Analysis of Tetramethylrhodamine-labeled Actin Polymerization and Interaction with Actin Regulatory Proteins*

Andrea Pelikan Conchaudron1, Dominique Didry2, Kim Ho Diep Le1, Eric Larquet3, Nicolas Boisset4, Dominique Pantaloni4, and Marie-France Carlier11

From the 1Dynamique du Cytosquelette, Laboratoire d'Enzymologie et Biochimie Structurale, CNRS, avenue de la Terrasse, 91198 Gif-sur-Yvette, France and the 4Institut de Minéralogie et de Physique de la Matière Condensée, Université Pierre et Marie Curie, 75252 Paris, France

The hydrolysis of ATP accompanying actin polymerization destabilizes the filament, controls actin assembly dynamics in motile processes, and allows the specific binding of regulatory proteins to ATP- or ADP-actin. However, the relationship between the structural changes linked to ATP hydrolysis and the functional properties of actin is not understood. Labeling of actin Cys374 by tetramethylrhodamine (TMR) has been reported to make actin non-polymerizable and enabled the crystal structures of ADP-actin and 5'-adenyl β,γ-imidodiphosphate-actin to be solved. TMR-actin has also been used to solve the structure of actin in complex with the formin homology 2 domain of mammalian Dia1. To understand how the covalent modification of actin by TMR may affect the structural changes linked to ATP hydrolysis and to evaluate the functional relevance of crystal structures of TMR-actin in complex with actin-binding proteins, we have analyzed the assembly properties of TMR-actin and its interaction with regulatory proteins. We show that TMR-actin polymerized in very short filaments that were destabilized by ATP hydrolysis. The critical concentrations for assembly of TMR-actin in ATP and ADP were only an order of magnitude higher than those for unlabeled actin. The functional interactions of actin with capping proteins, formin, actin-depolymerizing factor/cofilin, and the VCA-Arp2/3 filament branching machinery were profoundly altered by TMR labeling. The data suggest that TMR labeling hinders the intramolecular movements of actin that allow its specific adaptative recognition by regulatory proteins and that determine its function in the ATP- or ADP-bound state.

The regulated dynamics of actin assembly plays a pivotal role in cell motility. The hydrolysis of actin-bound ATP that accompanies actin polymerization is at the origin of treadmilling, which powers actin-based motility processes. Hydrolysis of ATP destabilizes actin-actin interactions in the filament and affects the structure of actin enough to specify the recognition of ATP- and ADP-actin by different regulatory proteins, yet the nature of the structural change that supports the functional importance of this reaction in actin following ATP hydrolysis remains a debated issue.

Crystallographic data indicate that the nucleotide-binding cleft of actin can be in an open or closed state; however, the open and closed states do not appear to logically correlate with the bound nucleotide being ADP (1–3) or ATP (4–9). Reconstructions of actin filaments indicate that the structure of ADP-F-actin is more consistent with the open state of actin (10, 11). The crystal structure of actin in complex with ciboulot, a Wiskott-Aldrich syndrome homology 2 (WH2) domain/β-thymosin repeat-containing protein that, like β-thymosin, specifically binds ATP-actin, shows that it is only in the closed state of actin that the conformation of the shear zone of actin between subdomains 1 and 3 allows the binding of WH2 domains (3). These two studies (3, 10) suggest that ATP-actin has a closed conformation, that ADP-actin has an open conformation, and that proteins that specifically bind ATP-actin maintain the closed conformation even though ATP has been hydrolyzed into ADP in the crystal.

In contrast with these conclusions, uncomplexed tetramethylrhodamine (TMR)-labeled actin harbors a closed structure in both the ADP- and AMPPNP-bound states; in addition, actin subdomain 2 adopts a novel helical fold in the ADP-bound state, whereas it displays the conventional disordered fold in the AMPPNP-bound state (2, 8). This observation suggests that the structural change in subdomain 2 is linked to ATP hydrolysis and is responsible for the weakening of longitudinal actin-actin bonds in the filament. This proposed structural effect of ATP hydrolysis was then questioned because the TMR probe, which locates at the shear zone linking subdomains 1 and 3 at the barbed face of actin, may interfere with the structural changes linked to the binding of ATP or ADP to unlabeled, fully functional actin (12).

Caution is required when trying to understand the functional properties of actin from the different crystal structures known.

* This work was supported by the Ligue Nationale contre le Cancer (Equipe Labellisée Ligue 2003–2006), Human Frontiers in Science Program Grant RGPO0712/2003-C, and the European Union Specific Targeted Research Project “Biomics.” The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed. Tel.: 33-1-6982-3465; Fax: 33-1-6982-3129; E-mail: carlier@lebs.cnrs-gif.fr.

‡ The abbreviations used are: WH2, Wiskott-Aldrich syndrome homology 2; TMR, tetramethylrhodamine; AMPPNP, 5’-adenyl β,γ-imidodiphosphate; ADF, actin-depolymerizing factor; FH2, formin homology 2; WASP, Wiskott-Aldrich syndrome protein.
First, crystal packing may introduce a bias favoring one of many structures that may exist in dynamic equilibrium in solution. To make things worse, if all these structures are energetically very similar, the structure favored by crystallization may not be the one that tends to predominate in solution upon binding a ligand like ATP or ADP. Second, crystal structures of actin are obtained by preventing actin polymerization by association with a polymerization-inhibiting protein or drug, by chemical modification, or by mutation. In all cases, the actin that is crystallized has lost its major functional property, which is to polymerize in filaments and to hydrolyze ATP upon assembly in filaments. Because actin is an efficient ATPase in the polymerized state only, the fact that TMR labeling renders actin unable to polymerize does not facilitate the correlation between the structural and functional properties of ADP- and ATP-actin.

