations between lots of IATR, but we are not yet certain of the explanation.

Although differences in critical concentrations between labeled and unlabeled actin could explain some results of this study (see Discussion), previous studies have shown that the critical concentrations of NBD–MalNet–actin (Detmers et al., 1981) and rhodamine–actin (Tait & Frieden, 1982a) are unlikely to differ much from the value of unlabeled actin. Using a pelleting assay, we rechecked the critical concentrations of actin, MalNEt–actin, NBD–MalNEt–actin, and rhodamine–actin in 1.5 mM $MgSO_4$ (20-h incubation at 20 °C). The y-axis intercepts of plots of supernatant actin concentration against total actin concentration were all approximately 0.03 mg/mL, confirming that labeling of actin had not grossly altered its critical concentration. However, for reasons that are now unclear, the slopes of these plots were greater for labeled than unlabeled actin. Wegner & Savko (1982), using MalNEt–actin, have shown that the nonzero

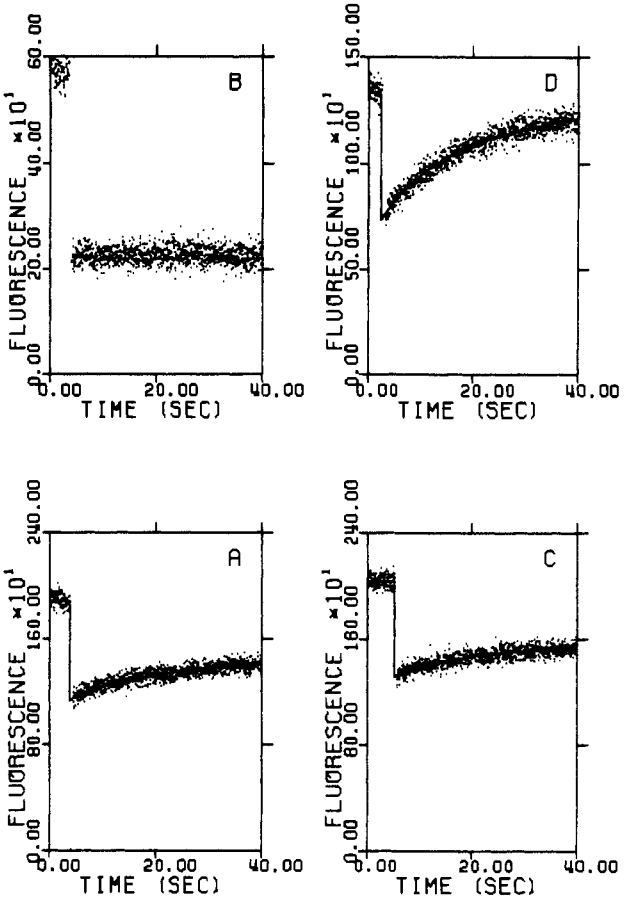

FIGURE 5: FPR data for rhodamine–actin and unlabeled actin. Measurements were made at room temperature as described under Materials and Methods. Polymerization was complete for all samples (judged by fluorescence enhancement) at the time of the measurements. Individual points represent measured fluorescence intensity; lines are the best fit of the data to the equation describing diffusive recovery from bleaching. (A) Rhodamine–actin (0.52 mol of rhodamine/mol of actin), 1 mg/mL, 28 min after addition of 1.5 mM $MgSO_4$. Laser power 0.1 μW, 400-ms bleach. Percent recovery = 37%; curve-fitting procedure gives t_d = 21 ± 2 s, mobile fraction = 0.58 ± 0.03. (B) Unlabeled actin, 1 mg/mL (0.1 mg/mL rhodamine–actin as tracer), 24 min after addition of 1.5 mM $MgSO_4$. Laser power 0.2 μW, 400-ms bleach. Percent recovery = 0.2%; curve fitting not possible. (C) Same sample as in (A), 570 min after addition of 1.5 mM $MgSO_4$. Percent recovery = 29%; t_d = 16 ± 2 s, mobile fraction = 0.46 ± 0.02. The prebleach intensity is not directly comparable with (A) due to a slight change in the laser power. (D) Rhodamine–actin (0.60 mol of rhodamine/mol of actin), 1 mg/mL, 21 min after addition of 1 mM $MgSO_4$. Laser power 0.2 μW, 600-ms bleach. Percent recovery = 80%; t_d = 15 ± 1 s, mobile fraction = 1.15 ± 0.02.

slopes of these plots cannot be simply attributed to the presence of denatured actin.

