Effect of Ca^{2+}-Mg^{2+} Exchange on the Flexibility and/or Conformation of the Small Domain in Monomeric Actin

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ABSTRACT A fluorescence resonance energy transfer (FRET) parameter, f' (defined as the average transfer efficiency, $\langle E \rangle$, normalized by the actual fluorescence intensity of the donor in the presence of acceptor, F_{DA}), was previously shown to be capable of monitoring both changes in local flexibility of the protein matrix and major conformational transitions. The temperature profile of this parameter was used to detect the change of the protein flexibility in the small domain of the actin monomer (G-actin) upon the replacement of Ca^{2+} by Mg^{2+}. The Cys-374 residue of the actin monomer was labeled with *N*-iodoacetyl-*N′*-(5-sulfo-1-naphthyl)ethylenediamine (IAEDANS) to introduce a fluorescence donor and the Lys-61 residue with fluorescein-5-isothiocyanate (FITC) to serve as an acceptor. The f' increases with increasing temperature over the whole temperature range for Mg-G-actin. This parameter increases similarly in the case of Ca-G-actin up to 26°C, whereas an opposite tendency appears above this temperature. These data indicate that there is a conformational change in Ca-G-actin above 26°C that was not detected in the case of Mg-G-actin. In the temperature range between 6°C and 26°C the slope of the temperature profile of f' is the same for Ca-G-actin and Mg-G-actin, suggesting that the flexibility of the protein matrix between the two labels is identical in the two forms of actin.

INTRODUCTION

During muscle contraction, the flexibility of actin filaments (F-actin) may have an important role in the actin-myosin interaction. It was shown recently that actin filaments polymerized from Mg-ATP-G-actin have fourfold increased flexibility compared with the flexibility of filaments polymerized from Ca-ATP-G-actin (Orlova and Egelman, 1993). The authors proposed that the origin of this difference was the different conformational states of the actin protomer. The change in the protomer conformation can alter the flexibility of the filament by modifying the intermonomer bonding and/or the intra-monomer flexibility. The investigation of the effect of divalent cations on the dynamic properties of monomeric actin seems to be an appropriate step to determine the dominant source concerning the filament flexibility.

The actin monomer is divided into two domains (the small and the large domains), and both of these domains consist of two subdomains (numbered 1 and 2 in the small domain and 3 and 4 in the large one; (Fig. 1) (Kabsch et al., 1990). It is well established that the structural and functional properties of the actin monomer depend on the nature of the bound cation (for review see, e.g., Carlier, 1990; Estes et al., 1992). It was shown that a major change occurs in the environment of Gln-41 with the conversion of Ca-G-actin to Mg-G-actin, and the distance between a dansyl probe (attached to Gln-41) and Trp-79 and/or Trp-86 is shorter in Mg-ATP-G-actin than that in Ca-ATP-G-actin (Kim et al., 1995). However, when other labels were used, the fluorescence energy transfer measurements detected essentially no change in the distance between Gln-41 and Cys-374 of the actin monomer on the replacement of Ca^{2+} by Mg^{2+} (Moraczewska et al., 1996). Strzelecka-Golaszewska and co-workers (1993) concluded from limited proteolytic digestion experiments in G-actin that the carboxyl-terminal segment becomes involved in some intramolecular interactions as a result of the exchange of the bound Ca^{2+} with Mg^{2+}. In agreement with their conclusion, we have shown recently that due to the replacement of the bound Ca^{2+} by Mg^{2+} the carboxyl-terminal region (located in subdomain 1) of actin monomer becomes more rigid (Nyitrai et al., 1997). Accordingly, the difference in the bending flexibility of the filaments of the Ca-actin and Mg-actin (Orlova and Egelman, 1993) is probably not related to the difference of the carboxyl-terminal region of the protomer. However, the possibility that the filament flexibility correlates with the intrinsic mechanical properties of the actin monomer cannot be excluded and requires additional investigations.

The small domain (involving the subdomains 1 and 2 (Fig. 1)) of the monomer plays an important role in the formation of intermolecular contacts between neighboring protomers in the actin filament (Holmes et al., 1990; Lorentz et al., 1993; Schutt et al., 1993). Therefore, the question appears whether the changes in flexibility of this domain correlate with the changes in the filament flexibility. To answer this question, we extend our previous study (Nyitrai et al., 1997) over the whole small domain of the actin monomer.

