Conformational and Dynamic Differences between Actin Filaments Polymerized from ATP- or ADP-Actin Monomers*

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Conformational and dynamic properties of actin filaments polymerized from ATP- or ADP-actin monomers were compared by using fluorescence spectroscopic methods. The fluorescence intensity of IAEDANS attached to the Cys374 residue of actin was smaller in filaments from ADP-actin than in filaments from ATP-actin monomers, which reflected a nucleotide-induced conformational difference in subdomain 1 of the monomer. Radial coordinate calculations revealed that this conformational difference did not modify the distance of Cys374 from the longitudinal filament axis. Temperature-dependent fluorescence resonance energy transfer measurements between donor and acceptor molecules on Cys374 of neighboring actin protomers revealed that the inter-monomer flexibility of filaments assembled from ADP-actin monomers were substantially greater than the one of filaments from ATP-actin monomers. Flexibility was reduced by phalloidin in both types of filaments.

Actin is one of the most abundant proteins in biological systems and responsible for a number of cell functions in vivo (1, 2). Two principal forms of actin exist in living cells, the monomeric and the filamentous. Actins biological function depends on the actual dynamic and conformational properties of the protein and on the dynamic equilibrium between the two principal forms (1, 2).

The polymerization of actin monomers with bound ATP is accompanied by the parallel hydrolysis of the nucleotide. However, the biological relevance of ATP hydrolysis by actin is still not well understood. Interestingly, ADP-monomeric actin also forms filaments (3), therefore, the presence of ATP and its hydrolysis are not essential for filament assembly. Previously, the hydrolysis of actin-bound ATP was assumed to play a key role in the steady-state treadmilling of actin filaments (4). Alternatively, Carlier (5, 6) suggested that ATP hydrolysis facilitated the rapid de-polymerization of actin.

Recently, Janmey and colleagues (7) provided evidence that actin filaments polymerized from ATP-actin monomers were significantly stiffer than the ones obtained from ADP-actin monomers. Considering that both types of filaments consist mainly of ADP-actin protomers, this observation was explained by the assumption that the ATP-actin monomers were conformationally trapped following the hydrolysis of ATP, and the energy released during the hydrolysis was stored as elastic energy (7). The authors proposed that this elastic energy could play an important role when the actin filament interacted with actin-binding proteins. Although this exciting observation was later supported by other laboratories, a number of experimental results were contradictory.

The direct effect of ATP on actin filaments was confirmed and extended to phalloidin-labeled actin by fluorescence microscopy experiments (8). Three-dimensional reconstructions from electron micrographs revealed similar effects of the nucleotides on the flexibility of actin filament (9, 10). The results of phosphorescence spectroscopic experiments apparently also supported the conclusion of Janmey’s group (7). By measuring steady-state phosphorescence anisotropy these authors found that filaments prepared from actin monomers in the presence of ADP were more flexible than the ones obtained from ATP-actin monomers (11). On the other hand, evidence provided from electron microscopy experiments and by measuring the dynamic elasticity and viscosity on actin solutions that filaments assembled from either ATP-actin or ADP-actin were indistinguishable (12). Rheometric studies on filament solutions reached a similar conclusion (13). Furthermore, fluorescence microscopy of individual rhodamine-labeled actin filaments revealed no difference between the persistence lengths of actin filaments (9 ± 0.5 μm) assembled from ATP-actin monomers or from ADP-actin monomers (14). These results suggest that the proper description of the effect of the monomer-bound nucleotide on the flexibility of generated filaments requires further investigations.

