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Articles

Transient Kinetic Analysis of Rhodamine Phalloidin Binding to Actin Filaments†

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ABSTRACT: We have characterized the binding of rhodamine phalloidin to actin filaments and actin filaments saturated with either myosin subfragment-1 or tropomyosin in 50 mM KCl, 1 mM $MgCl_2$ buffer at pH 7.0. Direct transient kinetic measurements of rhodamine phalloidin binding to actin filaments indicate an association rate constant of $2.8 \times 10^4\ M^{-1}\ s^{-1}$ and a dissociation rate constant of $4.8 \times 10^{-4}\ s^{-1}$. The ratio of the rate constants yields a dissociation equilibrium constant of 17 nM. From equilibrium measurements, the apparent affinity of rhodamine phalloidin for actin filaments is 116 nM. The difference between the affinities determined by equilibrium and kinetic experiments is attributed to the depolymerization of filaments at low actin concentrations in the equilibrium samples. The binding stoichiometry is one rhodamine phalloidin molecule per actin subunit. When myosin subfragment-1 and tropomyosin are bound to actin filaments, the rate constants for rhodamine phalloidin binding are the same as for actin alone and in agreement with the binding affinities measured in equilibrium experiments. Presumably these proteins stabilize the filaments. Neither substitution of $CaCl_2$ for $MgCl_2$ nor the inclusion of 20 mM phosphate altered the rate or equilibrium constants.

Phalloidin, a heptapeptide toxin from the mushroom *Amanita phalloides*, binds tightly and specifically (Vandekerckhove *et al.*, 1985) to polymerized actin (Estes *et al.*, 1981). Binding stabilizes actin filaments by preventing monomer dissociation at both the barbed and pointed ends of the polymer (Estes *et al.*, 1981; Coluccio & Tilney, 1984; Sampath & Pollard, 1991). Phalloidin also decreases the association rate constant of actin at the barbed end by about 50% (Coluccio & Tilney, 1984; Sampath & Pollard, 1991). Each phalloidin molecule binds to an actin subunit and two neighboring actin subunits through both hydrogen bonds and van der Waals interactions (Lorenz *et al.*, 1993). The fact that each phalloidin molecule contacts three actin subunits simultaneously provides a rationale for the high specificity of phalloidin binding to actin filaments and its ability to stabilize the polymer (Wieland & Faulstich, 1978; Le Bihan & Gicquaud, 1991).

Fluorescent derivatives of phalloidin are frequently used to visualize and quantitate actin filaments in cells (Howard & Oresajo, 1985; Cooper, 1987), in solution (Käs *et al.*, 1994), and in *in vitro* motility assays (Kron *et al.*, 1991). The most commonly used derivative is rhodamine phalloidin. Previous studies have shown that the dissociation equilibrium constant for the phalloidin–actin complex is between 40 and 400 nM (Faulstich *et al.*, 1977; Huang *et al.*, 1992; Cano *et al.*, 1992). The rates of interaction have been determined by a pelleting assay and indicate a slow association rate (Cano *et al.*, 1992). Since phalloidin inhibited the dissociation of subunits from actin filaments diluted below the critical concentration better than expected from the observed rate and equilibrium constant, Cano *et al.* postulated that phalloidin has a higher affinity and binding rate at actin filaments ends, where it might prevent the dissociation of the terminal subunits.

This study was initiated to reevaluate the affinity and kinetics of rhodamine phalloidin binding to actin filaments. We used the fluorescence enhancement assay described by Huang *et al.* (1992) to determine the affinity and kinetic rate constants of rhodamine phalloidin binding to actin filaments, tropomyosin-saturated actin filaments, and myosin subfragment-1-saturated actin filaments in 50 mM KCl, 1 mM $MgCl_2$ buffer at pH 7.0. We find that the association rate constant and binding affinity of rhodamine phalloidin for actin filaments are higher than previously reported (Cano *et al.*, 1992; Huang *et al.*, 1992). Our rate constants and affinities can account for the actin filament stabilizing properties of phalloidin observed by Cano *et al.* (1992) without postulating differential binding of phalloidin to polymer ends. We also report kinetic measurements of rhodamine phalloidin binding to actin filaments in the presence of 20 mM inorganic phosphate and when Ca^{2+} is substituted for Mg^{2+}.