The fluorescence probes *N*-iodoacetyl-*N′*-(5-sulfo-1-naphthyl)ethylenediamine (IAEDANS; attached to the Cys-374 residue in the monomer) and fluorescein-5-isothiocya-

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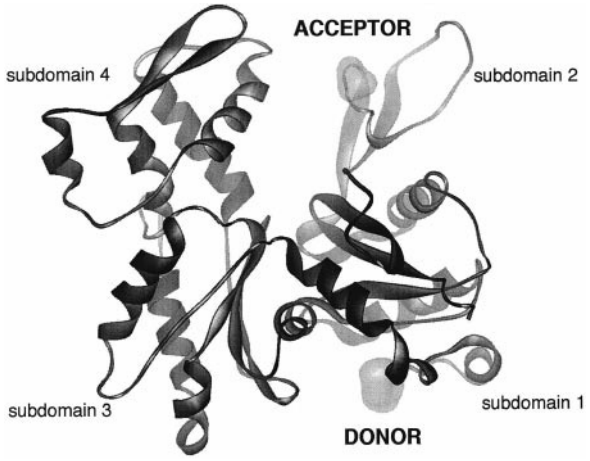

FIGURE 1 Schematic representation of the three-dimensional atomic structure of rabbit skeletal actin (Kabsch et al., 1990) showing the position of the two residues, Lys-61 (acceptor) and Cys-374 (donor), that were modified. Because the three COOH-terminal residues (involving Cys-374) are not resolved in the crystal structure (Kabsch et al., 1990), the labeled position of the donor molecule should be taken as an approximation. The four subdomains are also labeled (the coordinates were obtained from the Brookhaven Protein Data Bank, file 1ATN).

nate (FITC; Lys-61) (Fig. 1) make a suitable fluorescence resonance energy transfer (FRET) donor-acceptor pair to study spatial relationships within the actin monomer (Miki et al., 1987). It is also possible to detect the changes in conformational states and/or local fluctuations of the protein matrix between these probes by measuring the FRET parameter f' (defined as the average transfer efficiency ($\langle E \rangle$) normalized by the actual fluorescence intensity of the donor molecule in the presence of acceptor (F_{DA})) (Somogyi et al., 1984). In the present study, the inspection of the temperature profile of f' measured for Ca-G-actin and Mg-G-actin is used to monitor the effect of different metal ions (Ca^{2+} and Mg^{2+}) on the conformation and/or flexural rigidity of the small domain of the actin monomer.

MATERIALS AND METHODS

Reagents

KCl, $MgCl_2$, $CaCl_2$, Tris, sodium tetraborate (borax), IAEDANS, trypsin (porcine pancreas), quinine (hemisulfate salt), FITC, and EGTA were obtained from Sigma Chemical Co. (St. Louis, MO). ATP and α-mercaptoethanol (MEA) were obtained from Merck (Darmstadt, Germany), the Bradford protein assay reagent was purchased from Bio-Rad (München, Germany), and NaN_3 was from Fluka (Basel, Switzerland).

Protein preparation

Acetone-dried powder of rabbit skeletal muscle was obtained as described by Feuer et al. (1948). Rabbit skeletal muscle actin was prepared according to the method of Spudich and Watt (1971), with a slight modification introduced by Mossakowska et al. (1988), and stored in 2 mM Tris/HCl buffer (pH 8.0) containing 0.2 mM ATP, 0.1 mM $CaCl_2$, 0.1 mM MEA, and 0.02% NaN_3 (buffer A).

Fluorescence labeling of actin

The labeling of Cys-374 residue with IAEDANS was performed as described earlier (Miki et al., 1987). F-actin (2 mg/ml) was incubated with 10-fold molar excess of IAEDANS for 1 h at room temperature. The sample was centrifuged at 100,000 $\times$ g for 2 h at 4°C. The pellet was dissolved in buffer A and dialyzed overnight against the same buffer.

The G-actin concentration was determined spectrophotometrically using the absorption coefficient of 0.63 mg ml^{-1} cm^{-1} at 290 nm (Houk and Ue, 1974), with a Shimadzu UV-2100 spectrophotometer. In IAEDANS-labeled G-actin, the measured absorbance at 290 nm was corrected for the contribution of the fluorescence label (using absorbance at 290 nm (A_{290}) = 0.21 $\times$ A_{336} for IAEDANS bound to actin). The relative molecular mass of 42,300 Da was used for monomeric actin (Elzinga et al., 1973). Occasionally, the actin concentration was also determined by the Bradford (Coomassie blue) protein assay reagent (Bradford, 1976). The assay was calibrated as described by the manufacturer using unlabeled G-actin. The concentrations determined according to the two different methods were always identical within the limits of experimental error. The concentration of the IAEDANS in the protein solution was determined using the absorption coefficient of 6100 M^{-1} cm^{-1} at 336 nm (Hudson and Weber, 1973). The extent of labeling for IAEDANS was determined to be 0.85 $\pm$ 0.02 mol/mol of actin monomer (calculated from the results of five independent preparations).