Reduced Extent of Network Formation for Labeled Actins. FPR can be used to measure the gelation of F-actin because filaments become immobilized (unable to diffuse) when networks form (Tait & Frieden, 1982b). If gelation is partially or fully prevented, there will be a corresponding increase in the fraction of filaments which recover by diffusion after bleaching. Complete immobilization does not occur for rhodamine–actin at 1 mg/mL and 1.5 mM $MgSO_4$ (Figure 5A), even when the mass of F-actin has reached steady state, whereas unlabeled actin is fully immobilized under these conditions (Figure 5B). Rhodamine–actin shows partial recovery after bleaching for at least 10 h after addition of $MgSO_4$ (Figure 5C). The effect of modification is more pronounced at lower Mg^{2+} levels. At 1 mM Mg^{2+}, there is little or no immobilization, (Figure 5D) while in a control,

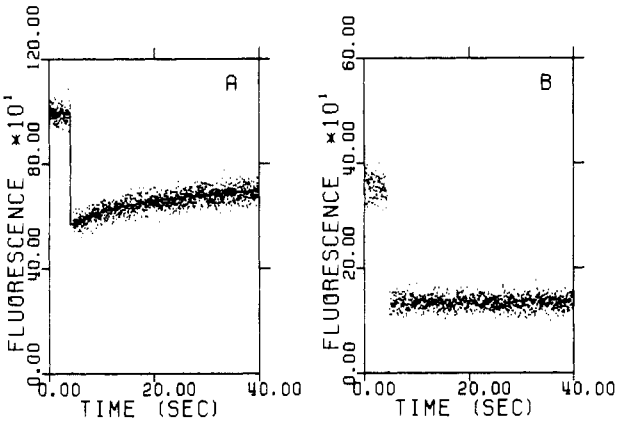

FIGURE 6: FPR data for NBD–MalNEt–actin and unlabeled actin at 1 mg/mL and 1.5 mM $MgSO_4$. Excitation wavelength was 476 nm. (A) NBD–MalNEt–actin (0.51 mol of NBD/mol of actin) 8 min after addition of 1.5 mM $MgSO_4$. Laser power 0.01 μW, 400-ms bleach. Percent recovery = 28%; t_d = 16 ± 2 s, mobile fraction = 0.45 ± 0.02. (B) Unlabeled actin (0.1 mg/mL NBD–MalNEt–actin as tracer) 8 min after addition of 1.5 mM $MgSO_4$. Laser power 0.03 μW, 400-ms bleach. Percent recovery = 0.4%; curve fitting not possible. Both samples were nearly completely polymerized (~90% of maximal fluorescence enhancement) at the time of the measurements.

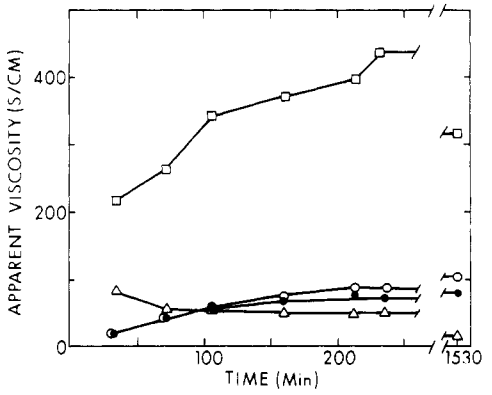

FIGURE 7: Low-shear viscometry for MalNEt–actin and sham-reacted actin with and without concurrent modification with IATR. MalNEt–actin (closed and open circles) and sham-reacted actin (squares, triangles) were prepared at 1 mg/mL with 2 mM $MgSO_4$ and 0.5% (v/v) *N,N*-dimethylformamide, 21.5 °C. Mg^{2+} was added at zero time, and samples were immediately mixed and transferred to capillaries. The ordinate gives the reciprocal of the velocity of the falling ball, which is directly proportional to the viscosity for a Newtonian fluid. One sample of MalNEt–actin (closed circles) and one of sham-reacted actin (triangles) also received 4 mol of IATR/mol of actin; these samples were protected from light between measurements.

immobilization is essentially complete (data not shown). Rhodamine–actin is therefore less capable of forming networks than is unlabeled actin.