MATERIALS AND METHODS

Reagents. Sigma Chemical Co. (St. Louis, MO) supplied all salts, buffers, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), NaN_3, ATP, Sephadex G-150, and phalloidin. Dithiothreitol was obtained from Boehringer Mannheim Corp. (Indianapolis, IN) or Sigma. Molecular Probes (Eugene, OR) provided rhodamine phalloidin which we stored as a 20 μM stock in ethanol.

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Protein Purification and Modification. Actin was purified from rabbit skeletal back and leg muscles by the method of Spudich and Watt (1971) and stored as Ca^{2+}−actin in continuous dialysis at 4 °C against buffer A (0.2 mM ATP, 0.5 mM DTT, 0.1 mM $CaCl_2$, 1 mM NaN_3, and 2 mM Tris, pH 8.0). Mg^{2+}−actin was made by adding 200 μM EGTA and 80 μM $MgCl_2$ to Ca^{2+}−actin in buffer A essentially as described (Gershman *et al.*, 1989; Kinosian *et al.*, 1991). Actin concentration was determined by the absorbance at 290 nm using $\epsilon = 2.66 \times 10^4\ M^{-1}\ cm^{-1}$. Tropomyosin was prepared from rabbit skeletal muscle (Bailey, 1948). Rabbit skeletal muscle myosin subfragment-1A2 prepared by chymotryptic digestion was kindly provided by Dr. Ed Taylor of the University of Chicago.

Equilibrium Binding Experiments. For equilibrium binding experiments, actin in buffer A was polymerized by adding 0.1 volume of 10× KMEI (500 mM KCl, 10 mM $MgCl_2$, 10 mM EGTA, and 100 mM imidazole, pH 7.0) to generate a stock solution of actin filaments at a concentration of 4−20 μM in 1× polymerizing buffer. After the actin was allowed to polymerize at room temperature for at least 2 h, the solution was diluted with 1× polymerizing buffer to the desired actin concentrations. Fifty microliters of rhodamine phalloidin stock solutions in 1× polymerizing buffer was added to 950 μL of actin, and the fluorescence intensity was allowed to reach steady-state. The final concentration of rhodamine phalloidin was 20 nM. Fluorescence intensity (λ_{ex} = 550 nm, λ_{em} = 580 nm) was recorded on a Photon Technology International Alphascan or a Hitachi Perkin Elmer 650−10S fluorescence spectrophotometer. We found a 20-fold fluorescence enhancement of rhodamine phalloidin when it bound to actin filaments. Huang *et al.* (1992) observed a 10-fold increase.

Actin filaments in 20 mM inorganic phosphate were made by adding 0.1 volume of 10× KPMEI (200 mM K_2HPO_4, 100 mM KCl, 10 mM $MgCl_2$, 10 mM EGTA, and 100 mM imidazole, pH 7.0) to Mg^{2+}−actin or Ca^{2+}−actin. At pH 7.0 and 20 mM phosphate, actin filaments are saturated with bound phosphate (Carlier & Pantaloni, 1988). Ca^{2+}−actin filaments were obtained by polymerizing actin in buffer A with 0.1 volume of 10× KCI (500 mM KCl, 10 mM $CaCl_2$, and 100 mM imidazole, pH 7.0). Acto−tropomyosin and actomyosin subfragment-1 filaments were made by polymerizing actin with 0.1 volume of 10× KMEI with subsequent addition of tropomyosin or myosin subfragment-1 to the indicated concentrations.

Transient Kinetic Experiments. The observed rate constant (k_{obs}) for rhodamine phalloidin binding to actin was determined by measuring the time course of fluorescence intensity increase after mixing equal volumes of either 20 or 100 nM rhodamine phalloidin with varying concentrations of actin filaments each in 1× polymerizing buffer. Measurements were made using a Photon Technology International Alphascan or a Hitachi Perkin Elmer 650-10S fluorescence spectrophotometer equipped with a Model SFA-12 Rapid Kinetics Stopped-Flow Accessory (Hi-Tech Scientific Ltd., Salisbury, U.K.) thermostated at 25 °C. The dead time of the mixer is 60 ms.

