Lysine 372 of N-ethylmaleimide actin was specifically (60%) labeled by 7-chloro-4-nitrobenzeno-2-oxa-1,3-diazole chloride (NBD-Cl), which also reacted with lysines on cyanogen bromide fragment 17 (20%) and other undeetermined residues (20%). Isolation of N-ethylmaleimide peptides and two-dimensional peptide mapping demonstrated that 90% of bound N-ethylmaleimide was attached to an adjacent residue, cysteine 373, independent of the polymerization state of actin during the labeling reaction. Formation of NBD cysteine severely inhibited lysine modification. After N-ethylmaleimide blockage of cysteine 373, lysine labeling with NBD was greatly accelerated. The kinetics of formation of fluorescent compounds were biphasic, with fluorescence decreasing upon prolonged incubation of actin in NBD-Cl. Lysine 372 of purified NBD actin reproducibly responded to polymerization by a 2.2- to 2.3-fold enhancement of fluorescence. By contrast, interaction of NBD actin with several actin-binding proteins caused only very small or undetectable changes in fluorescence intensity: 10% enhancement on myosin subfragment 1 binding, about 8% quenching by DNase I, and no change at all by tropomyosin-tropomyosin. Despite its sensitivity to polymerization the probe did not affect it. Native and modified actin polymerized randomly indicating that the rate constants for polymerization remained the same. Labeling actin with NBD did not diminish its cofactor activity for myosin ATPase activity. Contrary to previous reports we observed that myosin subfragment 1 (single myosin heads) caused actin polymerization in the absence of salt.

Fascination with the ease with which actin may change its state of polymerization dates from the discovery of the protein by Straub (1). Szent-Györgyi (2) was convinced that polymerization and depolymerization must play an important role in contraction of muscle, the only tissue in which actin had been found at that time. Although not for muscle this turned out to be true for nonmuscle cells (3), where changes in the polymerization state of actin have been observed, for instance monomeric actin polymerizes upon stimulation of platelets (4) and during elongation of the acrosomal process in Thyone sperm (5).

Current thought assumes that the polymerization state of cellular actin is regulated by its association with other proteins. Three proteins which influence actin polymerization in vitro have been investigated so far, DNase I (6), profilin (7-9), and myosin, including double (2) and single-headed myosin (10) as well as heavy meromyosin (11) (the double-headed digestion product of myosin). DNase I causes actin depolymerization and profilin inhibits polymerization (9), while myosin and heavy meromyosin have been shown to bring about polymerization in the absence of salt (11, 12). Knowledge of the detailed mechanism of action of these proteins or of other actin-binding proteins which may have similar specific effects is still quite rudimentary.

It would be useful for the understanding of the regulation of the state of polymerization to be able to clearly distinguish between elongation and nucleation, a distinction that is very difficult to make with viscosity measurements: first, because the protein concentrations needed are too high to prevent spontaneous nucleation, and second, because the filaments are constantly exposed to shear forces that alter the number of filament ends. Elongation measurements, preferably separately for each end, became especially important when it appeared that capping of filament ends is one of the mechanisms for regulation of the state of polymerization. Capping of filament ends influences the state of polymerization because polymerization does not reach equilibrium but a steady state that depends on ATP hydrolysis as first demonstrated by Wegner (17). During the steady state one filament end receives a net influx of monomers while the other end constantly loses monomers, a discovery made independently for microtubules by Margolis and Wilson (13). Margolis and Wilson (13) also demonstrated that capping of one microtubule end is the mechanism of action of colchicine. Recently it was shown that the cytochalasins affect the state of actin polymerization in a similar manner (14-16).

We describe a fluorescent probe for actin polymerization, 7-chloro-4-nitrobenzeno-2-oxa-1,3-diazole-labeled actin, first reported by Birkett et al. (18). The probe which has a sufficiently high quantum yield to be used at 10^{-6} M actin concentration has the advantage over cysteine 373-attached IAEDANS (19) in that it responds primarily to polymerization, while the binding of myosin or DNase I changes fluorescence intensity very little and that of tropoactin-tropomyosin has no effect at all. We have determined, by amino acid analysis of isolated NBD-labeled cyanogen bromide peptides, which residues are modified under optimal labeling conditions and we present evidence that is 60% of the attached NBD. We show that NBD-modified actin randomly copolymerizes with the unmodified actin.