None of the results cited above provided information regarding the nucleotide-dependent differences between the two forms of actin filaments with respect to molecular events occurring on a nanosecond time scale. Accordingly, the application of fluorescence spectroscopy seems to be reasonable for better understanding the biological importance of the nucleotide dependence of filament flexibility. A fluorescence resonance energy transfer (FRET) method first described by Taylor et al. (15) allows one to determine the radial coordinate (which is defined as the distance between the residue and the longitudinal filament axis) of a labeled side chain within the filament (Fig. 1b). In earlier studies this method was applied to determine the radial coordinate of a number of labeled amino acids in the actin filament (15–22). FRET spectroscopy was also

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1 The abbreviations used are: FRET, fluorescence resonance energy transfer; IAEDANS, N-(iodoacetyl)aminomethyl(ethyl)-5-naphthylamine-1-sulfonate; IAF, 5-iodoacetamidofluorescein; NaN3, sodium azide.
used to characterize the flexibility of the protein matrix between a donor and an acceptor molecule (23) and to study the dynamic properties of the actin filament (24).

The spectroscopic results presented here revealed local conformational differences around the Cys\textsuperscript{374} residue of the actin protomer during the comparison of filaments polymerized from ATP- or ADP-actin monomers. Furthermore, the inter-monomer flexibility was found to be greater in filaments from ADP-actin monomers than in the ones from ATP-actin monomers. Interestingly, some of these properties of the actin filament were influenced by phalloidin, while others were unchanged in the presence of this toxic peptide, which suggested that the overall effect of phalloidin on the actin filament was complex. Relying on these spectroscopic results we propose that the conformational and dynamic differences resolved here might appear due to the presence of nucleotide-induced conformational changes in the actin monomer (25).

**EXPERIMENTAL PROCEDURES**

**Chemicals**—KCl, MgCl\(_2\), CaCl\(_2\), Tris, IAEADANS, quinine (hemisulfate salt), dimethylformamide, phalloidin, hexokinase, glucose, and EGTA were obtained from Sigma. IAF was purchased from FLUKA (Switzerland). NaN\(_3\) was purchased from MERCK (Darmstadt, Germany). The actin was in a 2 mM Tris/HCl buffer containing 0.2 mM ATP, 0.1 mM CaCl\(_2\), 0.1 mM \(\beta\)-mercaptoethanol, and 0.005% NaN\(_3\), pH 8.0 (buffer A) at the end of this preparations. Fluorescence labeling was carried out in the same buffer. Cys\textsuperscript{374} of actin was labeled with the donor (IAEDANS) following polymerization as described earlier (28).

The solution of actin filaments (approximately 46 \(\mu\)M) was incubated in the presence of 10-fold molar excess of IAEADANS at 25 °C for 1 h. Then the samples were centrifuged at 100,000 \(\times\) g for 2 h at 4 °C so that free fluorophores could be separated from the protein. Acceptor labeling of Cys\textsuperscript{374} was carried out in separate samples by incubating monomeric actin with 15-fold molar excess of IAF for 20–25 h at 4 °C. Following IAF labeling actin was polymerized by adding 2 mM MgCl\(_2\) and 100 mM KCl, and centrifuged in a way similar to the one used during IAEADANS labeling. In the case of either donor or acceptor labeling the actin pellets were incubated for 2 h in buffer A, homogenized, and resuspended in buffer A. Subsequently, the samples were dialyzed overnight against the same buffer.

Actin concentration and labeling ratios were determined by measuring the absorption spectra of samples with a Shimadzu UV-2100 spectrophotometer. The following extinction coefficients were applied in the calculations: 1.11 mg ml\(^{-1}\) cm\(^{-1}\) at 280 nm or 0.63 mg ml\(^{-1}\) cm\(^{-1}\) at 290 nm (29) for monomeric actin with a relative molecular mass of 42,300 Da (30), 6,100 m\(^{-1}\) cm\(^{-1}\) at 336 nm for IAEADANS (31), and 77,000 m\(^{-1}\) cm\(^{-1}\) at 498 nm for IAF (32) at pH 8.0. When required, the measured absorption data at 280 or 290 nm were corrected for the contribution of fluorescent labels. The labeling ratios were determined by calculating the ratio of fluorophore concentration to the actin concentration. Accordingly, the labeling ratios were 0.8–0.9 for IAEADANS and 0.7–0.8 for IAF.