Actin fluorescently labeled with FITC at Lys-61 was prepared according to the method of Burtnick (1984) with a modification introduced by Miki et al. (1987). The pellet of F-actin (2 mg/ml) was incubated in buffer A for 1 h and gently homogenized with a Teflon homogenizer. The sample was then exhaustively dialyzed overnight against 2 mM borate (pH 8.5), 0.5 mM ATP, 0.1 mM $CaCl_2$, and 1 mM MEA. FITC was dissolved in a few microliters of 0.1 M NaOH and was added to the IAEDANS-G-actin solution to achieve a FITC/actin molar ratio of 20:1. The reaction mixture was stirred at room temperature for 3 h, maintaining the pH at 8.5 by the addition of an appropriate volume of 0.1 M NaOH. To polymerize unreacted actin, the salt concentration was increased to 100 mM KCl, 2 mM $MgCl_2$ and 20 mM Tris/HCl (pH 7.6), and the mixture was stirred for 2 h. The sample was centrifuged at 100,000 $\times$ g for 2 h at 4°C. To remove free FITC, the supernatant was applied to a column of Sephadex G-25 eluted with 100 mM KCl, 0.2 mM ATP, and 20 mM Tris/HCl (pH 7.6). Front peaks were collected and dialyzed exhaustively (twice) against 2 mM Tris/HCl buffer (pH 8.0), containing 100 mM KCl, 0.2 mM ATP, 0.1 mM $CaCl_2$, 0.1 mM MEA, and 0.02% NaN_3. The protein solution was further dialyzed against buffer A. The next dialysis was performed against buffer A in which the concentration of Ca^{2+} was decreased to 50 μM.

The concentration of FITC-actin was determined using the Bradford protein assay reagent. The FITC concentration was determined spectrophotometrically with an absorption coefficient of 74,500 M^{-1} cm^{-1} at 493 nm (Bernhardt et al., 1983). The extent of labeling was measured to be 1.00 $\pm$ 0.04 mol/mol of actin (calculated from the results of five independent labelings).

Cation exchange in G-actin

Mg-G-actin was prepared from Ca-G-actin according to the method of Strzelecka-Golaszewska and colleagues (1993). The protein solution was dialyzed exhaustively against buffer A in which the concentration of $CaCl_2$ was decreased to 50 μM. EGTA and $MgCl_2$ were added to the actin solution to reach a final concentration of 0.2 mM and 0.1 mM, respectively. The solution was incubated for 10 min at room temperature. The temperature was then adjusted to the desired value, and the fluorescence measurements were performed.

Fluorescence measurements

Fluorescence was measured with a Perkin-Elmer LS50B luminescence spectrometer. To calculate the FRET efficiency, the fluorescence intensities of IAEDANS were recorded in the presence and absence of its acceptor

(FITC) as a function of temperature in both Ca-G-actin and Mg-G-actin. The excitation monochromator was set to 360 nm. The fluorescence intensity of IAEDANS was monitored at 475 nm, where the contribution of FITC to the measured fluorescence is negligible. Both the excitation and emission slits were set to 2.5 nm. The fluorescence intensities were corrected for the inner filter effect using the following equation (Lakowicz, 1983):

$$F_{corr} = F_{obs}\,\text{antilog}[(OD_{ex} + OD_{em})/2], \quad (1)$$

where F_{corr} is the corrected fluorescence intensity, F_{obs} is the measured fluorescence intensity, and OD_{ex} and OD_{em} are the optical densities of the sample at the excitation and emission wavelengths, respectively.

The steady-state fluorescence anisotropy was calculated from the polarized fluorescence components (F_{VV}, F_{VH}, F_{HV}, and F_{HH}, where the first and second subscripts indicate the orientation of the excitation and emission polarizers, respectively) as

$$r = (F_{VV} - GF_{VH})/(F_{VV} + 2GF_{VH}), \quad (2)$$

where $G = F_{HV}/F_{HH}$. In the case of actin-bound IAEDANS, the excitation wavelength was 360 nm and the emission wavelength was 475 nm, whereas for FITC, the excitation monochromator was set to 493 nm and the emission was measured at 520 nm. The slits were set to 2.5 nm.

To determine the fluorescence quantum yield of the donor molecule, the corrected fluorescence emission spectrum of IAEDANS-G-actin was taken with an excitation wavelength of 360 nm. The quantum yield of quinine sulfate (0.53 in 0.1 N H_2SO_4) was used as a reference (Kouyama et al., 1985).

The concentration of the actin was adjusted to 2 μM in both the IAEDANS-G-actin and IAEDANS-FITC-G-actin samples. The IAEDANS-Mg-G-actin was freshly prepared from IAEDANS-Ca-G-actin, and all of the measurements were carried out within 30 min after the addition of EGTA/$MgCl_2$ to minimize the possibility of oligomer formation. In the case of FITC-labeled actin samples and IAEDANS-Ca-G-actin, the fluorescence measurements were performed using the same protein solution over the whole temperature range.

The temperature was maintained with a HAKE F3 water bath, and the temperature of the protein solution was continuously monitored in the sample holder. The errors of fluorescence data presented in this paper are standard errors of the means calculated from the results of at least four independent measurements.