Transients were fitted to single exponentials with a nonlinear least-squares curve-fitting program employing the Marquardt algorithm (Press et al., 1987). In some instances, the observed rate constant (k_{obs}) were calculated from the half-time ($t_{0.5}$) of the reaction, where $k_{obs} = 0.693/t_{0.5}$ The association rate constant (k_+) was determined from the dependence of k_{obs} on actin filament concentration.

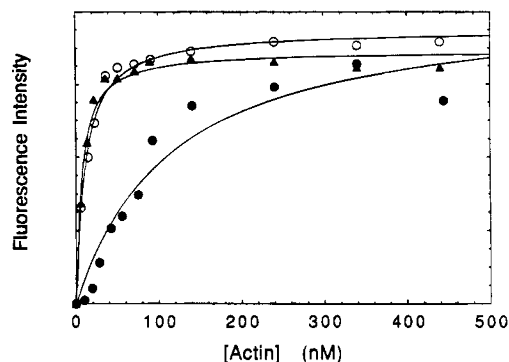


FIGURE 1: Titration of 20 nM rhodamine phalloidin with actin filaments (●), actin filaments in 1 μM tropomyosin (○), and actin filaments in 1 μM myosin subfragment-1 (▲) in 1× polymerizing buffer. Fluorescence emission intensities were recorded at 580 nm with excitation at 550 nm at 22 °C. The fluorescence intensity of 20 nM rhodamine phalloidin in the absence of actin was subtracted from each point. The data were fitted to hyperbolic curves using a nonlinear regression program (Press et al., 1987). The apparent K_d's for rhodamine phalloidin binding are 116.2 nM for actin, 11.0 nM for acto−tropomyosin, and 7.0 nM for acto−myosin subfragment-1.

Dissociation Experiments. The dissociation rate constant of the rhodamine phalloidin−actin complex was determined by displacing bound rhodamine phalloidin with excess unlabeled phalloidin. Actin samples were polymerized in 1× polymerizing buffer containing different concentrations of rhodamine phalloidin and equilibrated at room temperature for at least 4 h. The time course of the decrease in fluorescence intensity was monitored upon the addition of at least a 20-fold molar excess of phalloidin from a 2 mM stock in ethanol. The final ethanol concentration after mixing did not exceed 0.5%. Data were fitted to single-exponential decays. Samples containing Ca^{2+}, Mg^{2+}, Mg^{2+} and inorganic phosphate, tropomyosin, or myosin subfragment-1 were prepared as described above.

RESULTS

Affinity of Rhodamine Phalloidin for Actin, Acto−Myosin Subfragment-1, and Acto−Tropomyosin Filaments. The binding affinity of rhodamine phalloidin for actin filaments was determined by titrating 20 nM rhodamine phalloidin with actin filaments and recording the fluorescence intensity (Figure 1). When fit to a hyperbola, the apparent K_d is 116 nM, in agreement with Cano *et al.* (1992), who reported an affinity of 100−400 nM. However, the data fall on a sigmoidal rather than a hyperbolic curve. The sigmoidal curve is experimentally reproducible. Therefore, an accurate binding affinity of rhodamine phalloidin for actin cannot be determined from equilibrium binding experiments since the data do not fall on a hyperbola as is required for a true binding isotherm. In contrast, titrations of rhodamine phalloidin with actin filaments saturated with myosin subfragment-1 or tropomyosin are hyperbolic, giving K_d's of 11 nM for acto−S1 and 7 nM for acto−tropomyosin filaments (Figure 1). The actin concentration dependence of binding is consistent with a stoichiometry of 1 actin monomer:1 rhodamine phalloidin molecule and inconsistent with a stoichiometry of 2 actin monomers:1 rhodamine phalloidin molecule.