To clarify these issues, a biochemical analysis of TMR-actin was carried out (13). Nucleotide exchange is slower on TMR-actin than on unmodified actin, consistent with the permanently closed structure of TMR-actin. The susceptibility of the DNase I-binding loop to proteolytic cleavage is similarly affected for TMR-actin and unmodified actin by the nature of the bound nucleotide, supporting the view that a structural change in labeled as well as unlabeled actin (presumably the disordered-to-ordered transition of subdomain 2) accompanies the replacement of ATP by ADP. On the other hand, solution studies of actin and TMR-actin proved unsuitable to distinguish the open and closed structures. Earlier works using partially labeled TMR-actin had established that TMR-actin copolymerizes with unlabeled actin (14, 15), thus enabling monitoring of actin dynamics in live cells by microinjection and the VCA domain of human neural WASP and the VCA domain of human neural Wiskott-Aldrich syndrome protein (WASP) were bacterially expressed and purified at least three times. Recombinant human gelsolin, human ADF, thymosin 1, and spectrin-actin seeds were prepared from human erythrocytes (23). The His-tagged recombinant formin homology 2 (FH2) domain of mammalian Dia1 was expressed and purified as described (24).

A detailed analysis of the structure-function relationship of 100% labeled TMR-actin and its interactions with several actin-binding proteins is carried out here. We show that TMR-actin copolymerizes, with a 30-fold higher critical concentration than unlabeled actin, into very short filaments that are destabilized by ATP hydrolysis. The filament structure, the assembly dynamics, and the functional interactions of actin with regulatory proteins are profoundly affected by the nature of the regulatory proteins. This work was performed with 22 different batches of TMR-actin, and each experiment was reproduced at least three times.

Spectrin-actin seeds were prepared from human erythrocytes (23). The His-tagged recombinant formin homology 2 (FH2) domain of mammalian Dia1 was expressed and purified as described (24).

To make things worse, if all these structures are energetically similar, the one that tends to predominate in solution upon binding a ligand like ATP or ADP. Second, crystal structures of actin are obtained by preventing actin polymerization by association with a polymerization-inhibiting protein or drug, by chemical modification, or by mutation. In all cases, the actin that is crystallized has lost its major functional property, which is to polymerize in filaments and to hydrolyze ATP upon assembly in filaments. Because actin is an efficient ATPase in the polymerized state only, the fact that TMR labeling renders actin unable to polymerize does not facilitate the correlation between the structural and functional properties of ADP- and ATP-actin.

**EXPERIMENTAL PROCEDURES**

**Proteins**—Actin was purified from rabbit skeletal muscle and isolated in the monomeric CaATP-G-actin form by gel filtration chromatography on Superdex 300 in buffer A (5 mM Tris-Cl (pH 7.8), 1 mM dithiothreitol, 0.1 mM CaCl₂, 0.2 mM ATP, and 0.01% NaN₃). Actin was pyrenyl-labeled (22). TMR labeling of actin was carried out at a concentration of 50–70 μM G-actin in 5 mM HEPES (pH 7.5), 0.1 mM CaCl₂, and 0.2 mM ATP with overnight incubation at 0 °C in the dark in the presence of 3 molar equivalents of TMR-maleimide (Molecular Probes). Following arrest of the reaction and gel filtration of the adduct in buffer A, the extent of labeling was determined using an extinction coefficient of 106,000 M⁻¹ cm⁻¹ at 557 nm for rhodamine combined with the Bradford assay (50) for actin using unlabeled actin as a standard. The TMR/actin molar ratio was 0.9 to 1.2 (data from 24 independent experiments). The solution of TMR-G-actin was stored on ice and used within 1 week. Different batches of labeled material gave identical values of the critical concentration and parameters of interaction with regulatory proteins. This work was performed with 22 different batches of TMR-actin, and each experiment was reproduced at least three times.

Spectrin-actin seeds were prepared from human erythrocytes (23). The His-tagged recombinant formin homology 2 (FH2) domain of mammalian Dia1 was expressed and purified as described (24).

Recombinant human gelsolin, human ADF, thymosin 1, and the VCA domain of human neural Wiskott-Aldrich syndrome protein (WASP) were bacterially expressed and purified (25). The Arp2/3 complex was purified from bovine brain (25).

**Electron Microscopy**—Negatively stained samples of filaments of TMR-actin polymerized at 7 μM were observed under a JEOL JEM-2100F UHR microscope at 50,000-fold magnification.

**Actin Polymerization Assays**—Unlabeled or TMR-labeled actin was traced with 0.2% pyrenyl-labeled actin. Actin assembly was monitored by following the increase in pyrene fluorescence using a SPEX Fluorolog-2 spectrofluorometer.

Sedimentation assays were performed by centrifuging samples of polymerized actin for 30 min at 400,000 × g in a Beckman TL-100 ultracentrifuge. Supernatants and resuspended pellets were analyzed by SDS-PAGE.