THEORY

When two fluorophores form a FRET donor-acceptor pair, the transfer efficiency can be calculated as follows:

$$E = \{1 - ((F_{DA}/c_{DA})/(F_D/c_D))\}/\beta, \quad (3)$$

where F_{DA} and F_D are the fluorescence intensities of the donor molecule in the presence and the absence of the acceptor, respectively; β is the acceptor/monomer molar ratio; and c_D and c_{DA} are the concentrations of the donor molecule in the samples indicated by the subscripts. Knowing the energy transfer efficiency (E), the distance (R) between the donor and acceptor molecules can be calculated from the following equation:

$$E = R_o^6/(R_o^6 + R^6), \quad (4)$$

where R_o is the Förster's critical distance defined as the donor-acceptor distance at which the FRET efficiency is 50%. To calculate R from E, the value of R_o needs to be obtained as follows:

$$R_o^6 = (8.79 \times 10^{-11})n^{-4}\kappa^2\phi_D J, \quad (5)$$

where n is the refractive index of the medium, κ^2 is the orientation factor, ϕ_D is the fluorescence quantum yield of the IAEDANS in the absence of FITC, and J is the overlap integral given in $M^{-1}\,cm^{-1}\,nm^4$. The overlap integral (J) is defined as follows:

$$J = \int F_D(\lambda)\epsilon_A(\lambda)\lambda^4 d\lambda \Big/ \int F_D(\lambda)d\lambda, \quad (6)$$

where $F_D(\lambda)$ is the corrected fluorescence emission spectrum of the donor and $\epsilon_A(\lambda)$ is the absorption spectrum of the acceptor.

It was shown that the mean value of the energy transfer rate constant, $\langle k_{ti}\rangle$, is an appropriate parameter for monitoring intramolecular fluctuations and/or conformational changes of a macromolecule (Somogyi et al., 1984). To determine its value experimentally, the FRET parameter f' was introduced, which can be calculated as follows (Somogyi et al., 1984):

$$f' = E/F_{DA} \quad (7)$$

Taking into account that in a real experiment the fluctuations result in a distribution of the experimental parameters, only the average value of these parameters can be measured. It was shown using these average values (Somogyi et al., 1984) that, after appropriate spectral considerations,

$$f' = E/F_{DA} = \langle k_{ti}/k_f\rangle = C\langle R_i^{-6}\kappa^2\rangle, \quad (8)$$

where k_f is the rate constants for the fluorescence emission (natural emission, which is assumed to be constant during the experiment). The subscript i indicates the value of the given parameter for the ith population, taking a momentary picture, and C is a constant involving the refractive index (n) and the overlap integral (J), which were assumed to be constant (Somogyi et al., 1984). The change in f' (which is proportional to the value of $\langle k_{ti}\rangle$) can be related to the change in the $\langle R_i^{-6}\ \kappa^2\rangle$. Even if the equilibrium distance between the donor and acceptor molecules remains the same, the smallest distance between these molecules decreases when the amplitude of the fluctuation increases. Such an increase can be generated by increasing the temperature, increasing this way the average energy content of the molecule and therefore the average amplitude of the individual fluctuations. Taking into account that the value of the energy transfer rate constant depends upon the inverse sixth power of the donor-acceptor distance, a relatively small-distance fluctuation can result in a detectably large change in the transfer efficiency. It is therefore possible that the change in the value of the measured distance between the donor and acceptor falls within the range of the experimental error due to the limitation of the applied method and instrumentation, but the value of the f' parameter reflects the change in the protein fluctuation. The in-

tramolecular fluctuations appear as a superposition of different kinds of motions (from changing atomic positions on a picosecond time scale to relative changes in subdomain orientation and/or distance falling into the millisecond, or longer, time window). The parameter f' is sensing all of these motions as alterations in the donor-acceptor distance. Therefore, by obtaining the value of f', or its temperature dependence, it is not possible to resolve the contribution of individual groups of fluctuation, each resulting in an individual spatio-temporal distribution of the donor-acceptor distance. Accordingly, the temperature profile of f' reports the average flexibility of the protein matrix located between the two labels.

RESULTS

To determine the transfer efficiency, E, the fluorescence emission of IAEDANS was measured in both the presence and the absence of FITC using the excitation and emission wavelengths of 360 and 475 nm, respectively. The intensity of the donor decreased with increasing temperature between 5°C and 35°C in both Ca-G-actin and Mg-G-actin (Fig. 2), and the values of fluorescence intensities were larger for Mg-G-actin than those for Ca-G-actin at all temperatures. All of the temperature-induced changes in the fluorescence intensity were reversible. In the presence of acceptor, the intensity of the donor was lower than that in the absence of the acceptor for both Ca-G-actin and Mg-G-actin (Fig. 2).