This effect is also seen for other labeled actins. At 1 mg/mL and 1.5 mM Mg^{2+}, NBD–MalNEt–actin shows partial recovery after bleaching (Figure 6A) while unlabeled actin (containing trace amounts of NBD–MalNEt–actin) shows no recovery (Figure 6B). (The extent of recovery for NBD–MalNEt–actin cannot be directly compared to values obtained with rhodamine–actin because NBD–MalNEt–actin is more susceptible to bleaching by the observation beam than rhodamine. This means that the percent recovery is underestimated for NBD–MalNEt–actin.) The effect of modification with MalNEt is much less marked. At 1 mg/mL and 1.5 mM Mg^{2+}, differences in the percent recovery between MalNEt–actin and unlabeled actin are almost undetectable (not shown). However, at a lower $MgSO_4$ concentration (1 mM), the effects of modification become more pronounced. All samples (including unlabeled actin) have larger percent recovery values in 1 mM $MgSO_4$ than in 1.5 mM $MgSO_4$, and under these conditions, MalNEt–actin has a larger percent recovery than unlabeled actin. [For example, at 1 mg/mL and 1 mM $MgSO_4$, after 24 min of polymerization, the percent recovery is 15% for MalNEt–actin but 4% for unlabeled actin (data not shown).]

Several controls indicate that the FPR results do reflect actual differences in network formation due to chemical modification. Actin sham reacted under conditions used to prepare rhodamine–actin, NBD–MalNEt–actin, and MalNEt–actin immobilized to the same degree as unlabeled actin (not shown). The extent of immobilization also does not vary from one actin preparation to another. Rhodamine–actin gel filtered on Sephacryl S-300 had the same percent recovery as unrepurified rhodamine–actin, suggesting that incomplete immobilization was not due to actin fragments or high molecular weight impurities which, by enhancing nucleation, might transiently shorten filaments. It is also unlikely that the partial recoveries for rhodamine–actin are due to photoinduced effects, which have been suggested to occur for actin labeled at Cys-373 with 5-(iodoacetamido)fluorescein (Lanni et al., 1981). Rhodamine–actin recovers to the same extent at a level of illumination 10-fold less than that shown in Figure 5.

In conclusion, the FPR data show that the chemically modified actins do not become immobilized to the same extent as unlabeled actin. The different derivatives had the following relative potency in increasing percent recovery values at 1 mg/mL and 1–1.5 mM $MgSO_4$: rhodamine–actin ≥ NBD–MalNEt–actin > MalNEt–actin ≥ actin.

Falling-ball viscometry offers another way to measure network formation (MacLean-Fletcher & Pollard, 1980). Although the rate of shear in these measurements cannot be defined for a non-Newtonian fluid such as an F-actin solution, it is thought to be considerably less than in a capillary viscometer and is correspondingly more sensitive to the lengths of filaments and the interactions between them (MacLean-Fletcher & Pollard, 1980). Earlier evidence also suggests that viscometry at low rates of shear can measure network formation in solutions of F-actin (Kasai et al., 1960; Maruyama et al., 1974). However, we found that there were substantial differences in apparent viscosity between one actin preparation and the next, and sham-reaction procedures also altered the apparent viscosity of F-actin solutions. The apparent viscosity is also likely to be a nonlinear function of filament lengths and cross-link density. For these reasons, the results given below are only qualitative indicators of alterations in network formation.

Rhodamine–actin, NBD–MalNEt–actin, and MalNEt–actin always had lower apparent viscosities at 1 mg/mL and 2 mM $MgSO_4$ than sham-reacted controls [Figure 7: MalNEt–actin (open circles) vs. sham-reacted actin (squares); data not shown for rhodamine–actin and NBD–MalNEt–actin]. Wang & Taylor (1981) have also observed that MalNEt–actin has a lower apparent viscosity than unlabeled actin. The labeled actins have always had lower apparent viscosities than their controls for as long as we have followed them (routinely for 2–3 days, and in one instance for 20 days with rhodamine–actin). This suggests that reduction of network formation by chemical modification is not a transient phenomenon due only to the concomitant acceleration of polymerization (see Discussion).