The abbreviations used are: NBD, 7-chloro-4-nitrobenzeno-2-oxa-1,3-diazole; S-1, myosin subfragment 1; CB, cyanogen bromide fragment; IAEDANS, 5-(iodoacetamidoethyl)amino-2-naphthalene-1-sulfonic acid.
Acid Analysis—These have been previously described (25). Citro-
columns equilibrated with 50 mM imidazole, pH were concentrated by ammonium sulfate precipitation at 60% satu-
ration. Precipitation in this range removed remaining rod fragments (below
pound seemed to bind to actin and could not be removed by repeated
washing. Instead, actin was collected by centrifugation at 100,000
xg for 48 h against depolymerizing solution, initially containing
1 mM diithiothreitol, which was gradually reduced to 0.2 mM. Myosin
was extracted from rabbit back and leg muscles and purified according
to earlier reports (12).

MATERIALS AND METHODS

Protein Preparations—Actin was extracted from acetone powder
(1) of rabbit back and leg muscles with an ice-cold solution of 0.5M
ATP, 0.2 mM CaCl₂, 0.2 mM diithiothreitol, 10 mM Tris, pH 8.0 (at
25°C) (depolymerizing solution), and purified in a single step modified
from Spudich and Watt (20) by addition of KC1 to a final concentra-
tion of 0.8 M, and KCl, to a final pH of 8.2, to actin previously
elicated earlier in 0.1 M KCl and 1.0 mM MgCl₂. Actin-activation was avoided by holding the total extraction period to not more than 40
min. After sedimentation, the purified actin was depolymerized by dialysis for 48 h against depolymerizing solution, initially containing
1 mM diithiothreitol, which was gradually reduced to 0.2 mM. Myosin
was extracted from rabbit back and leg muscles and purified according
to Portehl et al. (21). Catatonic actomyosin was removed by precipitation at 0.28 M ionic strength before myosin precipitation at
0.05 M ionic strength. Two precipitation cycles produced actin-free myosin, which was stored at -20°C in 0.5 M KCl, 10 mM imidazole,
pH 7.0 (at 25°C), 50 μM EDTA, 0.2 mM diithiothreitol, and 50%
ethylene glycol. Heavy meromyosin was prepared by chymotryptic
(Sigma) digestion according to Weeds and Pope (23) from myosin in
60 mM NaCl, 1 mM MgCl₂, and 1 mM CaCl₂. After removal of the rods
at 0.05 M KCl, heavy meromyosin was precipitated with ammonium sulfate (Mann, Schwartz, ultrapure) at 55% saturation and dialyzed at 2°C against 10 mM imidazole, pH 7.0 (at 25°C), 1 mM diithiothreitol, and a protein concentration of not less than 20 mg/ml. Chymotryptic
S-1 was prepared according to Weeds and Taylor (23) from precipi-
tated myosin in 0.1 M Tris, 0.2 mM diithiothreitol, 20 mM phosphate,
pH 7.0, 1.0 mM EDTA. After removal of the rods, S-1 was collected by
ammonium sulfate precipitation between 50 and 55% saturation.
Precipitation in this range removed remaining rod fragments (below
50% saturation) and overdialysis S-1 which precipitated between 60 and
65% saturation. Column purification of the myosin fragments
when performed, was according to Weeds and Taylor (23) using DE52
columns equilibrated with 50 mM imidazole, pH 7.0 (at 25°C), 0.2 mM
dithiothreitol, 10 mM EDTA, and eluting with an increasing linear
KCl gradient (maximal concentration 0.5 M). The eluted proteins
were concentrated by ammonium sulfate precipitation at 60% satu-
ration.