Prior to inter-monomer FRET experiments the solutions of unlabeled, donor-labeled, and acceptor-labeled monomers were mixed so that the desired concentrations of donor, acceptor, and actin would be adjusted. The Ca\(^{2+}\) bound by the actin monomers was replaced by Mg\(^{2+}\) in these samples by adding EGTA and MgCl\(_2\) at final concentrations of 0.2 and 0.1 mM, respectively. The samples were incubated for 10–15 min at room temperature (33). To obtain filaments from ATP-monomeric actin the samples were polymerized with 2 mM MgCl\(_2\) and 100 mM KCl (final concentrations) after the cation exchange. When filaments were designed to assemble from ADP-monomers, ATP was replaced by ADP in Mg\(^{2+}\)-actin monomers prior to polymerization in a way described by Drewes and Faulstich (34). In the nucleotide exchange procedure hexokinase, glucose, and ADP were added to actin monomer solutions at final concentrations of 1.65 mg/ml, 0.5 mg/ml, and 1 mM, respectively, and the samples were then incubated for 1 h at 4 °C. To test the reliability of these exchange procedures, both the cation and the nucleotide exchanges were followed in control experiments by monitoring the kinetics of fluorescence emission of IAEADANS attached to the Cys\textsuperscript{374} residue of the actin monomer. During the preparation of filaments from ADP-actin monomers the polymerization was initiated similarly to the way described in the case of ATP-actin monomer polymerization after the nucleotide exchange procedure. The polymerization of ADP-actin is substantially slower than the one of ATP-actin (3). Therefore, to assure complete filament assembly, both types of actin were polymerized for 12 h at room temperature. Furthermore, the fluorescence experiments were also carried out after the incubation of the actin filaments for 4 h at room temperature in the presence of equimolar concentration of phalloidin, a cyclic peptide. Phalloidin is known to stabilize actin filaments and to shift the monomer-filament equilibrium toward the filamentous form (35).

**Fluorescence Experiments**—The fluorescence experiments were carried out in samples containing 30 \(\mu\)M actin at pH 8.0. Fluorescence was measured with a PerkinElmer LS55B luminescence spectrophotometer. The excitation wavelength was 350 nm for the IAEADANS. Considering that the absorption of the samples was not negligible at the excitation and emission wavelengths the fluorescence spectra were corrected for the inner filter effect over the emission wavelength range by using the measured absorption spectra as follows.

\[
F_{\text{corr}}(\lambda) = F(\lambda) \text{antilog}(OD_{B, \lambda} + OD_{A, \lambda})
\]

(Eq. 1)

where \(F_{\text{corr}}(\lambda)\) and \(F(\lambda)\) are the corrected and measured fluorescence intensities, respectively. \(OD_{B, \lambda}\) and \(OD_{A, \lambda}\) are the optical densities of the samples at the excitation wavelength and emission wavelengths, respectively. It should be noted that the optical densities were determined from the actual optical paths calculated from the geometrical parameters of the applied cuvettes. In energy transfer experiments the donor emission was detected between 400 and 470 nm so that the contribution of acceptor fluorescence to the measured intensities could be excluded. The optical slits were set to 5 nm in both the excitation and emission paths.

The efficiency of the FRET (E) is defined as follows,

\[
E = k_{\text{on}}/(k_{\text{on}} + k_{\text{off}})
\]

(Eq. 2)
where $k_d$ and $k_a$ are the rate constants of the fluorescence resonance energy transfer and the fluorescence transition, respectively, and $k_r$ is the sum of rate constants characteristic of all the other processes resulting in the de-excitation of the fluorophore.