**ATP Hydrolysis Measurements**—The G-actin solution in buffer A was equilibrated with [γ-32P]ATP. The cleavage of ATP was monitored by measuring acid-labile 32P (26). Polymerization was monitored in the spectrofluorometer, and aliquots were removed at time intervals and placed in an acid quench solution containing ammonium molybdate. The water-insoluble phosphomolybdate complex was extracted, and 32P radioactivity was measured in a scintillation counter.

**Actin Polymerization Modeling**—Polymerization of TMR-actin at different concentrations was modeled using Berkeley Madonna software. The following scheme and derived set of differential equations were used, where A is ATP-G-actin, F is the filament ends, and Aadp is ADP-G-actin: Step 1, A + A → F (nucleation); Step 2, F + A ↔ F (growth-depolymerization); Step 3, F ↔ 2F (fragmentation-reannealing); Step
Assembly of TMR-actin and Interaction with Regulatory Proteins

To elucidate the processes that underlie these puzzling observations, the ability of TMR-actin to polymerize was assayed by monitoring the change in fluorescence of pyrenyl-actin added at a very low percentage (0.1–0.2%) to TMR-actin. The polymerization of TMR-actin was characterized by a very slow nucleation process, followed by a rapid explosive polymerization with an overshoot and partial depolymerization to a lower steady-state fluorescence (Fig. 1A). The final value of pyrenyl-actin fluorescence was much lower than that of unlabelled actin fluorescence. The polymerization time courses obtained with different percentages of pyrenyl-actin (0.1–2%) superimposed when they were normalized (Fig. 1B), confirming that pyrenyl-actin traces the actual polymerization process of TMR-actin.

The overshoot polymerization of TMR-actin strikingly resembles the kinetics of ATP-actin polymerization under sustained fragmentation (22), in which filaments grow at a constant average length, and filament number increases in proportion to polymer mass. As a consequence of ATP hydrolysis and exposure of ATP-actin subunits at filament ends, it was shown that partial depolymerization occurs because ADP-actin polymerizes at a high critical concentration, and the exchange of ADP for bound ATP on TMR-G-actin is not very fast. Notably, addition of a low amount of TMR-actin to unlabeled actin induces an acceleration of the polymerization process and shortening of the filament length, also suggesting a severing effect of TMR-actin upon incorporation in a standard filament (17).

The view that the overshoot results from the partial depolymerization of TMR-F-actin following ATP hydrolysis was challenged as follows. TMR-actin was polymerized in the presence of BeF$_3^-$, a structural analog of P$_i$ that binds to the site of the γ-phosphate of ATP immediately following release of P$_i$ and that mimics the transition state F-ADP-P$^*$ of ATP hydrolysis on F-actin (31). In the presence of BeF$_3^-$, the overshoot disappeared, and a higher fluorescence level was reached (Fig. 1D, cyan curve). The same level was reached upon addition of BeF$_3^-$ to TMR-F-actin assembled at steady state in ATP (Fig. 1D, black curve). When the 1:1 TMR-labeled ATP-actin complex (31.5 μM) was polymerized in the absence of free ATP, the polymerization was transient and was followed by 90% depolymerization. Addition of BeF$_3^-$ again restored the high level of TMR-F-actin (Fig. 1D, red curve). These data indicate that filaments assembled from TMR-ATP-actin are greatly destabilized by ATP hydrolysis and that BeF$_3^-$ recapitulates a stable ATP-like state of TMR-F-actin, as already demonstrated for unlabeled F-actin.

RESULTS

Analysis of Filament Assembly from TMR-actin—G-actin was labeled with TMR-maleimide as described (2, 8). The extent of labeling was $1 \pm 0.1$ mol of TMR/actin, in agreement with the previous reports. As reported, after 1 night of incubation in the presence of salt at physiological concentration, 90% of the TMR-actin was essentially found in the supernatant when it was centrifuged. However, two puzzling observations were made. First, much less TMR-actin (typically 10 μM, i.e. 25% of the total actin) was found in the supernatant after 15 min of polymerization than after 18 h. Second, when TMR-F-actin was centrifuged after 1 night of incubation under physiological ionic conditions, the supernatant, once re-equilibrated in buffer A by gel filtration, was again able to polymerize.

4, F + Aadp ⇔ F (growth-depolymerization of ADP-actin); and Step 5, Aadp → A (nucleotide exchange). The following values of the rate constants corresponding to Steps 1–5 were used to obtain a satisfactory fit of experimental spontaneous assembly curves recorded over an order of magnitude span in TMR-actin concentrations. $K_1$ and $K_2$ refer to the forward and reverse rate constants for Step 1.