Actin Modification—N-Ethylmaleimide actin was prepared by the addition of N-ethylmaleimide (Sigma) to a final concentration of 0.3
mM in excess over 0.2 mM diithiothreitol and KC1 and MgCl₂, to respective final concentrations of 0.1 M and 1 to 2 mM to actin (2
mM dithiothreitol) depolymerizing solution. The instantly polymerizing actin was kept at room temperature for 15 min; then at 15°C for 15 min,
followed by 2.5 h at 2°C before termination of the reaction by addition of diithiothreitol to 1 mM and a sedimentation-depolymerization cycle.
NBD-N-ethylmaleimide actin was prepared by incubation of pol-
ymered N-ethylmaleimide actin (1 mg/ml in 10 mM Tris, pH 8.0, at
25°C, 0.2 mM CaCl₂, 0.5 mM ATP, 0.1 mM KCl, 2 mM MgCl₂) for 5 h at
15°C with 0.4 mM NBD-Cl (Pierce) dissolved in 95% ethanol (spec-
trograde) (final ethanol 2%). The incubation was not terminated by
the addition of diethiothreitol because the NBD diethiothreitol com-
pond seemed to bind to actin and could not be removed by repeated
dialyzed against 0.4 mM NBD-Cl (Pierce) dissolved in 95% ethanol (spec-
trograde) (final ethanol 2%). The incubation was not terminated by
the addition of diethiothreitol because the NBD diethiothreitol com-
pound seemed to bind to actin and could not be removed by repeated
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trograde) (final ethanol 2%). The incubation was not terminated by
the addition of diethiothreitol because the NBD diethiothreitol com-
pound seemed to bind to actin and could not be removed by repeated

Cyanogen Bromide Cleavage, Separation of Peptides, and Amino
Acid Analysis—These have been previously described (25). Citro-
conic anhydride treatment was performed by adding a 9-fold molar
excess of citroconic anhydride to peptides in 0.5% ammonium bicar-
bonate, maintaining the pH at 8.0 to 8.2, and incubating until the pH
was stable. For the subsequent tryptic digestion an enzyme-substrate
ratio (1:50) was used with incubation for 4 h at 25°C.

ATPase Assays—The quality of the myosin fragments was re-
sessed by K⁺-activated ATP hydrolysis at pH 8.0, 5 mM ATP, 1 mM
EDTA, and 0.6 M KC1. Inorganic phosphate was determined according
to Taussky and Schorr (26). Rates of 16-20 mol/mol of heads x s at
25°C were considered satisfactory. For the evaluation of the cofactor
activity of modified actin, actin and myosin were mixed in 0.3 M KCl
before dilution into the assay mixture, to a final concentration of 30
mM KC1, 10 mM imidazole, pH 7.3, 1 mM MgCl₂, 4 mM MgATP, 2 μM
myosin (i.e. 4 μm heads), 25°C. Protein determinations were made
according to Lowry et al. (27), calibrated for myosin and actin by
Kjeldahl, and standardized with albumin. Fluorescence measure-
ments were made in a Perkin-Elmer MFP-3L fluorescence spectro-
photometer with the temperature controlled by a circulating water
bath. For the studies of the effect of myosin fragments on actin
polymerization light-scattering artifacts were kept to a minimum by
using an emission cut off filter (Corning color specification CS37, 70,
glass 3584). For further light-scattering correction, light reaching the
phototube at an emission setting of 530 nm with an excitation wave-
length of 400 (minimum for fluorescence excitation) was subtracted
from the values obtained with 470 nm excitation. At 2 μM N-ethyl-
_maleimide-NBD actin the inner filter correction was about 7%. Flu-
orescence was standardized with quinine sulfate in 0.1 N sulfuric acid.