This transfer efficiency can be calculated from the under-curve areas of the donor fluorescence in the following way,

$$E = 1 - (\phi_d/\phi_0)$$  \hspace{1cm} (Eq. 3)$$

where $\phi_d$ and $\phi_0$ are the fluorescence quantum yields of the donor measured in the presence and in the absence of acceptor, respectively. The Förster's critical distance ($R_0$) is defined as the donor-acceptor distance where the FRET efficiency is 0.5. In order to calculate the donor-acceptor distance ($R$) from transfer efficiency ($E$) the value of $R_0$ needs to be obtained as follows,

$$R_0^6 = (8.79 \times 10^{-11})k^2\phi_d\phi_aJ$$  \hspace{1cm} (Eq. 4)$$

where $n$ is the refractive index of the medium, $k^2$ is the orientation factor, $\phi_a$ is the fluorescence quantum yield of the donor in the absence of acceptor, and $J$ is the overlap integral given in $\text{m}^{-3} \text{ cm}^{-1} \text{ nm}^3$. In this study the refractive index ($n$) and the orientation factor ($k^2$) were taken to be 1.4 and 2/3, respectively. In the determination of the fluorescence quantum yield of the donor the fluorescence intensity was detected over a wide range of emission wavelengths (between 400 and 700 nm). In these experiments the quantum yield was calculated by comparing the emission of the donor to the one of a reference substance (quinine sulfate in 1 N NaOH). The fluorescence quantum yield of the reference was taken to be 0.53 under these experimental conditions (36). The overlap integral ($J$) is defined as follows,

$$J = \int F_D(\lambda)\alpha(\lambda)\lambda^4 d\lambda/\int F_D(\lambda)\lambda^4$$  \hspace{1cm} (Eq. 5)$$

where $F_D(\lambda)$ is the fluorescence emission spectrum of the donor and $E_r(\lambda)$ is the absorption spectrum of the acceptor. If the transfer efficiency ($E$) and Förster's critical distance ($R_0$) are known the distance between the donor and acceptor molecules ($R$) for a single donor-single acceptor system can be calculated from the following equation.

$$E = R_0^6/R^6 + R^6$$  \hspace{1cm} (Eq. 6)$$

Characterization of Protein Flexibility—The mean value of the rate constant for the fluorescence resonance energy transfer, $<k_r>$, is an appropriate parameter used to monitor local fluctuations of a macromolecule (23). The normalized efficiency of FRET, $f$, was introduced in a following way so that the value of $<k_r>$ could be approximated experimentally (23).

$$f = El/\phi_d$$  \hspace{1cm} (Eq. 7)$$

It should be noted that in our present FRET experiments a single donor usually transferred energy to more than one acceptors. In such a complex system the measured normalized energy transfer parameter is the sum of the normalized energy transfer efficiencies that characterize the individual donor-acceptor systems (37). Furthermore, after appropriate spectral consideration (23),

$$f = <k_nR_n> = c <R_0^6k_r^2>$$  \hspace{1cm} (Eq. 8)$$

where $c$ is a constant involving the refractive index ($n$) and the overlap integral ($J$). The subscript ‘$n$‘ refers to the value of the given parameter for the $n^{th}$ population, taking a momentary picture, and the brackets ($< >$) denotes average values.

In the analysis of the temperature dependence of FRET efficiency the presence of the donor molecules without a fluorescence energy transfer pair on neighboring protomers should also be considered due to the incomplete acceptor labeling (the acceptor labeling ratio was 0.66 in these experiments). If one takes into account the probability that a monomer is not labeled with an acceptor (0.34), the probability that none of the four closest monomers is acceptor labeled around a donor labeled monomer is only 0.013 (0.34). Accordingly, we considered the concentration of the donors, which did not have an acceptor pair, as negligible in the interpretation of fluorescence measurements. The fluorescence parameters used for the calculation of FRET parameters were routinely measured after heating the samples to appropriate temperatures. To exclude the effects of either a possible time dependence of the FRET parameters, or the presence of irreversible temperature-induced changes in the protein structure, control experiments were carried out. The fluorescence parameters were re-measured after overnight incubation of the samples at 4 °C, or by obtaining these parameters by cooling back the samples from the highest to the lowest temperature.