Nucleation is a slow, thermodynamically unfavorable step and, for simplicity, is represented as a single slow irreversible reaction of rate constant $K_{1f}$. (The reverse reaction was assumed to be extremely slow compared with the rate of assembly.) The modeled curves were unchanged if a reverse reaction (dimer dissociation) with a rate constant as high as 4 × 10$^{-3}$ s$^{-1}$ was included because the dimer has a higher probability to elongate than to dissociate at the range of actin concentrations investigated. $K_{1f} = 4.0 \times 10^{-8}$ μM$^{-1}$ s$^{-1}$; critical concentration ($C_c$) = 0.5 μM; $K_{2f} = 0.6$ μM$^{-1}$ s$^{-1}$; $K_{2r} = K_{2a}C_a$; $K_{3f} = K_{3a}(A_0 - A_{total})/(F)$; $K_{3r} = 0.088$ μM$^{-1}$ s$^{-1}$; $C_{cd} = 30$ μM (where $C_{cd}$ is the critical concentration for assembly of ADP-TMR-actin); $K_{4f} = 0.03$ μM$^{-1}$ s$^{-1}$; $K_{4r} = K_{4c}C_{cd}$; and $K_{5f} = 0.1$ s$^{-1}$. It was found that the fragmentation rate constant $K_{3f}$ varied with the square root of the average length of the filaments that presented an exponential length distribution: $K_{3f} = K_{3a}(A_0 - A_{total})/F^{1/2}$, where $F$ is the concentration of filaments. The concentration of total actin was $A_0$. The concentration of unassembled actin was $A_{total} = A + Aadp$. The concentration of assembled actin was $Pol = A_0 - A_{total}$. The observed fluorescence was computed as follows: $F = f_0 + (A_0 - A_{total})f(F) + Aa$f(A) + Aa$dp$f(Aa$dp$), where $f_0$ is the fluorescence signal for buffer alone, $f(F)$, $f(A)$, and $f(Aa$dp$)$ are the specific fluorescence of F-actin, ATP-G-actin, and ADP-G-actin, respectively.

The values of $K_{1f}$, $K_{2f}$, $K_{3f}$, and $K_{4f}$ were adjusted to provide the best fit to the data, whereas the values of all other parameters were experimental values that introduced a constraint. The global fit for all curves was performed step by step by first adjusting the parameters for each curve individually. Then, the best fit values of a given parameter were averaged over all the curves, and the average value was taken as a constraint in the subsequent fit of all curves individually. The cycle was repeated for the next parameter. The first parameter to be adjusted was the one that displayed the smallest S.D.
Negatively stained samples of filaments assembled from TMR-actin were observed under the electron microscope (Fig. 2A). Consistent with the view that the rapid assembly results from extensive fragmentation, only very short filaments were seen in a noisy background because of the high amount of TMR-G-actin (4–5 μM; see below). The average length was 30 nm, which corresponds to ~15 actin subunits (Fig. 2B). Upon addition of BeF$_3^-$, the length of TMR-actin filaments increased, consistent with BeF$_3^-$-induced stabilization (Fig. 2A).

The very short length of TMR-ADP-F-actin filaments and their lengthening in the ADP-BeF$_3^-$ state suggest that the filaments fragment often because of the fragility of the longitudinal interactions between ADP-actin subunits and must be very dynamic at steady state. This view was strengthened by meas-

---

**FIGURE 1.** **Polymerization of TMR-actin.** A, TMR labeling affects actin assembly kinetics. Regular actin (black curve) or TMR-actin (red curve) at 13.7 μM was traced with 0.2% pyrenyl-actin and polymerized by addition of 0.1 M KCl, 1 mM MgCl$_2$, and 0.25 mM EGTA, a.u., arbitrary units. B, polymerization of TMR-actin (10 μM) traced with 0.2, 0.5, 1, or 2% pyrenyl-actin (PYR). The inset shows that, once normalized, the assembly curves are superimposable, i.e. the fluorescence change truly traces the assembly of TMR-actin. C, the nucleus of TMR-actin assembly is a dimer. TMR-actin (0.2% pyrenyl-labeled) was assembled at 7.8, 10.3, 12.9, 15.5, 20.6, and 25.8 μM (bottom to top). Thin lines were calculated using the model described under "Discussion" and in Fig. 9, with the same values for parameters as in Fig. 9. Inset, dependence of the lag time on TMR-actin concentration. The slope of the log/log plot is ~1.0. D, ATP hydrolysis destabilizes TMR-F-actin. TMR-ATP-G-actin (31.5 μM, 0.5% pyrenyl-labeled) was polymerized in the absence (red curve) or presence (black and cyan curves) of free ATP. BeF$_3^-$ was added at the times indicated by the arrows.
uring ATP hydrolysis during the assembly of TMR-actin and at steady state in comparison with unlabeled actin at the same concentration (Fig. 2C). ATP was hydrolyzed in a burst phase accompanying the polymerization of TMR-actin, followed by a steady rate. The rate of ATP hydrolysis at steady state was 26-fold faster for TMR-actin than for unlabeled actin, in agreement with the large increase in filament number. A steady rate of 5 μM P_i released per min was measured in a solution of 20 μM TMR-F-actin. From these numbers, we infer that, in the conventional method of preparation of TMR-actin, all the ATP present initially in the actin solution must have been hydrolyzed into ADP upon overnight incubation under physiological ionic conditions.

Critical concentration measurements for assembly of TMR-actin in ATP and ADP were carried out using pyrenyl-actin fluorescence and sedimentation assays. Under physiological ionic conditions (0.1 M KCl and 1 mM MgCl_2), the critical concentrations for assembly of TMR-actin were 3 ± 0.5 μM in ATP and 30 ± 3 μM in ADP (Fig. 3 and Table 1). The same critical concentration in ATP was obtained using TMR-actin isolated from either the pellet or supernatant of TMR-actin centrifuged overnight in the presence of salt and then re-equilibrated in buffer A.