RESULTS

Reaction of NBD Chloride with Cysteine 373—Native F-
actin labeled with NBD had a significant absorbance shoulder in the region between 405 and 440 nm (Fig. 1, Curve 1) which could be eliminated by diithiothreitol treatment (Fig. 1, Curve 2), and the formation of which could be prevented by pretreat-
ing F-actin with N-ethylmaleimide (Fig. 1, Curve 3), behavior expected for an NBD cysteine adduct. The labeled cysteine was residu 373 since we ascertained that about 90% of the blocking N-ethylmaleimide was attached to cysteine 373 by
isolating the N-ethylmaleimide containing cyanogen bromide peptide (28) by column chromatography (Fig. 2). Cysteine 373 is
the most reactive cysteine in both G- and F-actin, contrary to earlier reports using N-ethylmaleimide nitroxide labels (29) since Elzinga and Collins (28) have previously shown that N-
ethylmaleimide reacts specifically also with Cys 373 on G-
actin. This conclusion was confirmed by autoradiography of tryptic peptide maps of [²³CN]-N-ethylmaleimide actin (Fig. 3).

Identification of Lysine Residues Reacting with NBD
Chloride—The wavelength maxima at 340 and 480 nm of the two major absorbance peaks of NBD actin (Fig. 1, Curves 2 and 3) which are not affected by diethiothreitol treatment are similar to those reported for NBD lysine compounds (31)
which have been described as resistant to reducing agents (32). The corresponding fluorescence excitation and emission

![Fig. 1. Absorption spectra of NBD actin. Curve 1, NBD actin; Curve 2, NBD actin treated with diethiothreitol after removal of excess NBD; Curve 3, NBD-N-ethylmaleimide actin.](image-url)
spectra are shown in Fig. 4 (lower curves). The observation that dithiothreitol treatment did not diminish the fluorescence emission at 530 nm indicates that the NBD lysine adducts are the major fluorescent species in actin, not the NBD cysteine 373 adduct.

To determine the identity of the lysine residues labeled by NBD, 120 mg of F-actin with cysteine 373 blocked by N-ethylmaleimide were incubated with NBD chloride for 5 h as described under "Materials and Methods." Following cyanogen bromide cleavage the labeled actin was chromatographed first on Sephadex G-50 and then on SP-Sephadex, and the NBD lysine content of the fractions was followed by absorbance at 480 nm (Fig. 5A). About 62% of the NBD lysine absorbance was associated with CB 9. The modified lysine apparently reduced the binding of NBD CB 9 to SP-Sephadex, so that the labeled peptide was eluted before CB 9.

To distinguish which of the 2 lysine residues, lysine 358 and 372, contained the NBD, trypsinization was performed following citroconilation of unlabeled lysines to limit proteolysis to one position (arginine 371). All 480 nm absorbance was eluted from Sephadex G-25 in a single peak (Fig. 5B), containing equimolar amounts of free lysine, S-succinylcysteine (the N-ethylmaleimide adduct), and phenylalanine. Apparently NBD lysine had been hydrolyzed since we could show that the ε-amino-NBD bond in ε-N-butyloxycarbonyllysine NBD could not withstand acid hydrolysis. About 60% of the NBD is then bound to lysine 372, the residue next to the reactive cysteine.

A large fraction (about 22% of the total absorbance) of the remaining NBD lysine absorbance was found in one peak (Peak A, Fig. 5A) consisting of a mixture of cyanogen bromide peptides 11, 13, 15, and 17. Although NBD lysine fluorescence in labeled actin is sensitive to polymerization, no NBD was bound to lysine 113 in CB 11, a residue that had previously been shown to respond to polymerization by increased reactivity towards 1-fluoro-2,4-dinitrobenzene (25, 33). Instead the NBD in this fraction was bound to one or more of the lysines of CB 17.