To confirm the stoichiometry of the phalloidin−actin complex, actin filaments were titrated with rhodamine

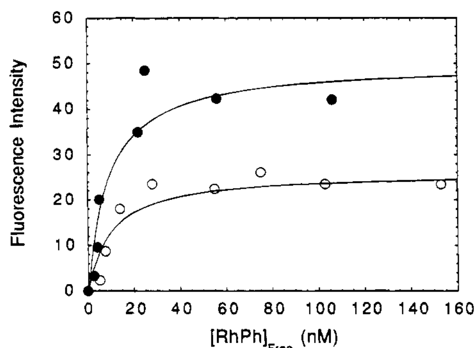
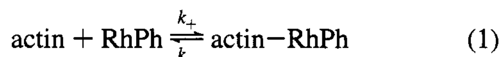


FIGURE 2: Titration of actin with rhodamine phalloidin. Stock solutions of actin filaments in 1× polymerizing buffer were diluted with a range of rhodamine phalloidin concentrations to give final actin concentrations of 50 (○) or 100 nM (●). After 2 h at 22 °C, fluorescence was measured. Absolute fluorescence values were corrected by subtracting the background fluorescence of the corresponding rhodamine phalloidin concentrations in the absence of actin. The curves represent the best fits through the data points. The K_d's are 10.15 nM for the 50 nM actin data set and 9.11 nM for the 100 nM actin data set. The data points are the average of two experiments.

phalloidin (Figure 2). When the free rhodamine phalloidin concentration is calculated assuming a stoichiometry of 1 rhodamine phalloidin molecule per actin monomer (Wieland & Faulstich, 1978; Cano *et al.*, 1992; Huang *et al.*, 1992; Naber *et al.*, 1993), the estimated K_d's are 10.2 and 9.1 nM for the 50 and 100 nM actin data sets, respectively.

Kinetic Analysis of Rhodamine Phalloidin Binding to Actin. (a) Determination of the Association Rate Constant. The interaction of rhodamine phalloidin with actin can be described as a simple second-order binding process:



$$\text{actin} + \text{RhPh} \underset{k_-}{\overset{k_+}{\rightleftharpoons}} \text{actin}-\text{RhPh} \quad (1)$$

where k_+ is the second-order association rate constant and k_- is the dissociation rate constant. A second-order reaction can be analyzed as a first-order process if the molar concentration of one species, in this case actin, is far greater than the second. Under such pseudo-first-order conditions

$$k_{obs} = k_+[A] + k_- \quad (2)$$

where k_{obs} is the experimentally observed rate constant and [A] is the actin concentration.

The time course of the fluorescence change generated from rapidly mixing rhodamine phalloidin and actin filaments (Figure 3A) fit a single exponential, shown as solid lines through the data points. The transients are not affected by addition of 2 μM tropomyosin or by a 1:1 molar ratio of myosin subfragment-1 to actin subunits (not shown).

The observed rate constant, k_{obs}, depends linearly on the actin filament concentration over a wide range (Figure 3B). The slope of the line yields a k_+ of 2.8×10^4 M^{-1} s^{-1}. The association rate constant (k_+) is not significantly affected by 2 μM tropomyosin or myosin subfragment-1 at a 1:1 ratio with actin (data not shown).

(b) Determination of the Dissociation Rate Constant. The dissociation rate constant (k_-) of rhodamine phalloidin from actin filaments was determined from the time course of fluorescence decay when 200-fold molar excess of unlabeled phalloidin was added to an equilibrium sample of 300 nM polymerized actin and 100 nM rhodamine phalloidin (Figure