In the absence of KCl and in the presence of 1 mM MgCl_2, the critical concentration was 8 ± 1 μM in ATP and was too high (>50 μM) to be measured in ADP. In the presence of ATP and BeF_3 at physiological ionic strength, the critical concentration decreased to 0.5 μM. Sedimentation assays indicated that TMR-ADP-actin did not accumulate at steady state (data not shown). The amounts of sedimented F-actin and TMR-F-actin at 20 μM were identical. Still, the slopes of the fluorescence of unlabeled F-actin and TMR-F-actin in the presence of BeF_3 were not identical (Fig. 3). In conclusion, the fluorescence of pyrenyl-F-actin in a filament made from TMR-actin was 2.5-fold lower than in a standard filament. This effect cannot be due to simple quenching of pyrenyl fluorescence by TMR because addition of free fluorophore to a solution of pyrenyl-actin did not affect the fluorescence. We conclude that the environment of the pyrenyl probe is different in F-actin and TMR-F-actin because of the different structures of the two filaments.

Overall, the data demonstrate that, in contrast with previous views, TMR-actin can polymerize. A plausible interpretation of
Assembly of TMR-actin and Interaction with Regulatory Proteins

Barbed and Pointed End Assembly Parameters of TMR-actin—Previous reports indicate that, during the labeling reaction, exhaustion of ATP due to filament rapid turnover yielded TMR-ADP-actin. We showed above that TMR-ADP-actin has a high critical concentration for assembly, especially at low ionic strength. TMR-actin was thus essentially unassembled and was found in the supernatant of centrifuged TMR-F-actin (2, 8, 19). Our data show, however, that, once re-equilibrated in ATP-containing buffer A, TMR-actin found in the supernatant can be polymerized again, with the same characteristics as the originally labeled material.

TABLE 1

| Thermodynamic and kinetic parameters for filament assembly from TMR-actin |
|--------------------------------------------------|
| Parameter                        | Unlabeled actin | TMR-actin |
|----------------------------------|-----------------|-----------|
| Critical conc in ATP             |                 |           |
| Both ends free                   | 0.1 μM          | 3 ± 0.5 μM|
| Pointed ends                     | 0.6 μM          | 6 μM      |
|----------------------------------|-----------------|-----------|
| Critical conc in ADP             | 1.5 μM          | 30 μM     |
|----------------------------------|-----------------|-----------|
| $k_B^+$                          | 10 μM s⁻¹       | 0.65 μM s⁻¹|

The rate parameters for association of TMR-G-actin with filament ends cannot be derived from the analysis of spontaneous assembly because the slow nucleation is not followed by association of G-actin with the ends of preformed nuclei that would remain in constant number. The continuous fragmentation of filaments introduces a supplementary variable in the system. The rate of barbed end assembly of TMR-G-actin from spectrin-actin seeds was measured at different concentrations of TMR-actin (Fig. 4). To observe a significant barbed end growth rate, spectrin-actin seeds were used at 20-fold higher amounts than used in standard assays. A value of 0.7 μM⁻¹ s⁻¹ was derived for the association rate constant $k_B^+$ for TMR-actin and barbed ends under physiological ionic conditions (Fig. 4, inset).

This value was 20-fold lower than for unlabeled actin. Therefore, it is essentially the extent of fragmentation of filaments that supports the fast polymerization of TMR-actin. Structural studies (8) and DNase I binding assays (13) indicate that the pointed face of TMR-ATP-G-actin that associates with a filament barbed end is very similar to the pointed face of unlabeled actin. Therefore, the structural change in the barbed end of TMR-F-actin is mainly responsible for the large decrease in the $k_B^+$.

To obtain insight into the polymerization parameters of TMR-actin at pointed ends, TMR-actin was polymerized in the presence of increasing concentrations of either gelsolin or capping protein to determine the saturation amount of capping protein needed to block all barbed ends generated by continuous fragmentation. Addition of increasing amounts of either one or the other capping protein led to an increased lag time, a gradual disappearance of the overshoot, and a decrease in the extent of fluorescence change (Fig. 5A). A saturating effect was reached at very high amounts of capping protein (0.2 μM). The time course of polymerization then remained constant and reflected spontaneous assembly at pointed ends. A value of 6 μM was derived for the pointed end critical concentration from analysis of the extent of pyrenyl-actin fluorescence change upon assembly (Fig. 5B and Table 1). This value was only twice higher than that measured with both ends free (3 μM). The energetic difference between the two ends of TMR-actin filaments at steady state in ATP thus appears to be lower than for unlabeled actin.