### Determination of Radial Coordinates—
Taking advantage of the helical symmetry of the actin filament, a method had been developed to determine the radial coordinate (Fig. 1b) of a residue in the actin filament (15). In the present coordinate, FRET experiments were carried out to determine the radial coordinate of Cys734 at 22 °C. In these experiments, Cys734 was labeled with either a donor or an acceptor molecule (Fig. 1a) on different actin protomers. The solutions of labeled monomers were mixed so that the appropriate value of the donor and acceptor concentrations would be adjusted. After polymerization the radial coordinate can be determined from the dependence of the measured FRET efficiency on the acceptor molar ratio. In such an analysis it is assumed that the polymerization of monomers forming long actin filaments is random. It has been shown that polymerization of actin monomers into filaments is random in the presence of phallolidin (20).

An arrangement can be defined by considering five actin monomers as a donor in the central monomer and $m$ acceptors on neighboring monomers (where $m$ can take integer values from 0 to 4). The permutation of the $m$ acceptors on the four neighboring monomers does differentiate between arrangements. Accordingly, under the geometric conditions applied here, 16 arrangements can be considered. Assuming an acceptor molar ratio of $\gamma$, the probability of an arrangement with $m$ acceptor can be calculated as follows.

$$p_m = \gamma^{m+1}(1-\gamma)^{5-m}$$  \hspace{1cm} (Eq. 9)$$

At a given acceptor molar ratio ($\gamma$) the total efficiency of the transfer ($E$) can be taken as a weighted sum of the individual efficiencies.

$$E = \sum_{m=0}^{4}p_mE_m$$  \hspace{1cm} (Eq. 10)$$

where $E_m$ is the transfer efficiency characteristic of the $m^{th}$ arrangement. During the calculation of this parameter it should be taken into account that a single donor could potentially transfer energy to more than one acceptors. In this case Equation 6 transforms to (37, 38),

$$E = \sum_{m=0}^{4}(R_m/R_0)^6/[(1 + \sum_{m=0}^{4}(R_m/R_0)^6]^6$$  \hspace{1cm} (Eq. 11)$$

where $R_m$ denotes the appropriate individual donor-acceptor distances. The individual distances ($R_m$) between the donor ($n = 0$) and adjacent acceptors ($n = -2, -1, 1, 2$, see Fig. 1a) should be calculated so that the value of assembled transfer efficiency can be obtained. In this calculation the geometric parameters of the actin filament need to be considered. In this study the half-pitch of the genetic helix was taken to be 27.5 Å, and the relative rotation of the genetic helix was assumed to be 166° (Fig. 1b and c). In these calculations it was also assumed that the efficiency of transfer occurring between the donor and acceptors located in more distant monomers was negligible. Accordingly, if we have a radial distribution of $r$, the individual distances ($R_m$) can be calculated as follows (15):

$$R_m = [r^2(1 - \cos(n \times 166°))^2 + r^2\sin^2(n \times 166°) + (n \times 27.5\gamma)^2]^{1/2}$$  \hspace{1cm} (Eq. 12)$$

Using these equations an algorithm was constructed to fit the simulated data to the experimental results. In these fitting procedures the only free parameter is the radial coordinate, which allows the determination of its value. The fitting algorithm was written in MATLAB 5.2 (MathWorks Inc., Natick, MA).

### RESULTS

Characterization of Samples—Previous reports provided evidence that the fluorescence emission of IAEADS (attached to the Cys734 residue) was an appropriate parameter to follow Ca$^{2+}$-Mg$^{2+}$ (39) or ATP-ADP (25) exchange in actin monomers. Accordingly, in this study the kinetics of fluorescence intensity of IAEADS was monitored in monomeric actin samples (Fig. 2) during the exchange procedures in control experiments. The results obtained in these experiments were in good agreement with the ones published earlier (25, 39), which indicated that both the cation and nucleotide exchanges were appropriately completed during the sample preparation. On the other hand, according to these results the first, relatively fast conformational change in the actin monomer, which occurs simultaneously with the nucleo-
otide exchange (25) was already complete by the time when polymerization was initiated (Fig. 2).