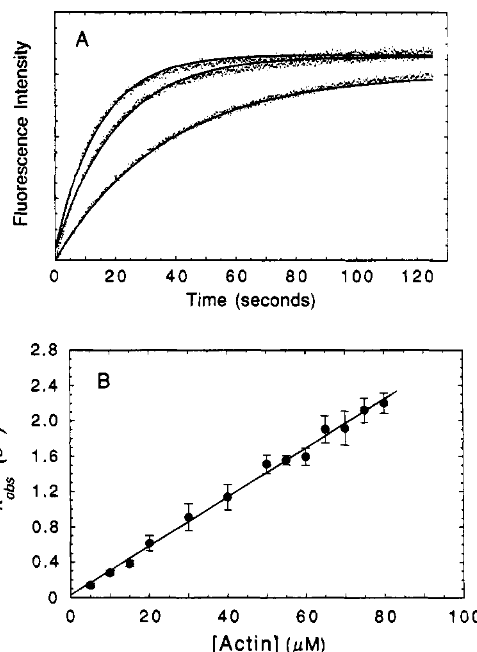


FIGURE 3: Kinetics of rhodamine phalloidin binding of actin filaments. (A) Time course of the fluorescence change after mixing 10 nM rhodamine phalloidin with 4 μM (left), 3 μM (middle), and 2 μM (right) actin filaments in 1× polymerizing buffer. The final steady-state fluorescence intensities were the same for all samples. The solid lines represent the best fits to single exponentials. (B) Dependence of the observed rate constant (k_{obs}) for association of actin and rhodamine phalloidin as a function of actin concentration. The association rate constant (k_+) for the reaction is 2.8×10^4 M^{-1} s^{-1}. Vertical bars represent ±1 standard deviation from the mean ($n = 4-16$).

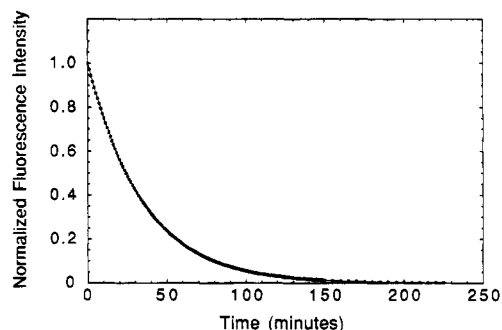


FIGURE 4: Time course of the dissociation of rhodamine phalloidin from actin filaments. A mixture of 300 nM polymerized actin and 100 nM rhodamine phalloidin was equilibrated in 1× polymerizing buffer at room temperature (22 °C) for 4 h. Changes in fluorescence intensity were monitored after the addition of 20 μM phalloidin. The solid line represents the best fit to a single exponential yielding a dissociation rate constant (k_-) of 4.6×10^{-4} s^{-1}.

4). As expected for a first-order process, k_- is independent of the actin concentration. From a series of experiments varying the actin, rhodamine phalloidin, and phalloidin concentrations, k_- is calculated to be 5×10^{-4} s^{-1} (SD = $\pm 1 \times 10^{-4}$, $n = 5$). The time course of dissociation of rhodamine phalloidin from actin is not affected by a 5-fold molar excess of tropomyosin or a 2-fold molar excess of myosin subfragment-1 over actin (data not shown). From the ratio of the rate constants, we calculate K_d's of 17 nM for rhodamine phalloidin binding to actin filaments, 17 nM for actin filaments saturated with tropomyosin, and 14 nM for actin filaments saturated with myosin subfragment-1. This is in close agreement with the equilibrium data for tropomyosin and myosin subfragment-1 shown Figure 1 (7 and

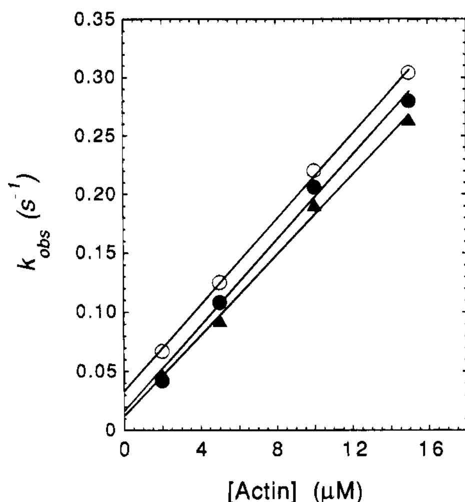


FIGURE 5: Effects of bound divalent cation and phosphate on the kinetics of rhodamine phalloidin binding to actin filaments. Mg^{2+}–actin was polymerized with 50 mM KCl, 10 mM imidazole (pH 7.0), and 1 mM $MgCl_2$ in the presence (○) or absence (●) of 20 mM inorganic phosphate; or Ca^{2+}–actin in buffer A was polymerized with 50 mM KCl, 10 mM imidazole (pH 7.0), and 1 mM $CaCl_2$ (▲). The association rate constants (k_+) are 0.017 s^{-1} for Mg^{2+}–actin, 0.018 s^{-1} for Ca^{2+}-actin, and 0.018 s^{-1} Mg^{2+}–actin in 20 mM phosphate in this series of experiments.