The efficiency (E) of the FRET was calculated from these intensities using Eq. 3 containing corrections (c_D and c_{DA}) for the possible difference between the donor concentrations in IAEDANS-G-actin and IAEDANS-FITC-G-actin samples. The resonance energy transfer was inhibited by incubating the protein with trypsin (Miki et al., 1987). The fluorescence intensities of the digested samples were taken to be proportional to the IAEDANS concentrations and were used to obtain the ratio of c_D and c_{DA}. The value of the transfer efficiency (E) decreased with increasing temperature in both Ca-G-actin and Mg-G-actin (Table 1).

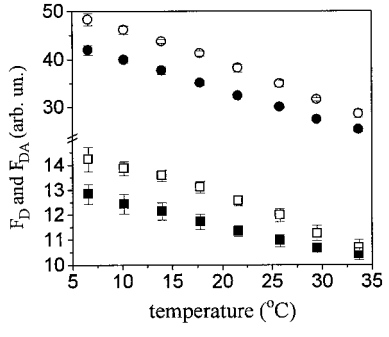

FIGURE 2 The fluorescence intensity of IAEDANS in Ca-G-actin and Mg-G-actin as a function of temperature in the presence (■ and □) and absence (● and ○) of FITC in Ca-G-actin (■ and ●) and Mg-G-actin (□ and ○). The excitation and emission wavelengths were 360 and 475 nm, respectively. The optical slits were set to 2.5 nm in both the excitation and emission sides. (The error bars at many points are within the area of the symbols.)

The value of f' (calculated according to Eq. 7) shows a different tendency in Ca-G-actin and Mg-G-actin (Fig. 3 *a*). Below 26°C, the f' is larger for Ca-G-actin than that for Mg-G-actin (Fig. 3 *a*). In this temperature range, the f' increased with increasing temperature for both Ca-G-actin and Mg-G-actin. More importantly, the slope of the f' versus temperature curves are identical for Ca-G-actin and Mg-G-actin (below 26°C; Fig. 3 *b*). Above 26°C, the tendency in Mg-G-actin remains to increase, although in Ca-G-actin, the value of f' follows an opposite tendency (Fig. 3, *a* and *b*).

The interpretation of these data requires additional spectral considerations. The absorption spectrum of FITC proved to be sensitive to the change of the temperature (data are not shown). The effect of Ca^{2+}-Mg^{2+} exchange on this parameter was negligible (data not shown). The shape of the fluorescence emission spectrum of IAEDANS is different for Ca-G-actin and Mg-G-actin (Fig. 4 *a*), and it is sensitive to the temperature in both forms of the monomer (Fig. 4, *b* and *c*). It should be noted here that the sensitivity of the fluorescence emission spectrum of IAEDANS to the change in either the temperature or the type of bound cation does not invalidate the calculation of the FRET efficiency, because the shape of the donor emission spectrum is the same in the presence and absence of acceptor between 420 and 480 nm (data not shown). Accordingly, the fluorescence intensities measured at the emission wavelength of 475 nm can be used to properly determine the value of the transfer efficiency.

The invariance of the overlap integral (J) was an important assumption used for the derivation of the Eq. 8 (Somogyi et al., 1984). However, based upon our data, this parameter cannot be treated as a constant. Due to the temperature dependence of both the absorption spectrum of FITC and the fluorescence emission spectrum of IAEDANS (Fig. 4, *b* and *c*), the fluorescence quantum yield of the donor molecule (ϕ_D), the overlap integral (J), and the value of R_o for the IAEDANS-FITC pair were calculated as a function of temperature in both Ca-G-actin and Mg-G-actin (Table 1). In the calculation of R_o, the assumption was made that the relative rotation of the donor and acceptor molecules is rapid, and therefore the κ^2 value of ⅔ was used (see Eq. 5). The refractive index of the medium was taken to be 1.4 in all cases. The donor-acceptor distances (R) were determined according to Eq. 4. The differences of calculated donor-acceptor distances in Ca-G-actin and Mg-G-actin are within the limits of experimental error (Table 1), and the value of this parameter is apparently temperature independent in both forms of the actin monomer.

The steady-state fluorescence anisotropy values of the donor and acceptor molecules are provide information about the sensitivity of the orientation factor to the type of the bound cation in the monomer. This parameter is cation independent within the limits of experimental error at any temperature (Fig. 5, *a* and *b*). The apparent limiting anisot-

TABLE 1 The measured and calculated FRET parameters for the IAEDANS-FITC pair in Ca- and Mg-G-actin