Comparison of Filaments from ATP- or ADP-Actin Monomers—The fluorescence intensity of IAEDANS was measured in actin filaments as well. This intensity was greater in actin filaments polymerized from ATP-actin monomers than in filaments from ADP-actin monomers (Fig. 3). The spectra of the latter type of filaments were red-shifted as compared with that of the former ones (Fig. 3). In the presence of acceptor (IAF) the fluorescence of the donor (IAEDANS) was decreased due to the presence of fluorescence resonance energy transfer (Fig. 3).

FRET efficiency was measured as a function of acceptor molar ratio so that the radial coordinate of Cys 374 would be determined. The tendency of FRET efficiency of filaments from ATP- and ADP-actin monomers was different (Fig. 4). On the other hand, in the presence of phalloidin the FRET efficiencies were identical within the limits of experimental error with the ones measured in its absence in the case of both types of actin filaments (inset in Fig. 4). The independence of FRET efficiencies from the presence of phalloidin indicates that the contribution of nonrandom monomer assembly to the measured FRET parameters can be excluded (20). The radial coordinates were determined from the experimental data by using the mathematical treatment described earlier (15) and a fitting algorithm written in MatLab 5.2. To carry out these calculations it was necessary to determine the Förster's critical distance ($R_0$) for the IAEDANS-IAF pair in both types of filaments (Equation 4). This parameter was determined to be 47.74 and 40.83 Å for filaments from ATP- or ADP-actin monomers, respectively. According to these data, the radial coordinate of Cys 374 was found to be 17.3 ± 1.1 Å in filaments from ATP-actin monomers and 16.7 ± 1.4 Å in filaments assembled from ADP-actin monomers. These results are in good agreement with the radial coordinate range of 12–23 Å, which was published recently for the Cys 374 (20).

The inter-monomer flexibility of actin filaments was characterized by measuring the temperature profile of the normalized FRET efficiency, $f'$. In the case of actin filaments obtained from ATP-actin monomers the increase of $f'$ was relatively small (20%) between 6 and 34 °C (Fig. 5). When the filaments were polymerized from ADP-actin monomers, however, $f'$ increased progressively over the same temperature range (Fig. 5) providing a total change of more than 100% (Fig. 5). Neither of these temperature-dependent tendencies of $f'$ was changed in control experiments designed to estimate the time dependence of the FRET parameters and the effect of irreversible temperature induced denaturation of the protein (see “Experimental Procedures”). Furthermore, the value of relative $f'$ was only slightly temperature dependent in the presence of equimolar amount of phalloidin in the cases of actin filaments polymerized from either ATP- or ADP-actin monomers (see inset in Fig. 5).

**DISCUSSION**

According to earlier results the actin monomer with bound ADP can denature faster than the monomer in the presence of ATP (34). Therefore, in our experiments the ADP-actin was desired to be in a monomeric form for only the shortest required
Fo¨rster's critical distance (concentration). The ratio of the acceptor concentration to the total actin monomers as a function of acceptor molar ratio (which is defined as acceptor was IAF attached to the Cys374 residue of actin. The total actin concentration was 30 μM, the acceptor molar ratio was 0.66 and the donor labeled monomer concentration was 3 μM in the filaments. The inset shows the effect of phalloidin on the temperature dependence of FRET parameters in filaments obtained from ATP- (filled squares) or ADP-actin protomers (empty squares). The solid and dashed lines represent the experimental data measured in the absence of phalloidin in the case of ATP-monomer and ADP-monomer filaments, respectively.

Therefore, the filaments from both ATP- and ADP-actin monomers were assembled following a Ca\textsuperscript{2+}-Mg\textsuperscript{2+} exchange procedure with bound Mg\textsuperscript{2+} so that the time, which the actin monomers spend in ADP form, could be minimized and also the known effect of different cations could be excluded (10, 24, 40, 41).