Formin Is a Poor Nucleator of TMR-actin Assembly and Destabilizes Filaments of TMR-actin—Formins are barbed end nucleators that catalyze rapid processive barbed end assembly from the profilin-actin complex. The FH2 and FH1-FH2 domains of mammalian Dia1 are sufficient to nucleate filament barbed ends and to bind barbed ends with equilibrium dissociation constants of 5–20 nM in a leaky capping protein fashion (24). Neither the FH2 nor FH1-FH2 domains at 0–100 nM affect the assembly of TMR-actin, whereas a prominent nucleation of unlabeled actin is observed in that concentration range (24, 32). At 0.2–1.4 μM, formin caused a shortening of the lag phase and lowered the extent of fluorescence change, indicating that less TMR-actin was assembled at steady state (Fig. 6A). Although the critical concentration was unchanged, the slope of the critical concentration plot was decreased by formin, suggesting that a larger amount of TMR-ADP-G-actin was present at steady state in the presence of formin, most likely because of the larger depolymerization flux of the formin-nucleated filaments (Fig. 6B). This view was confirmed by measuring the increase in the slope in the presence of BeF₃. A sedimentation assay confirmed these conclusions and showed that the FH2 domain cosedimented with TMR-F-actin at an approximate molar ratio of 1:10 (Fig. 6C). In conclusion, the FH2 domain is a very poor nucleator of TMR-actin and destabilizes the TMR-F-actin filaments, thus increasing the concentration of unassembled TMR-ADP-actin at steady state.

ADF Stabilizes Filaments Assembled from TMR-actin by Binding TMR-ADP-actin in the F Form Exclusively—ADF/co-filins are small proteins that bind ADP-actin specifically in both the G and F forms. When added to a solution of unlabeled...
F-actin, ADF binds cooperatively to the sides of filaments. The resulting structural change promotes an increased twist of the two-strand helix (33). The associated destabilization of longitudinal actin-actin interactions weakens the filament. ADF has been proposed to favor an intrinsic structural state of the actin filament (34, 35). Accordingly, the steady-state concentration of G-actin increases, and filaments display a shorter average length (26, 36–38).

To further understand how actin-actin contacts in the filament are affected by TMR labeling, polymerization of TMR-actin was monitored in the presence of ADF (Fig. 7A). ADF affected the kinetics of TMR-actin assembly in several aspects: the overshoot progressively decreased and eventually disappeared, and the extent of pyrenyl-actin fluorescence change was lowered because of the quenching of pyrenyl-actin fluorescence by ADF (26). The effect of ADF on the assembly of TMR-actin at steady state was therefore more conveniently appreciated using a sedimentation assay (Fig. 7B). ADF bound to TMR-F-actin exclusively at a 1:1 molar ratio and with a $K_d$ of 0.1 $\mu$M, similar to unlabeled F-actin. The amount of TMR-G-actin in the supernatant decreased to a limit value of 0.5 $\mu$M upon increasing ADF, indicating that the preferential binding of ADF to TMR-F-actin shifted the monomer-polymer equilibrium toward the polymer form, thus stabilizing the filament. This conclusion was validated by assays in which TMR-actin at different concentrations was polymerized in the presence of a saturating concentration of ADF and sedimented. ADF bound to F-actin exclusively. The concentration of TMR-actin was 0.5 $\mu$M in all supernatants; TMR-ADP-G-actin did not accumulate at steady state. This result confirms that ADF lowers the critical concentration of TMR-actin by binding exclusively to TMR-ADP-F-actin. Consistent with this conclusion, ADF did not bind to TMR-actin filaments in the presence of BeF$_3$ (data not shown), and in electron microscopy, the filaments appeared thicker and ~10-fold longer (Fig. 2).

In conclusion, ADF displays opposite effects on the assembly of unlabeled and TMR-labeled actin: it destabilizes standard F-actin filaments and binds ADP-G-actin better than ADP-F-actin. In contrast, ADF stabilizes the filaments of TMR-actin by binding TMR-ADP-F-actin specifically. The recognition by ADF of the structural change of F-actin following ATP hydrolysis is maintained following TMR labeling. On the other hand, the specificity of binding of ADF to actin is altered by TMR labeling. ADF binds TMR-ADP-F-actin, but not TMR-ADP-G-actin.

**ADF Does Not Polymerize in Branched Filaments with VCA and the Arp2/3 Complex, and VCA-TMR-actin Does Not Participate in Barbed End Assembly—**

WASPs catalyze filament branching in a complex reaction (25, 39). The C-terminal domain of WASP, VCA, binds one molecule of G-actin and one molecule of the Arp2/3 complex. This ternary complex, also called the “branching complex” or “activated Arp2/3,” then associates with a filament to initiate the formation of a daughter filament growing off the mother filament. Daughter and mother filaments grow and branch again, promoting actin assembly in a dendritic array.

In the branching complex, actin displays different functional interactions with the different partners of the reaction. G-actin and the WH2 domain of VCA form a complex that behaves as a functional homolog of profilin-actin, *i.e.* VCA poisons actin nucleation, but the VCA-actin complex participates in barbed end growth (25, 39).

Polymerization of TMR-actin was not stimulated by VCA and the Arp2/3 complex added together (up to 0.5 $\mu$M each), indicating that VCA-Arp2/3 did not form branched filaments.
with TMR-actin (Fig. 8A). The effect of VCA alone on polymerization of TMR-actin was examined. VCA decreased the extent of actin assembly in a TMR-G-actin-sequestering fashion. The same effect was obtained with thymosin β4, a standard G-actin-sequestering protein (Fig. 8B). From the linear decrease in the extent of pyrenyl-actin fluorescence change, values of 0.45 and 5.5 μM were derived for the equilibrium dissociation constants of the complexes of TMR-actin with VCA and thymosin β4, respectively (Fig. 8C). The affinities of these two proteins are only 2–5-fold lower for TMR-actin than for unlabeled actin (25, 40).