Labeling Kinetics and Optimal Conditions for Lysine Labeling—On incubation of actin with NBD-Cl, adduct forma-
tion with cysteine proceeded rapidly as indicated by the 400 nm absorbance. (The difference in absorbance with and without cysteine labeling, not shown here, showed the cysteine NBD peak to be at 420 nm.) Lysine NBD formation (= absorbance increase at 490 nm) was very slow initially and accelerated when the 400 nm absorbance was reaching its plateau value (Fig. 6A). With exciting wavelengths of 355 and 470 nm, fluorescence emission at 530 nm increased in parallel with the absorbance at 490 nm (Fig. 6, compare curve 2 in A with curve 4 in C). The relationship between the kinetics of the absorbance changes at 400 and 490 nm suggests that the rapidly formed cysteine adduct may have been the precursor for NBD lysine as previously described for other proteins (34, 35). The potential for transfer from cysteine to lysine was demonstrated by labeling native F-actin with NBD for 60 min, quickly freeing it from excess NBD by centrifugation, and monitoring the changes in the absorption spectrum with time (Fig. 7). The actin continued to gain NBD lysine absorbance at the expense of the NBD cysteine compound, the absorbance of which steadily decreased. However, smearing of the isosbestic point and a comparison of the loss of NBD cysteine with the gain of NBD lysine indicates that not all of the NBD modification considerably (Fig. 6, B and C, Curves 1 to 3), suggesting that NBD cysteine inhibited the much more rapid direct interaction between NBD and some of the lysines. The increase in the ratio of fluorescence emission $\lambda_{exc}/A_{430}$ (in arbitrary units) after N-ethylmaleimide pretreatment (Table I) indicates first, that not all lysine NBD adducts fluoresced with the same quantum yield, and second, that NBD cysteine interfered preferentially with the formation of fluorescent NBD lysine adducts which exhibit the highest quantum yields. The rate of formation of the fluorescent lysine NBD adducts did not increase proportionally with the concentration of NBD-C1 above 0.4 mM (Fig. 6C, Curves 1 to 3), independent of N-ethylmaleimide blocking of cysteine 373 absorbance and fluorescence emission did not increase in parallel (Table I) and labeling did not exhibit pseudo-first order kinetics. Instead the maximal level of fluorescent labeling was lower after incubation with 2 mM than after incubation with 0.4 mM NBD-C1, and at NBD-C1 concentrations above 0.4 mM the fluorescence after reaching a maximum declined again with

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**Fig. 4.** Excitation and emission spectra of polymerized NBD-N-ethylmaleimide actin and a comparison of the shape of the emission spectra between G- and F-actin. Excitation spectrum measured with 530 nm emission, and the emission spectrum excited with 470 nm. S-1 added in 10 $\mu$M excess over actin; O, G-actin scaled up 2.2 times.

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**Fig. 5.** Chromatography of cyanogen bromide cleavage products of NBD-N-ethylmaleimide actin. A, Sephadex G-50 in 25% acetic acid; B, Sephadex G-25 chromatography (25% acetic acid) of Peak B after it had been chromatographed on SP-Sephadex (elution by gradient of 0 to 10% pyridine—25% acetic acid); C, Sephadex G-10 chromatography of Peak A; sample in 70% formic acid column developed in 0.01 M pyridine-acetate.

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**Fig. 6.** Time course of NBD labeling of actin. A, a comparison between cysteine 373 (400 nm) and lysine (490 nm) labeling during incubation of native actin (1 mg/ml) in 0.4 mM NBD-C1 at 15°C. Curve 3, extrapolation of the initial rate of lysine labeling to show what fraction of lysines could have been maximally labeled directly as distinct from transfer from cysteine 373. B, lysine labeling of N-ethylmaleimide actin, conditions as for A. C, lysine labeling of N-ethylmaleimide actin and native actin with increasing NBD-C1 concentrations: 0.4 mM (Curves 1 and 4), 0.8 mM (Curves 2 and 5), 2.0 mM (Curve 3). Arbitrary units of emission calculated for 0.1 mM actin.
Fig. 7. Lysine labeling by NBD transfer from NBD Cys 373 as indicated by transfer of absorbance from 420 to 480 nm (4 of 0.1 mM actin). NBD labeling of native actin for 60 min at 15°C, removal of excess NBD by centrifugation and washing, Curves 1, after final dilution (about 90 min after addition of NBD-C1); Curve 2 = 30 min; Curve 3 = 60 min; Curve 4 = 120 min; Curve 5 = 180 min; Curve 6 = 300 min later.