In the presence of ADP the nucleotide content of the generated actin filaments were homogeneous. The filaments polymerized in the presence of ATP also consist mainly of ADP-actin protomers. However, there were ATP and ADP-P\textsubscript{i} protomers at the barbed end of them (6). The presence of similar “cap” of ATP and ADP-P\textsubscript{i} protomers at the pointed end is practically negligible (6). To estimate the relative concentration of ATP- and ADP-P\textsubscript{i} protomers one needs to consider the treadmilling rate (2 μm/h (42)) and the mean length of the actin filament. According to earlier results the mean length of the filament ranges between 1.7 and 7.2 μm (43–46). Using a consensus value of 5 μm for the mean length the turnover time is 2.5 h. The rate constants characteristic for the conversion of ATP to ADP-P\textsubscript{i} in actin filaments is 0.02–0.05 s\textsuperscript{-1} (5, 47), while for the conversion of ADP-P\textsubscript{i} to ADP is 0.006 s\textsuperscript{-1} (48–50). Accordingly, one can calculate that less than 2% of the actin protomers contained ATP or ADP-P\textsubscript{i} in our present experiments, while the remaining actin population bound ADP when the filaments were generated in the presence of ATP. Therefore, the effect of this ATP and ADP-P\textsubscript{i} actin protomer cap at the barbed end on the dynamic properties of the filament is very likely negligible.

To properly interpret the spectroscopic results it seems to be important to discuss whether the actin was in a filament form in the samples during the measurements. The preparation procedures applied in this study involved a long polymerization time (10–12 h) at room temperature, which should be sufficient to reach the appropriate monomer-filament equilibrium (3). However, a portion of actin, equal to the critical concentration, was in a monomer form during the experiments and the relative concentration of monomers was very likely different in filament samples obtained from ATP- or ADP-actin monomers (3). To decrease the effect of actin monomers the actin concentration was adjusted to a relatively large value, 30 μM. Furthermore, the FRET efficiency was also measured in the presence of phalloidin, so that the undesired effect of the contribution of actin monomers on the measured fluorescence parameters could be estimated. The stabilizing effect of phalloidin on actin filaments was described earlier by using a number of experimental methods (20, 35, 52–56). It was shown that phalloidin decreased significantly the rate of monomer dissociation and shifted the monomer-filament equilibrium toward the filamentous form. The observation according to which the presence of phalloidin did not appreciably modify the transfer efficiency at 22 °C in filaments polymerized either from ATP- or ADP-actin monomers at any acceptor molar ratios suggests, that the effect of the presence of actin monomers on the FRET parameters was negligible.

The nucleotide dependence of the fluorescence spectra of IAEADANS (Fig. 3) indicates that the conformation of the protein matrix around the Cys\textsuperscript{374} residue is different in filaments from ATP- and ADP-actin monomers. In actin filaments the effect of nucleotides on the IAEADANS intensity was in good agreement with the results obtained recently on actin monomers (25). Interestingly, the conformational difference between the two types of filaments is not associated with a change in the axial position of Cys\textsuperscript{374} relative to the longitudinal filament axis as the radial coordinate of this residue was found to be identical in the two kinds of actin filaments.

To interpret the results of temperature-dependent FRET...

![Fig. 4. The efficiency of the FRET in actin filaments polymerized from ATP- (filled circles) or ADP- (empty symbols) monomers as a function of acceptor molar ratio (which is defined as the ratio of the acceptor concentration to the total actin concentration). The fitting was carried out with MatLab 5.2 using the Förster's critical distance (R\textsubscript{0}) of 47.74 Å and 40.83 Å for the IAEDANS-IAF pair in ATP- and ADP-actin filaments, respectively. The solid and dashed lines were calculated on the basis of fitting results. Standard deviations were calculated from the results of four independent experiments. The inset shows the acceptor molar ratio dependence of the FRET efficiency in the presence of equimolar amount of phalloidin in the cases of filaments assembled from ATP- (filled squares) or ADP-actin (empty squares) monomers as compared with the experimental results in its absence (solid and dashed lines indicate these data in filaments from ATP- or ADP-actin monomers, respectively).](image1)