11 nM) but is nearly an order of magnitude greater than the apparent affinity of rhodamine phalloidin for actin alone (116 nM). We suggest that this discrepancy arises from depolymerization of actin filaments at low concentrations in the absence but not the presence of tropomyosin or myosin subfragment-1. As a result, the actin filament concentration is overestimated and the K_d is overestimated for actin alone.

Effect of Bound Divalent Cation and Phosphate on the Association and Dissociation Rates of Rhodamine Phalloidin with Actin. The association of rhodamine phalloidin in independent of the divalent cation bound to actin (Figure 5). The association rate constants (k_+) of rhodamine phalloidin binding to Mg^{2+}–actin filaments or Ca^{2+}–actin filaments are the same. The dissociation rate constant (k_-) obtained from displacement of bound rhodamine phalloidin with phalloidin is not significantly different either (5×10^{-4} s^{-1} for Mg^{2+}–actin and 4×10^{-4} s^{-1} for Ca^{2+}–actin; data not shown). The ratio of the dissociation and association rate constants gives a K_d of 17 nM.

Dancker and Hess (1990) reported that phalloidin decreases the rate of inorganic phosphate release during actin polymerization, so arguing from detailed balance, it is possible that phosphate bound to an actin filament might influence phalloidin binding. However, under conditions where the actin is saturated with phosphate (Carlier & Pantaloni, 1988), neither the k_+ (Figure 5) nor the k_- (5×10^{-4} s^{-1}, data not shown) of rhodamine phalloidin are significantly altered. Identical results were obtained for Mg^{2+}–actin and Ca^{2+}–actin polymerized in the presence of 1 mM $MgCl_2$.

DISCUSSION

Summary of Results. Rhodamine phalloidin binds to actin filaments with an association rate constant of 2.8×10^4 M^{-1} s^{-1} and a dissociation rate constant of 4.8×10^{-4} s^{-1} in 50 mM KCl, 1 mM $MgCl_2$ buffer at pH 7.0. The linear concentration dependence of k_{obs} on the actin concentrations examined indicates that if a conformational change of rhodamine phalloidin occurs after binding to actin, must occur at a rate greater than 2.5 s^{-1}. This could not be tested at higher actin concentrations, as the high viscosities of samples containing greater than 80 μM actin filaments prevented efficient mixing of rhodamine phalloidin and actin.

The association and dissociation rate constants of rhodamine phalloidin binding to actin are not affected by saturating the filaments with myosin subfragment-1 or tropomyosin. The dissociation equilibrium constant calculated from the ratio of the rate constants is 14–17 nM. From equilibrium studies, at affinity of 116 nM is determined for actin, 7 nM for actin saturated with tropomyosin, and 11 nM for actin saturated with myosin subfragment-1. We believe that the apparent lower affinity for actin filaments without tropomyosin or myosin subfragment-1 arises from the depolymerization of the filaments when they are diluted sufficiently far below the critical concentration of actin. At very low actin concentrations, the actual concentration of filaments in the sample is much less than the total actin concentration. The hyperbolic dependence of binding to actin filaments seen in Figure 1 reflects the absence of filaments at low actin concentrations. Since the ligand concentration (actin filaments) is overestimated, the binding affinity is underestimated. Inclusion of tropomyosin or myosin subfragment-1 inhibits depolymerization of the filaments and permits a more accurate determination of binding affinity. Since the association and dissociation rate constants are not affected when filaments are saturated with tropomyosin or myosin subfragment-1, these two proteins must not affect rhodamine phalloidin binding to actin filaments.