TABLE I

| Incubation time (min) | Emission (nm) | 480 nm | 530 nm |
|-----------------------|--------------|--------|--------|
| 30                    | 1.6          | 3.3    |        |
| 45                    | 2            | 3.3    |        |
| 60                    | 2.1          | 3.3    |        |
| 90                    | 2.1          | 3      |        |
| 120                   | 2.5          | 2.8    |        |
| 180                   | 2.2          | 2.3    |        |
| 240                   | 2            | 2.1    |        |
| 300                   | 1.7          | 1.8    |        |

Actin preparations

| Emission/Actin | Emission/F-Actin |
|---------------|-----------------|
| NBD           | 2.4             |
| NBD + dithiothreitol | 1.8 | 2.3 |
| NBD N-ethylmaleimide | 3     |
| NBD N-ethylmaleimide + dithiothreitol | 2.9 | 2.3 |

⁴ 0.4 mM NBD-Cl.
⁵ In arbitrary units.
⁶ A 5-h incubation in 0.4 mM NBD-Cl followed by removal of NBD by polymerization cycles and dialysis.
⁷ Dithiothreitol treatment after NBD removal.

Time, i.e., on prolonged incubation fluorescence intensity was lost. This loss in fluorescence intensity was associated with a decline in the yield of polymerizable actin with increasing incubation periods at high NBD-Cl concentrations.

From these studies we concluded that the following conditions are optimal for preparing actin with maximal specific fluorescence, responding to polymerization with about a 2-fold increase in emission: 5-h incubation of N-ethylmaleimide-labeled polymerized actin with 0.4 mM NBD-Cl at 15°C for 5 h. Under these conditions a maximal, plateau, level of fluorescence was reached with an extent of labeling of 6.7 NBD/actin molecule, based on the reported extinction coefficient for NBD lysine of 2.6 × 10⁴ M⁻¹ cm⁻¹ at 480 nm (34). Since radioactive NBD is very difficult to prepare and could be obtained commercially only at enormous price, we did not determine the extinction coefficient for actin. If it is the same as the literature value, the fluorescence emission of actin was maximal when lysine 372 of 40% and the lysines of CB 17 of 14% of the actin molecules had been labeled. Response of NBD Lysine to Polymerization, Activity of NBD Actin, and S-I-induced Polymerization—Polymerization of actin approximately doubled the fluorescence intensity but did not cause any red or blue shift in the emission spectrum (Fig. 4). There was also no significant change in the shape of the excitation spectrum other than a very small reduction in the 400 to 440 nm region. The increase in fluorescence intensity was the same for NBD actin which had a low total emission due to the inhibitory effect of NBD Cys 373 on lysine labeling (after removal of NBD from cysteine by treatment with dithiothreitol) together with a low ratio of emission/Actin (Table I) and for NBD-N-ethylmaleimide actin with its higher fluorescence intensity and emission/Actin ratio.

Fig. 8, Curves 1 and 2, compares the fluorescence of N-ethylmaleimide-NBD actin with increasing actin concentrations in depolymerizing medium (0.5 mM ATP, 0.2 mM CaCl₂, 10 mM Tris, pH 8.0, 0.2 mM dithiothreitol) and after the addition of 0.1 mM KCl and 2.0 mM MgCl₂, optimal salt concentrations for polymerization.

NBD labeling did not interfere with polymerization. The critical concentrations for modified actin (Fig. 8) were somewhat lower than those reported in the literature for native actin (36, 37) and very similar to our own critical concentrations of native actin, measuring actin resistant to sedimentation. Furthermore, NBD actin apparently randomly copolymerized with native actin. This was not unexpected but does not necessarily follow from the similarity of their critical concentrations. The copolymerization is shown by the experiment of Fig. 8. Native and NBD-N-ethylmaleimide actin were mixed in increasing ratios, and the apparent critical concentration of only NBD-N-ethylmaleimide actin was measured by following the intensity change of fluorescence emission at 530 nm as just described. After polymerization the values for the apparent critical concentrations of the NBD-N-ethylmaleimide actin became lower in direct proportion to...
leimide actin for myosin ATPase. NEM, pure NBD-N-ethylmaleimide actin. Critical concentration calculated for total (NBD-N-ethylmaleimide actin) and NBD lysine adducts formed during polymerization, however. The reactions between actin and NBD are complex. 1) Fluorescence reached a peak value when a maximum of only 50% of the actin molecules contained one NBD lysine compound (using the extinction coefficient for NBD lysine previously reported (34)). 2) Fluorescence was lost with prolonged incubation periods. 3) The rate of appearance of fluorescence became independent of the NBD-C1 concentration and NBD concentrations above 0.4 mM, and the saturating NBD concentration range was the same for direct lysine labeling and for labeling by transfer from NBD cysteine 373.