Chemical modification with IATR also reduces the apparent viscosity of preexisting solutions of F-actin. In Figure 7, the

apparent viscosity of actin (squares) was followed at 1 mg/mL and 2 mM $MgSO_4$. In addition, IATR (4 mol/mol of actin) was added to an identical sample of actin (triangles) at the same time that $MgSO_4$ was added to start polymerization. Thus, actin becomes labeled with rhodamine only during or after polymerization. [Under these conditions, polymerization is rapid (half-time ~5 min), so the actin should be largely polymerized before much has been modified by IATR.] The apparent viscosity of the control increases over time, while the apparent viscosity of the sample containing IATR progressively decreases. However, reduction of viscosity by IATR is largely prevented by prior labeling of the actin with MalNEt (Figure 7, closed circles), indicating that the reduction in viscosity is due to reaction of IATR with Cys-373. In summary, these results provide further evidence that chemical modification of actin at Cys-373 reduces network formation independently of its effect on the rate of polymerization.

Discussion

This study shows that chemical modification of actin with MalNEt, IATR, and NBD-Cl has two effects: acceleration of polymerization and partial inhibition of network formation. There are a number of different explanations for each finding and at least one hypothesis (stabilization of nuclei) which gives a single explanation for both findings. Before considering possible explanations for our results, we should briefly review existing theoretical studies of how the length distribution of actin filaments is affected by properties of the nucleation and polymerization reactions.

The length of actin filaments is likely to be an important determinant of their ability to form networks. Large polymer networks begin to form when the fraction of cross-linked subunits exceeds the reciprocal of the weight-average degree of polymerization (Flory, 1953). This theory is consistent with experimental studies of gelation of actin filaments by actin-binding protein (Hartwig & Stossel, 1979) and by filamin (Nunnally et al., 1981). We have presented indirect evidence that this theory also applies to the gelation of actin filaments in the absence of added cross-linking proteins (Tait & Frieden, 1982b). Chemical modification of actin could therefore inhibit gelation if it reduced the average length of actin filaments.

Theoretical Analysis of Filament Length Distributions. Oosawa & Kasai (1962) analyzed the polymerization of actin as a cooperative process in which the addition of monomeric actin to existing polymers was thermodynamically much more favorable than the initial formation of polymer nuclei. They obtained an expression for the number-average degree of polymerization which shows that average polymer length is decreased if nucleation is made more favorable; average polymer length can also be reduced at constant actin concentration if the equilibrium constant for addition of monomer to polymer is reduced. Winklmair (1971) considered a very similar model in which formation of trimers and all higher order polymers is governed by an equilibrium constant K, while formation of dimers is governed by an equilibrium constant σK, with $\sigma < 1$:

$$A + A \rightleftharpoons A_2 \qquad K_{eq} = \sigma K$$

$$A_i + A \rightleftharpoons A_{i+1} \qquad K_{eq} = K \ (i \geq 2)$$

where A represents an actin monomer. Plots of number- and weight-average polymer sizes (Winklmair, 1971) as a function of σ show that polymer sizes decrease as nucleation becomes less difficult (i.e., as σ increases); average polymer size also decreases as the value of K decreases.

These studies show that filament lengths at equilibrium can be reduced by stabilizing nuclei. It is important to recognize that stabilization of nuclei can reduce the equilibrium filament length quite apart from any transient shortening which might result from the increased concentration of nuclei during polymerization. One physical interpretation of this result is that the ends of an actin filament are similar to nuclei in that not all possible monomer–monomer contacts have been formed. Therefore, a change which results in greater stability of nuclei will also result in greater stability of filament ends. The relative energetic penalty of an end is reduced, resulting in shorter average filament lengths at equilibrium.

Explanations for the Effect of Chemical Modification. It is apparent that stabilization of nuclei by chemical modification is a simple hypothesis which could explain all our results. The greater stability of nuclei would both accelerate polymerization and shorten filaments, resulting in reduced network formation. A reduction in average filament length would reduce the probability that a given filament would be cross-linked to other filaments, since this probability is proportional to the length of the polymer. Some fraction of the labeled polymer would thus escape cross-linking into a network and would be able to diffuse, accounting for the partial recoveries after bleaching seen in Figures 5 and 6. But stabilization of nuclei (within certain limits) would not alter the apparent critical concentration [see graphs in Winklmair (1971)], also in agreement with our results. The low-shear viscosity data suggest that reduction of network strength is permanent on a time scale of days, and the low-shear viscosity of preformed filaments is reduced by modification with IATR; both findings are consistent with a true equilibrium shortening of the filaments by chemical modification. There is also a correlation between the effects of polymerization rate and on network formation: MalNEt causes smaller changes in the rate of polymerization than do NBD and rhodamine, and this corresponds with MalNEt's more modest effects on network formation.