The actin concentration dependence of rhodamine phalloidin binding (Figure 1) is consistent with a stoichiometry of 1 actin monomer:1 rhodamine phalloidin molecule and inconsistent with a stoichiometry of 2 actin monomers:1 rhodamine phalloidin molecule. To confirm the binding stoichiometry of the rhodamine phalloidin–actin complex, actin was titrated with rhodamine phalloidin (Figure 2). When the free rhodamine phalloidin concentration was calculated assuming a stoichiometry of 1 actin monomer:1 rhodamine phalloidin molecule (Cano *et al.*, 1992; Huang *et al.*, 1992; Naber *et al.*, 1993), the K_d's obtained were 9 and 10 nM. This is in close agreement with the binding affinity determined from equilibrium experiments (Figure 1) and from the ratio of kinetic rate constants. We conclude that there is one rhodamine phalloidin bound per actin monomer incorporated into a filament as previously reported (Wieland & Faulstich, 1978; Cano *et al.*, 1992; Huang *et al.*, 1992; Naber *et al.*, 1993).

The association and dissociation rate constants are the same for Mg^{2+}–actin filaments and Ca^{2+}–actin filaments, and for actin polymerized in the presence of 20 mM inorganic phosphate. This suggest that minor structural changes in the nucleotide and divalent cation binding cleft of actin known to affect the polymerization properties of actin do not alter the rhodamine phalloidin binding site on the filament in a way that we could detect.

Relation to Earlier Work. Binding affinities between 37 and 400 nM have been reported for rhodamine phalloidin and actin filaments. Our range of affinities, 10–20 nM, indicates slightly greater binding affinity than reported by Huang *et al.* (1992) and Faulstich *et al.* (1977), who obtained K_d's of 40 and 37 nM using fluorescence enhancement and

equilibrium dialysis, respectively. Cano *et al.* (1992) reported a dissociation equilibrium constant of 200 nM for tetramethylrhodamine phalloidin. As noted by Huang *et al.* (1992), a dissociation equilibrium constant greater than 100 nM cannot account for the efficient actin filament staining at 20 nM rhodamine phalloidin observed by Yanagida *et al.* (1984). Our determined binding affinity of 10–20 nM accounts for the efficient labeling.

The association and dissociation rate constants obtained by Cano *et al.* for tetramethylrhodamine phalloidin are smaller than our determined values for rhodamine phalloidin. It is important to note that the binding affinity determined by Cano *et al.* from the ratio of the rate constants is in agreement with the affinity determined from their equilibrium experiments. The differences between their values and ours may reflect the addition of four methyl groups on the probe. In addition, there are some differences in both of the assays employed. The assay used by Cano *et al.* requires the pelleting of tetramethylrhodamine phalloidin stained actin filaments followed by methanol extraction of the phalloidin for 24–48 h at which point the fluorescence intensity of the extracted supernatant is compared to known standards. The fluorescence enhancement assay relies on the increase in fluorescence quantum yield of rhodamine phalloidin when it binds to actin filaments and can be used to measure the reaction in real time. Since the fluorescence enhancement assay directly measures the time course of the binding reaction without any other experimental procedures, measurement of kinetic transients generated from fluorescence enhancement may be more accurate for obtaining kinetic parameters for rhodamine phalloidin binding to actin filaments than the pelleting assay.

Implications for Actin Filament Structure. Our determined association rate constant for rhodamine phalloidin binding to actin filaments is orders of magnitude smaller than one expects for protein–ligand association reactions (Northrup & Erickson, 1992). Theoretical values for the maximum association rate constant of a diffusion-limited reaction can be calculated from the Smoluchowski equation [see Berg and von Hippel (1985)], which defines the collision rate constant, $k_{\text{collision}}$, for two spheres diffusing randomly in solution:

$$k_{\text{collision}} = 4\pi(D_A + D_B)(r_A + r_B)N_0 10^{-3}$$

where N_0 is Avogadro's number, 10^{-3} is the ratio of L cm^{-3}, D_A and D_B are the translational diffusion coefficients (in cm^2 s^{-1}) for the two reaction species, and r_A and r_B are their interaction radii (in cm). We will use the interaction radius of a single actin monomer, since one rhodamine phalloidin binds per actin subunit incorporated into a filament (Figure 2; Cano *et al.*, 1992; Huang *et al.*, 1992; Naber *et al.*, 1993; Lorenz *et al.*, 1993). The interaction radius of an actin monomer is taken to be 3×10^{-7} cm, based on the crystal structure (Kabsch *et al.*, 1990). We will consider an actin filament to be a collection of immobilized monomers since actin filaments have a translational diffusion coefficient of $<10^{-9}$ cm^2 s^{-1} (Tait & Frieden, 1982). An interaction radius of 5.7×10^{-8} cm for phalloidin was estimated on the basis of the refined actin filament model of Lorenz *et al.* (1993) and the NMR solution structure of phalloidin (Kessler & Wein, 1990). Using the Stokes–Einstein relation:

$$D = \frac{k_B T}{6\pi\eta r}$$

the diffusion coefficient of phalloidin in water at 22 °C was calculated to be 4×10^{-6} cm^2 s^{-1}. These parameters give $k_{\text{collision}}$ a value of 1.1×10^{10} M^{-1} s^{-1}, which is about 6 orders of magnitude greater than the association rate constant of rhodamine phalloidin binding to actin filaments. The "steric factor" takes into account the probability of the two species colliding in an orientation that leads to productive association (von Hippel & Berg, 1989). In the case of rhodamine phalloidin binding to actin filaments, the steric factor has a value 2.5×10^{-6}. This means that approximately 1 out of every 400 000 random encounters between rhodamine phalloidin and an actin subunit results in successful binding. This is a much lower binding frequency than expected for protein–protein interactions, in particular when microcollisions and rotational reorientation of rhodamine phalloidin and actin are considered [see Northrup and Erickson (1992)].

A small value for the steric factor suggests that not all of the rhodamine phalloidin binding sites in an actin filament are available at a given moment in time but that filament "breathing" (*i.e.*, when thermal forces perturb the regular structural organization of the filaments) is required to open cryptic binding sites for the ligand. Measurements on the flexural rigidity of actin filaments show that it is a rather flexible polymer displaying large thermal fluctuations in shape [see Oosawa (1993), Nagashima and Asakura (1980), Yanagida *et al.* (1984), and Gittes *et al.* (1993)] which are not affected by addition of rhodamine phalloidin (Gittes *et al.*, 1993) and only marginally affected by binding of myosin subfragment-1 (Yanagida *et al.*, 1984; Gittes *et al.*, 1993). Such a binding mechanism based on polymer breathing seems plausible from a structural point of view, since the phalloidin binding site is at the interface of three actin subunits within the filament (Lorenz *et al.*, 1993).

Conclusions. Rhodamine phalloidin binds to actin filaments assembled in Mg^{2+} and KCl with a 1:1 stoichiometry and a dissociation equilibrium constant of 10–20 nM. The kinetic rate constants for binding are $k_+ = 2.8 \times 10^4$ M^{-1} s^{-1} and $k_- = 4.8 \times 10^{-4}$ s^{-1}. Neither the binding affinity nor the kinetic rate constants are affected when the filaments are saturated with tropomyosin or myosin subfragment-1, indicating that the binding sites of the two proteins and phalloidin are exclusive. Furthermore, this indicates that there are no drastic structural perturbations of filament structure when tropomyosin or myosin subfragment-1 binds to actin. Similarly, Ca^{2+}–actin filaments and Mg^{2+}–ADP–P_i filaments bind rhodamine phalloidin with identical rates and affinities as Mg^{2+}–actin filaments, demonstrating that changes in the nature of the nucleotide and divalent cation which are known to affect polymerization do not disrupt the rhodamine phalloidin binding site on the filament. The slow association rate constant suggests that most of the rhodamine phalloidin binding sites on a filament are not available at one time but become accessible as the filaments experience thermal fluctuations in shape ("breathing").

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