	$J \times 10^{-14}$ [M^{-1} cm^{-1} nm^4]		ϕ_D		R_o (nm)		E (%)		R (nm)	
Temperature (°C)	Ca^{2+}	Mg^{2+}	Ca^{2+}	Mg^{2+}	Ca^{2+}	Mg^{2+}	Ca^{2+}	Mg^{2+}	Ca^{2+}	Mg^{2+}
6.6	21.65	21.28	0.59	0.67	5.19	5.28	69.39 (0.42)	70.53 (0.50)	4.53 (0.08)	4.56 (0.08)
10.2	20.98	20.78	0.57	0.64	5.13	5.23	68.85 (0.45)	69.90 (0.39)	4.50 (0.08)	4.54 (0.08)
14.0	20.63	20.38	0.54	0.62	5.06	5.17	67.78 (0.40)	68.96 (0.38)	4.49 (0.08)	4.53 (0.08)
17.8	20.25	19.83	0.52	0.59	5.02	5.11	66.54 (0.38)	68.21 (0.36)	4.48 (0.09)	4.50 (0.09)
21.5	19.94	19.31	0.49	0.56	4.96	5.04	64.94 (0.23)	67.14 (0.28)	4.48 (0.09)	4.47 (0.09)
25.8	19.42	19.12	0.46	0.52	4.90	4.98	63.44 (0.32)	65.69 (0.35)	4.47 (0.09)	4.46 (0.09)
29.5	19.19	18.72	0.44	0.49	4.84	4.90	61.08 (0.42)	64.31 (0.41)	4.49 (0.09)	4.45 (0.09)
33.7	19.05	18.59	0.41	0.45	4.78	4.84	58.71 (0.79)	62.56 (0.86)	4.51 (0.09)	4.44 (0.09)

Standard errors are shown in parentheses.

ropy (r_o) values of the donor and acceptor were calculated from the plot of $1/r$ against T/η (where η is the viscosity) by extrapolating to $T/\eta = 0$ (plots not shown). The r_o values are 0.31 ± 0.02 and 0.29 ± 0.02 for IAEDANS and 0.25 ± 0.02 and 0.25 ± 0.02 for FITC in Ca-G-actin and Mg-G-actin, respectively.

DISCUSSION

The fluorescence intensity of the donor is larger in the presence of Mg^{2+} than that in the presence of Ca^{2+}, in agreement with the results of Frieden and co-workers (1980). The shape of the fluorescence emission spectrum of the donor is different in Ca-G-actin and Mg-G-actin, and also different at different temperatures in both forms of the monomer. Both the increase in the temperature and the replacement of Ca^{2+} by Mg^{2+} result in a small but significant blue shift of the emission spectrum, suggesting that the microenvironment of the donor molecule becomes more hydrophobic due to these changes.

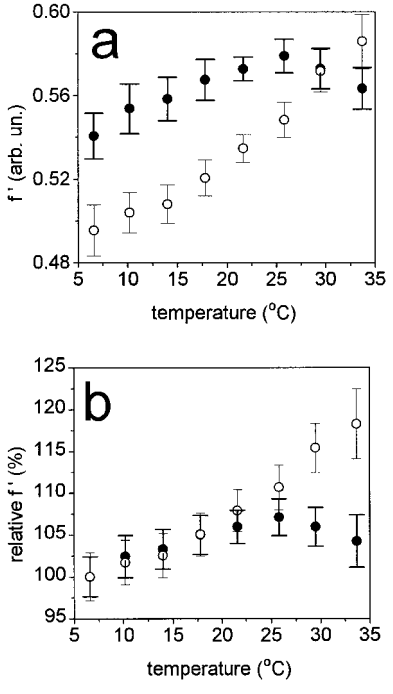

FIGURE 3 The temperature profile of f′ (*a*) and the relative f′ (*b*) in Ca-G-actin (●) and Mg-G-actin (○).

The donor-acceptor distance was determined to be 4.49 ± 0.09 nm at 21.5°C in Ca-G-actin (Table 1), which is in good agreement with the value measured by Miki and co-workers (1987) (4.6 ± 0.01 nm). According to recent studies, the distance between Gln-41 and Trp-79 and/or Trp-86 is smaller in Mg-ATP-G-actin than that in Ca-G-actin (Kim et al., 1995), whereas the distance between Gln-41 and Cys-374 residues was found to be essentially cation (Ca^{2+} and Mg^{2+}) independent in ATP-G-actin (Moraczewska et al., 1996). The latter group concluded that the apparent discrepancy of these results might arise from either the different ways of obtaining the Mg-G-actin or because the probes applied by the two groups report changes in the conformation of different regions of the monomer. Using the same cation exchange method as was used by Moraczewska and co-workers, we found that the distance between Lys-61 and Cys-374 is independent of the cation content (Ca^{2+} or Mg^{2+}) of the actin monomer. Therefore, our results seems to support the observation of Moraczewska and co-workers (1996). However, taking into account that the protein segment investigated in our experiments is essentially the same as the protein segment in Moraczewska's study, it is still not possible to exclude that the different conclusions obtained by Kim and co-workers (1995) is the result of the difference in either the investigated protein region or the nature of the applied reporter molecules.