There is a parallel between these results and the effects of cytochalasin. Cytochalasin shortens actin filaments (Hartwig & Stossel, 1979; Maruyama et al., 1980), and we previously observed that cytochalasin D partially inhibited immobilization of actin filaments in FPR (Tait & Frieden, 1982b). Cytochalasin also accelerates the polymerization of actin, which has been attributed to its ability to enhance nucleation (Brenner & Korn, 1980; Dancker & Low, 1979; Howard & Lin, 1979; Tellam & Frieden, 1982). The two findings may well be manifestations of the same phenomenon, namely, stabilization of nuclei. [This interpretation could also reconcile the apparent conflict between the high concentrations of cytochalasin (usually >0.1 μM) which seem to be necessary to accelerate polymerization (Brenner & Korn, 1980; Dancker & Low, 1979; Howard & Lin, 1979; Tellam & Frieden, 1982) and the low values (5–10 nM) which have been reported for the binding constant of cytochalasin B to F-actin (Flanagan & Lin, 1980; Hartwig & Stossel, 1979). The end of a filament is a relatively stable structure in a favorable conformation for binding cytochalasin, so the K_d is low; but a nucleus is relatively unstable, so some binding energy must be used to stabilize the nucleus, resulting in a higher apparent K_d for the effect on nucleation during polymerization.]

Several other explanations for our results seem less consistent with the data. It is unlikely that the acceleration of polymerization is due to alterations in the rate constants for the addition of labeled monomeric actin to polymer ends. Since the rate of fluorescence change during polymerization is governed by the nature of the predominant actin species and

not by the fluorophore on the tracer species (Figures 1–4), we can rule out the possibility that labeled monomeric actin elongates more rapidly onto any existing filament (whether labeled or unlabeled). NBD–MalNEt–actin and rhodamine–actin also give the same normalized time course of fluorescence increase when they are used as tracers for the polymerization of unlabeled actin (Tait & Frieden, 1982a); the normalized rate of fluorescence change is also the same whether one follows the fluorescence of NBD or of rhodamine for polymerization of NBD–MalNEt–actin with trace quantities of rhodamine–actin (see Results). Measurement of the rate of polymerization by FPR also shows that MalNEt–actin polymerizes more rapidly than unlabeled actin (data not shown). Finally, the association rate constants for addition of G-actin to F-actin are probably already at the diffusion limit (Pollard & Mooseker, 1981), so it is unlikely that the rate acceleration due to chemical modification could be due to further increases in these rate constants. We conclude that the observed rate acceleration reflects a true increase in the rate of accumulation of polymer mass.

Reduction of the equilibrium constant for the monomer–polymer reaction is another possible explanation for our results. This would explain the partial inhibition of network formation because filaments would be on average shorter at a given actin concentration (see above). However, we and others have not detected differences in the critical concentrations of the labeled actins we have used (see Results); since the critical actin concentration is a measure of monomer–polymer affinity (Oosawa & Asakura, 1975), the postulated differences must be smaller than the usual error in the determination of the critical concentration. On the other hand, a relatively modest change in monomer–polymer affinity, too small to be easily detected, could cause noticeable changes in gelation due to the sensitivity of gelation to small changes in average polymer lengths or in the length distribution (Flory, 1953).