Fluorescence emission excited at 470 nm was not significantly affected by the binding of the troponin-tropomyosin complex to the actin polymer and by binding of DNase I to the monomer (less than 6% quenching). Binding of S-1 produced a slight red shift and intensity increase in the emission spectrum of NBD-N-ethylmaleimide actin (compare Curves 1 and 2 of Fig. 4). This was similar to, although smaller than, the red shift and intensity increase observed upon S-1 binding to actin with IAEDANS attached to cysteine 373 (19). The emission increase was small compared to that caused by polymerization, however.

Fig. 9 shows that S-1 is also capable of lowering the critical concentration to 0.2 μM actin in the absence of KCl and Mg.

**Discussion**

Not all of the various NBD lysine adducts fluoresce with the same quantum yield since NBD lysine adducts formed after N-ethylmaleimide pretreatment fluoresced with a greater apparent quantum yield than NBD lysine compounds formed without N-ethylmaleimide pretreatment. The NBD lysine 372 adduct must be responsible for most of the fluorescence emission since it is the only single NBD lysine species that can account for the doubling to tripling of the total NBD lysine labeling after N-ethylmaleimide pretreatment and since it is the lysine most likely to be affected by the state of cysteine 373. Our data do not allow us to decide whether any of the other NBD lysine compounds, e.g. any of those in CB 17, are fluorescent since we do not know the extinction coefficients for any of the different NBD lysine adducts of actin. It is likely that the major fluorescent species, NBD lysine 372, is responsible for the fluorescence enhancement on polymerization although it cannot be ruled out completely that the response to polymerization could instead be due to a large increase in quantum yield of a minor fluorescent species. This would require that this minor species represent the same fraction of the total fluorescence under all conditions of labeling independent of N-ethylmaleimide pretreatment of the actin since the fluorescence increment was the same for NBD-labeled native actin and N-ethylmaleimide actin in spite of the differences in extent of labeling and the emission/A(373) ratios (Table 1). The conclusion that NBD lysine 372 is responsible for the fluorescence enhancement on polymerization agrees with the observation that IAEDANS attached to the neighboring cysteine 373 reacts to polymerization in a similar manner: fluorescence increase (although smaller than that of NBD lysines) without any change in the shape of the emission spectrum (19). Furthermore, cysteine 373 has been shown to be at an actin-actin interface by the cross-linking experiments of Knight and Offer (38) and the apposed surface of an adjacent actin monomer could well influence the fluorescence of a monomer located at that interface. That the modified cysteine 373 is more sensitive to myosin binding and lysine 372 to polymerization, suggests that the lysine may be more deeply inserted in the actin-actin interface than the cysteine.

The reactions between actin and NBD are complex. 1) Fluorescence reached a peak value when a maximum of only 50% of the actin molecules contained one NBD lysine compound (using the extinction coefficient for NBD lysine previously reported (34)). 2) Fluorescence was lost with prolonged incubation periods. 3) The rate of appearance of fluorescence became independent of the NBD-C1 concentration and NBD concentrations above 0.4 mM, and the saturating NBD concentration range was the same for direct lysine labeling and for labeling by transfer from NBD cysteine 373.

Loss of fluorescence during prolonged incubation with NBD-C1, but not during storage of NBD actin in the absence of NBD-C1, may be caused by two kinds of adduct formation that influence actin fluorescence in an antagonistic way. One adduct may be responsible for fluorescence emission and the other adduct for quenching of this fluorescence. The quenching could well be the result of structural changes in the actin molecule. Whether fluorescence reached a maximum before 1 NBD/actin had been attached or whether the reported extinction coefficient (34) does not apply to actin cannot be decided.