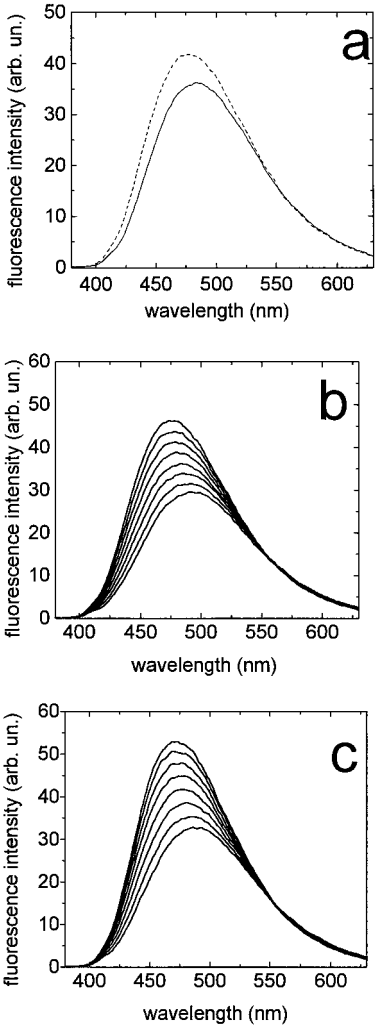

FIGURE 4 The corrected fluorescence emission spectrum of IAEDANS (*a*) in Ca-G-actin (——) and Mg-G-actin (- - -) at 21.5°C. (*b* and *c*) The emission spectrum of IAEDANS as a function of temperature (6.6, 10.2, 14.0, 17.8, 21.5, 25.8, 29.5, and 33.7°C) in Ca-G-actin (*b*) and Mg-G-actin (*c*). The excitation wavelength was 360 nm, and the optical slits were set to 2.5 nm in both the excitation and emission sides. The peak intensity in *b* and *c* increases with decreasing temperature.

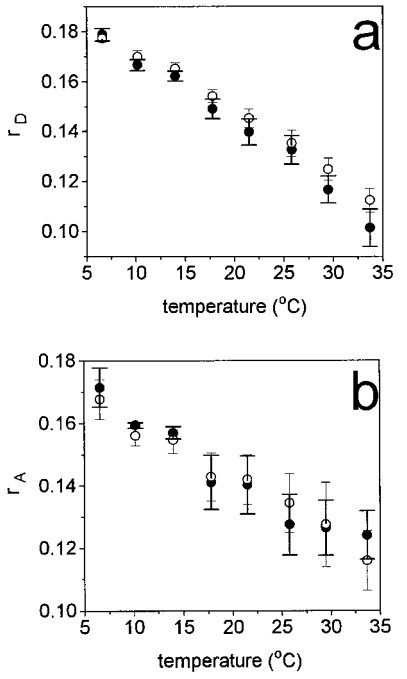

FIGURE 5 The fluorescence anisotropy of the donor (*a*) and acceptor (*b*) molecules as a function of temperature in Ca-G-actin (●) and Mg-G-actin (○). The excitation wavelengths were 360 nm and 493 nm, and the emission was monitored at 475 nm and 520 nm for IAEDANS and FITC, respectively. The slits were set to 2.5 nm.

The temperature profile of the energy transfer parameter f' (Eq. 7) may provide information about the flexibility of the protein matrix between the donor and acceptor molecules. Two major processes can contribute to the change in this parameter. Generally, the dominant effect is the change in the relative fluctuations of the protein segment between the donor and acceptor molecules. Comparing the two forms of the macromolecule, the distribution that describes the distances between the donor and acceptor molecules can be different in the width of distribution, whereas the mean distance value is the same. This kind of difference is clearly the sign of the different flexibility of the protein matrix in the two forms of protein. However, the major conformational change in the protein that would result in different mean distances between the donor and acceptor molecules could also contribute to the change in the value of the temperature profile of f'.

The change in the temperature affects the energy content of the protein matrix. The increased thermal energy at higher temperature usually results in an increase of the amplitude of local fluctuations inside the protein matrix. Considering the dependence of the k_t on the donor-acceptor distance (R), the larger amplitude of the relative fluctuation of the donor and acceptor molecules would result in a larger $\langle k_{ti} \rangle$ value. The experimentally obtained parameter, f', was shown to be proportional to the average value of the rate constant ($\langle k_{ti} \rangle$) characterizing the FRET mechanism (Somogyi et al., 1984). Accordingly, one would expect f' to increase with increasing temperature if the effect of major conformational changes can be excluded. Furthermore, in a more rigid form, of the protein the slope of the temperature profile of the f' should be smaller.

The tendency of f' to increase in Mg-G-actin over the whole temperature range, and in Ca-G-actin below 26°C (Fig. 3) is in agreement with the above considerations. However, f' displays an opposite tendency in Ca-G-actin above 26°C, indicating that a temperature-induced conformational change is present in this form of the monomer above room temperature. This kind of conformational transition was not detected in Mg-G-actin.