In conclusion, although VCA bound TMR-actin almost as well as unlabeled actin, the functional properties of the complex were dramatically altered. The VCA-TMR-actin complex failed to productively associate with barbed ends like VCA-actin, profilin-actin, and other WH2 domains (3). VCA merely sequestered TMR-actin. This change in function of VCA-actin may be responsible for the failure of TMR-actin to polymerize in branched filaments.

DISCUSSION

The versatile structure of the actin filament is probably one of the main functionally relevant features of actin in motility
In adopting a variety of conformational states, the actin filament can be directed to different tasks by regulatory proteins that stabilize different structures associated with specific dynamic properties.

We have shown here that when Cys$^{374}$ of actin is labeled with TMR-maleimide, 100% of the TMR-actin still polymerizes, although less well than unlabeled actin; polymerization of TMR-ATP-actin is accompanied by hydrolysis of ATP and subsequent destabilization of longitudinal actin-actin bonds in the filament. The energetic change in stability due to hydrolysis of ATP corresponds to a 10-fold increase in critical concentration.
similar to standard actin. Because TMR-actin keeps these major functional properties of unlabeled actin, it is an interesting tool to understand which changes in the structural properties of actin linked to ATP hydrolysis (opening of the nucleotide cleft or structural reorganization of subdomain 2) correlate with distinct dynamic states of the filament.

TMR-actin polymerizes into short dynamic filaments <0.05 μm long. The fragmentation-reannealing processes of these very short filaments are more extensively involved in assembly dynamics compared with standard long filaments. This property introduces strong biases in analyses of actin filament assembly dynamics performed using fluorescence microscopy of TMR-actin (18).

TMR-actin nucleates very slowly, and the nucleus is a dimer, whereas it is a trimer in unlabeled actin. The nucleus is defined as the first oligomer that can interact with G-actin as a filament end by exposing the actin surfaces that build a lateral and a longitudinal bond upon association with G-actin. Nucleation of unlabeled actin implies the initial formation of a dimer and then a trimer. The two actin monomers in the initial dimer nucleus are much likely to be connected by lateral interactions, parallel to the filament axis, than by longitudinal interactions, considering the additional buried 400 Å² in a longitudinal contact (30, 42). The third subunit that associates with the longitudinal dimer makes lateral contacts with the two actin monomers. This trimer is a nucleus because it associates with a fourth G-actin by making both a lateral and a longitudinal contact identical to those formed during filament growth. The longitudinal dimer does not have this property and thus is not a nucleus. In contrast, a lateral dimer can be a nucleus of the helix because the third subunit that adds to such a dimer makes both a lateral and a longitudinal contact with the two actin subunits of this dimer. The very slow nucleation of TMR-actin, the finding that the nucleus is a dimer, and the view that longitudinal contacts between TMR-actin are weakened by the probe all support the view that lateral contacts, which are not affected by the TMR label, are made in this dimer. The low stability of the lateral contacts explains the energetically unfavored nucleation of TMR-actin. In this view, the lateral contacts along the short pitch helix maintain the stability of the filament assembled from TMR-actin, in contrast to filaments assembled from unlabeled actin, whose stability is due mainly to strong longitudinal contacts. As a result, filaments assembled from TMR-actin break easily and are expected to display a greater angular disorder compared with standard filaments.

Polymerization of TMR-ATP-actin was modeled based on the above findings and using the experimentally determined values of kinetic and thermodynamic assembly parameters (see “Experimental Procedures”). A perfect agreement was obtained between experimental and modeled polymerization curves at different concentrations of TMR-actin (Fig. 9). Strong constraints in the modeling were incurred by the overshoot in polymerization curves; hence, the fit is nonequivocal. The calculated average length of the filaments of TMR-actin at steady state was in good agreement with experimental measurements. In agreement with the slow nucleation, the on-rate of TMR-ATP-actin association with barbed ends was 20-fold lower than that of regular actin. Similarly, the critical concentrations for assembly of TMR-actin in ATP and ADP were 20–30-fold higher than for unlabeled actin. Despite its lower ability to polymerize, TMR-actin polymerizes very fast because of the frequent fragmentation of filaments following ATP hydrolysis. In turn, filaments of TMR-actin are more stable and longer in the ADP-P* transition state. The polymerization of TMR-ATP-actin thus displays the qualitative features of polymerization
of unlabeled actin, but the thermodynamic/structural effects of ATP hydrolysis are enhanced. We conclude that the rotation of the two domains of actin that leads to opening of the cleft following ATP hydrolysis is not involved in the destabilization of the ADP filament because this movement is blocked in TMR-actin. On the other hand, if we assume that the structural change in subdomain 2 from a disordered loop to a helix is not due solely to crystal packing features specific to TMR-actin, this change may be linked to the destabilization of longitudinal contacts in unlabeled and TMR-labeled actin.

Given the ability of TMR-actin to polymerize practically as well as unlabeled actin, it is surprising that crystals of uncomplexed TMR-actin could be obtained. It is possible that TMR labeling enables the formation of the large crystal packing contacts made between the TMR moieties of two actin monomers (2). The solution conditions used for crystallization may also inhibit polymerization (even possibly for unlabeled actin). In this respect, we determined that unlabeled actin at 25 μM does not polymerize in the solutions used to crystallize TMR-actin (8). Although the structure of TMR-actin is extremely similar to the structure of unlabeled actin in complex with several actin-binding proteins, subtle differences must exist to account for the large differences in the functional interactions of TMR-actin with actin-binding proteins.