A reduction in monomer–polymer affinity by chemical modification might also explain the acceleration of polymerization if reactions between polymers were also affected. If the rate of fragmentation were increased, or the rate of annealing decreased, the net effect would be an acceleration of polymerization due to the larger number of polymer ends. Arisaka et al. (1975) found that they needed to include polymer–polymer reactions in order to model the kinetics of polymerization adequately. More recently, Wegner (1982) and Wegner & Savko (1982) also found it necessary to take account of polymer fragmentation in order to describe the kinetics of polymerization at 37 °C under some ionic conditions. However, they found that the kinetics could be adequately described without fragmentation for polymerization in 40 mM KCl (Wegner & Savko, 1982). Since we have observed a large rate acceleration for polymerization of NBD–MalNEt–actin in KCl (not shown), this suggests that differences in fragmentation do not explain our results. In addition, the polymerization time courses we have measured generally show a slow final approach to a plateau rather than the relatively abrupt achievement of the plateau which is said to be characteristic of kinetics governed by fragmentation (Wegner & Savko, 1982). In contrast, when the sample is stirred continuously during polymerization, thereby increasing the rate of fragmentation (Tait & Frieden, 1982a), the shape and time course of fluorescence increase resemble the results of Wegner & Savko (1982) much more closely. In conclusion, although we cannot rule out the possibility that fragmentation contributes to our results, there are several aspects of the data that argue against it.

Network formation could also be affected by chemical modification if the modified region of the actin monomer participates in the interfilament bonds which form the cross-links in the network. At this point, we have no evidence for or against this hypothesis; it might contribute to our results but is not necessary to explain them.

Use of Labeled Actin To Study Polymerization. There have been many studies of the polymerization of actin and the properties of actin polymers which have used derivatives of actin labeled at Cys-373 (e.g., Stone et al., 1970; Cheung et al., 1971; Lin, 1978; Ikkai et al., 1979; Thomas et al., 1979; Taylor et al., 1981; Wang & Taylor, 1980,1981) or at Cys-373 and Lys-372 (Detmers et al., 1981). These studies have assumed that labeling does not alter the properties of the actin. We have now shown that this is not necessarily correct, at least for actin labeled with MalNEt, IATR, or NBD-Cl. It will be necessary to see whether other derivatives of Cys-373 also polymerize more rapidly and form networks less well, and whether the strength of the effect varies from one derivative to another. In the meantime, it is probably best to use trace quantities of fluorescently labeled actin to follow the polymerization of unlabeled actin; we have previously provided evidence that this approach is valid for rhodamine–actin in both FPR and static fluorescence measurements (Tait & Frieden, 1982a,b).

Conclusion. We have shown that two properties of actin—its rate of polymerization and its ability to form networks—can be altered by chemical modification. Theoretical analysis indicates that both these effects could be explained if the modification stabilized nuclei, and there is a parallel between these results and the effects of cytochalasins and capping proteins. However, we recognize that this explanation is only tentative and that other phenomena may well contribute to the effects we have observed. Further work should reveal whether this effect occurs for other labeled actins as well, and what its mechanism might be.

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Hexa- and Pentapeptide Extension of Proalbumin: Feedback Inhibition of Albumin Synthesis by Its Propeptide in Isolated Hepatocytes and in the Cell-Free System†

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ABSTRACT: Addition of the chemically synthesized proalbumin hexapeptide in a concentration of 110 μM to the medium of isolated rat hepatocytes decreased net albumin synthesis by 12%. The synthesis of other secretory proteins was not altered. A weaker inhibitory effect on albumin synthesis was found for a tetrapeptide, a possible degradation product of the proalbumin hexapeptide. For the uptake of the hexa- and tetrapeptide into the cells, bovine serum albumin is required. In a reticulocyte and in a wheat germ cell-free system a propeptide concentration of 600 μM inhibited albumin synthesis by 50%, whereas total protein synthesis was inhibited by 19% only, and the synthesis of α_1-antitrypsin was not inhibited. These results suggest that the synthesis of preproalbumin is regulated by a feedback mechanism with its propeptide as inhibitor.

Proalbumin is the immediate intracellular precursor of albumin, differing from serum albumin by a hexapeptide extension at the N-terminal end. The amino acid sequence of the rat (Quinn et al., 1975; Russel & Geller, 1975) and bovine (Patterson & Geller, 1977) albumin propeptide has been shown to be NH_2-Arg-Gly-Val-Phe-Arg-Arg, and probably the propeptide of human albumin has the same structure (Brennan & Carrell, 1978). This peptide is split off in the Golgi apparatus (Edwards et al., 1976; Ikehara et al., 1976) by an enzyme with the characteristics of cathepsin B (Judah & Quinn, 1978; Quinn & Judah, 1978). Multiple possible functions of the basic hexapeptide extension have been discussed (Peters & Reed, 1980). It may be involved in the formation of the tertiary structure of the albumin molecule, it may facilitate transport through or export out of the cell,

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