![Fig. 5. Temperature dependence of the relative f’ measured in actin filaments polymerized from ATP- (filled circles) or ADP-actin (empty circles) monomers. The donor was IAEADANS and the acceptor was IAF attached to the Cys\textsuperscript{374} residue of actin. The total actin concentration was 30 μM, the acceptor molar ratio was 0.66 and the donor labeled monomer concentration was 3 μM in the filaments. The inset shows the effect of phalloidin on the temperature dependence of FRET parameters in filaments obtained from ATP- (filled squares) or ADP-actin monomers (empty squares). The solid and dashed lines represent the experimental data measured in the absence of phalloidin in the case of ATP-monomer and ADP-monomer filaments, respectively.](image2)
experiments it was assumed that the temperature-induced increase of the amplitude of relative motion of the donor and acceptor molecules was greater in a flexible form of the protein than in its more rigid form (23). Accordingly, the temperature profile of $<k_\lambda>$ and thus the experimentally obtained $f'$ (see Equation 8) should be steeper in the more flexible form of macromolecule (23). Such a consideration involves the assumption that the presence of major temperature-induced conformational changes can be excluded. It is usually very difficult to test this assumption experimentally. However, the smooth temperature dependent tendency of the relative $f'$ in both types of filaments (Fig. 5) suggests that this assumption was fulfilled in the present experiments (23). Furthermore, it was necessary to take it into account that the temperature dependence of the overlap integral ($J$) could have an undesired effect on the temperature dependence of $f'$. Therefore, the overlap integral characteristic of the IAEDANS-IAF pair was determined in both types of the filaments as a function of temperature, and corrections were carried out by normalizing the value of $f'$ with the appropriate overlap integral. Throughout this article these corrected values of $f'$ are presented. The orientation factor ($\kappa^2$) can also modify the value of $f'$. Although, the exact value of $\kappa^2$ cannot be determined experimentally, its effect might be taken as negligible because of two reasons. First, in most cases the results of FRET experiments assuming a value of $2/3$ for the orientation factor were in good agreement with the distances calculated on the basis of x-ray diffraction data (57). Second, theoretical considerations imply that the temperature-induced change for assumptions of the donor-acceptor distances dominates over the effect of the orientation factor in temperature-dependent FRET experiments (23).

Accordingly, the results of temperature-dependent FRET experiments (Fig. 5) indicate that the inter-monomer connections between neighboring actin protomers are looser in filaments polymerized from ADP-actin than in filaments from ATP-actin monomers. This observation seems to support recent results, which report nucleotide-induced flexibility differences in actin filaments (7–11). However, care should be taken when comparing results obtained by using experimental methods characteristic for motions on substantially different time scales (40). The methods applied in previous experiments (7–14) to describe the nucleotide dependence of the dynamic properties of actin filaments reported earlier (7–11) and resolved in this study is probably the consequence of conformational differences between ATP- and ADP-actin monomers. To support this conclusion further investigations should be carried out.

CONCLUSIONS

Relying on spectroscopic data presented here one may conclude that there are local conformational and inter-monomer flexibility differences between actin filaments polymerized from ATP- or ADP-actin monomers. To provide a possible explanation for these differences one needs to consider that the fluorescence intensity of IAEDANS was found to be smaller in filaments from ADP-actin monomers than in filaments polymerized in the presence of ATP, and similar nucleotide dependence of IAEDANS fluorescence was reported on actin monomer (25). Accordingly, it is reasonable to assume that due to the nucleotide-induced conformational differences of actin monomers the connection sites of the filaments assembled from these actin monomers are also different, which modifies the properties of inter-monomer interactions within the filament. Relying on these considerations we may conclude that the nucleotide dependence of the dynamic properties of actin filaments reported earlier (7–11) and resolved in this study is probably the consequence of conformational differences between ATP- and ADP-actin monomers. To support this conclusion further investigations should be carried out.

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