The FH2 domain of mammalian Dia1, which is a potent nucleator of unlabeled actin, is a poor nucleator of TMR-actin, indicating that functional protein-protein contacts are impaired in the FH2 domain-TMR-actin complex. Nevertheless, TMR-actin has been crystallized in complex with the FH2 domain of mammalian Dia1 (18). In the crystal structure, the two actins bound to the FH2 bridge are related by a 180° rotation in a pseudo-short pitch geometry, and three consecutive actin subunits are bound to each pair of the FH2 bridge element. This structure, in which the barbed end is sterically blocked, cannot accommodate the catalysis of processive assembly by the FH2 domain. Whether the blockage reflects the altered function of the FH2 domain when it binds TMR-actin or represents a functionally relevant intermediate in processive filament assembly is an open issue.

Analysis of TMR-actin provides insight into the structural basis for the function of β-thymosin/WH2 domains in actin assembly. WH2 domains interact with different regions of G-actin. An amphipathic N-terminal helix binds at the cleft between subdomains 1 and 3 and recognizes the ATP-bound form of actin specifically; the central consensus actin-binding motif binds the side of subdomain 1 in an extended fashion; the C-terminal variable region binds subdomain 2 in a more or less dynamic fashion. The C-terminal regions of G-actin-sequestering proteins such as thymosin β4 (3, 43, 44) and WASP-interacting protein (45) bind tightly to subdomain 2, thus preventing association of the complex with barbed ends, whereas the C-terminal regions of the WH2 domains of VCA and ciboulot are in loser contact with subdomain 2 (3, 45); thus, their complex with G-actin, like profilin-actin, associates productively with barbed ends (3). We have found here that TMR does not weaken the interaction of thymosin β4 or VCA with G-actin, but switches the function of VCA from assembly-promoting (like profilin) to G-actin-sequestering (like β-thymosins). This result strongly suggests that the blockage of the rotation movement of the shear zone by the TMR probe is coupled to a structural change in subdomain 2 or freezes the structure of subdomain 2 in a conformation that cannot adapt to the appropriate functional interaction with the C-terminal segment of the WH2 domain.

The fact that the VCA·TMR-actin complex does not productively associate with barbed ends may be at the origin of the failure of TMR-actin to polymerize in branched filaments. We proposed that the productive association of VCA-actin with the barbed end of the mother filament is required to initiate the branch (39). In this view, the inability of the VCA-TMR-actin complex to interact with a filament barbed end suffices to abolish filament branching.

ADF specifically binds TMR-ADP-F-actin, not TMR-ADP-G-actin. Whereas the longitudinal actin-actin contacts in unlabeled F-actin are destabilized by ADF binding, they appear to be stabilized in TMR-F-actin by ADF binding. ADF is known to destabilize the longitudinal interactions in filaments assembled from unlabeled actin and to induce a tilted orientation of the actin subunits by bridging two adjacent monomers along the long pitch helix. It is possible that this structure, the thermodynamic stability of which is intermediate between that of F-actin and TMR-F-actin, is also induced by the binding of ADF to TMR-F-actin.

The structural basis for the loss of affinity of ADF for TMR-ADP-G-actin remains to be explained. Different regions of ADF are involved in F-actin and G-actin binding (46). The present results support the view that ADF contacts the barbed face of ADP-G-actin as do profilin and gelsolin segment-1 (47, 48). This contact would be hindered by TMR. It is also possible that the helical structure of subdomain 2 in TMR-ADP-G-actin does not allow ADF binding or that the changes in orientation of subdomain 2 linked to the opening of the cleft are crucial for ADF binding to ADP-G-actin, but would be dispensable for binding to ADP-F-actin. ADF has been shown to modify the structure of subdomain 2 in F-actin, making it accessible to proteolytic cleavage (49). Biochemical and structural investigations are required to address these issues. A large number of crystal structures of actin will be required to understand the structural basis of the functional properties of native or modified actin derived from biochemical studies in solution.

REFERENCES
1. Kabsch, W., Mannherz, H. G., Suck, D., Pai, E. F., and Holmes, K. C. (1990) Nature 347, 37–44
2. Otterbein, L. R., Graceffa, P., and Dominguez, R. (2001) Science 293, 708–711
3. Hertzog, M., van Heijenoort, C., Didry, D., Gaudier, M., Coutant, J., Gigant, B., Didecot, G., Preat, T., Knossow, M., Guittet, E., and Carlier, M.-F. (2004) Cell 117, 611–623
4. Schutt, C. E., Myslik, J. C., Rozycki, M. D., Goone sekere, N. C., and Lindberg, U. (1993) Nature 365, 810–816
5. McLaughlin, P. J., Gooch, J. T., Mannherz, H. G., and Weeds, A. G. (1993) Nature 364, 685–692
6. Chilk, J. K., Lindberg, U., and Schutt, C. E. (1996) J. Mol. Biol. 263, 607–623
7. Vorobiev, S., Strokopytov, B., Drubin, D. G., Frieden, C., Ono, S., Condee, J., Rubenstein, P. A., and Almo, S. C. (2003) Proc. Natl. Acad. Sci. USA 100, 12323–12328

3 A. Pelikan Conchaudron, unpublished data.