Saturation of the rate of fluorescence development at NBD concentrations above 0.4 mM is easily explained by assuming a nonfluorescent actin NBD-precursor complex. However, the rate of fluorescence development was saturated at the same NBD-C1 concentrations before and after N-ethylmaleimide pretreatment, although NBD cysteine 373 could have been the precursor only in the first and not in the second case. The similarity in the saturating concentration range suggests that even in the presence of NBD cysteine 373 a significant fraction of the lysine did not receive its NBD from NBD cysteine 373. Reaction of lysine 372 with actin-bound NBD rather than NBD in solution may also explain the relatively high rate of labeling observed with actin as compared with the much slower reaction rates reported for amino groups in solution below pH 8 (39, 34) or observed by us with sulfhydryl-blocked tropomyosin which was barely labeled at all in 5 h.

NBD actin is especially useful as a probe if it copolymerizes randomly with native actin. Similarity of critical concentration does not guarantee copolymerization, but the two actin species must be said to copolymerize when polymerization occurs at concentrations that are above critical for the sum of both species but below critical for either species alone. However, that does not necessarily indicate that the two species copolymerize randomly, i.e. that the same rate constants govern all possible actin-actin interactions. It is possible that three different sets of constants determine binding of NBD
actin to NBD actin, binding of native to native, and binding of NBD actin to native actin, so that the polymers are not completely random. However, in that case one would not have expected the apparent critical concentration of NBD actin to have been altered in exact proportion to the ratio of native to NBD actin over a wide range of ratios. That it did change in exact proportion strongly suggests that there is no difference between native and NBD actin with respect to polymerization.

Monitoring polymerization by fluorescence has the advantage over viscosity measurements and centrifugation studies in that the polymerized actin is not subject to shearing forces or high pressure. Although bleaching of the probe does not permit continuous recording sampling can be more frequent than with viscosity measurements. Changes in size and aggregation of filaments interfere with a fluorescence signal much less than they distort light-scattering measurements. Furthermore, because of the specificity of the NBD response to actin the presence of actin-binding proteins does not interfere with the signal. The high quantum yield of the NBD actin allows measurements at very low actin concentrations, with about 5 \times 10^{-7} M as the limiting actin concentration.

NBD actin has the following advantages over other spectroscopic probes sensing polymerization. 1) It can be used in very much lower concentrations (5 \times 10^{-8} M) than spin-labeled actin (40), in an open cuvette which permits addition of reagents as compared to the closed compartment for EPR measurements. 2) It bleaches less than 5-dimethylaminophthalene-1-sulfonyl dansyl ethanoamine probes (19, 41) especially IAEODANS (19) which is very important for measurements under conditions of high viscosity where the bleached molecules in the exciting beam are not readily replaced by unbleached molecules. NBD actin has a higher quantum yield than the 5-dimethylaminophthalene-1-sulfonyl dansyl ethanoamine actins and therefore allows measurements at lower actin concentrations. 3) In contrast to Cys 373-based probes, NBD lysine responds only insignificantly to a large variety of binding proteins.

The observation that S-1 causes polymerization in the absence of KC1 and Mg2+ confirms earlier observations made in this laboratory by Beschomer and by Marston using different methods of measurement, i.e. activation of S-1 ATPase activity and centrifugation, respectively. Marston actually measured critical concentrations and found them to be in a similar range to those reported here. Our experiments differ from those of others in that we checked the critical concentration of actin alone in the absence of KC1 and Mg2+. It appeared that the absence of KC1 and Mg2+ slowed down nucleation more than elongation. Although at concentrations above 30 \mu M, actin at pH 8.0 and 15-25°C remained monomeric for hours, it polymerized slowly after the addition of nuclei. Below 30 \mu M it did not polymerize under these conditions. Thus we established that both heavy meromyosin and S-1 truly lowered the critical concentration as distinct from accelerating nucleation which was not the case in some of the earlier determinations (12). A decrease of the critical concentration by 100-fold or more indicates that both myosin fragments bind much more strongly to actin filaments than to monomeric actin. We are in the process of finishing up a detailed study of the effect of myosin fragments on the kinetics of polymerization.

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2 Unpublished results.