The comparison of the f' values measured in the two forms of the protein can provide information about the cation-dependent change in protein flexibility. Due to the temperature-induced conformational transition in Ca-G-ac-

tin at temperatures above 26°C, the comparison of the flexibilities is possible only below this temperature. A few spectral considerations should be taken into account. The invariance of the rate constant for the fluorescence emission (k_f) is an important assumption used for the derivation of the final equations (Somogyi et al., 1984). This assumption is likely satisfied according to earlier publications that describe a fairly constant value of k_f under a wide variety of different experimental conditions (Badley and Teale, 1969).

The fluorescence anisotropy of both the donor and the acceptor molecules (Fig. 5) and the apparent limiting anisotropy of the donor and acceptor are independent of the cation content of the actin monomer. These results of the fluorescence anisotropy measurements suggest that the value of the orientation factor (κ^2) is unchanged during the cation exchange (this conclusion is likely valid even if the initial assumption of freely rotating donor and acceptor molecules (and therefore $\kappa^2 = ⅔$) is not reliable).

Although it was assumed in the derivation of the final equation that the value of the overlap integral (J) is constant (Somogyi et al., 1984), in our experiments the value of this parameter proved to be sensitive to the change of both the temperature and the type of the bound cation. The value of f' depends on the value of the overlap integral. Therefore, to interpret the temperature profile of f', the sensitivity of J to the kind of the bound cation should be taken into account. The ratio of f' and J was calculated as a function of temperature in both Ca-G-actin and Mg-G-actin (Fig. 6 *a*). The slope of the f'/J versus temperature curve is identical for Ca-G-actin and Mg-G-actin (Fig. 6 *b*). According to all these data, the flexibility of the small domain seems to be identical in Ca-G-actin and Mg-G-actin.

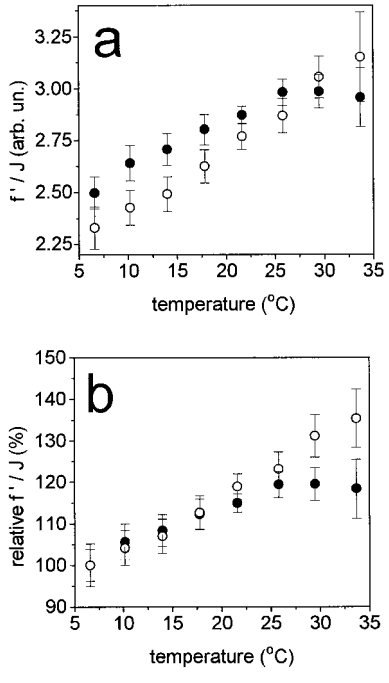

FIGURE 6 The ratio of f′ and the overlap integral (*a*) and the relative change of this ratio (*b*) as a function of temperature in Ca-G-actin (●) and Mg-G-actin (○).

The fluorescence parameters of a reporter molecule attached to the protein might be sensitive to the change in the association state of the protein, and therefore it is necessary to investigate the possibility of actin oligomer formation under the experimental conditions applied here. The ability of Mg-G-actin to form short oligomers was reported by several laboratories (Newman et al., 1985; Attri et al., 1991). The formation of oligomers is believed to occur above a certain concentration of actin. This limiting concentration was determined to be 12.5 μM in the presence of 100 μM $MgCl_2$ at low ionic strength (Attri et al., 1991). In the present work, the concentration of actin monomers was well below this critical concentration (2 μM), and the IAEDANS-Mg-G-actin samples were freshly prepared from IAEDANS-Ca-G-actin for each temperature to minimize the possibility of oligomer formation. In this concentration range, the formation of oligomers can be excluded.

The absence of these oligomers is further supported by the results of our fluorescence anisotropy measurements. Considering the precision of fluorescence anisotropy measurements (the usual error is ~0.005), even the presence of 10% actin dimers is detectable as stated by Kasprzak (1994). In our measurements, the anisotropy of IAEDANS was found to be 0.140 ± 0.005 and 0.145 ± 0.003 for Ca-G-actin and Mg-G-actin, respectively, at 21.5°C, indicating that the investigated actin was monomeric (Kasprzak, 1994). Furthermore, the self-association ability of FITC-labeled actin monomers is negligible (Burtnick, 1984), an observation that also corroborates the conclusion that the properties of monomeric actin were investigated in this report.

CONCLUSIONS

According to our results, the distance between the labels attached to Lys-61 and Cys-374 of the actin monomer is not sensitive to temperature and remains the same after the replacement of Ca^{2+} by Mg^{2+}. There is a temperature-induced conformational change in Ca-G-actin above 26°C, which is not present in Mg-G-actin. More importantly, the inspection of the temperature profile of f' measured in the two forms of actin monomer could not detect any difference between the flexibility of the protein matrix in Ca-G-actin and Mg-G-actin between residues Lys-61 and Cys-374.

The microenvironment of the Cys-374 residue was shown to be more rigid in Mg-G-actin than that in Ca-G-actin (Nyitrai et al., 1997). The extent of this difference around the carboxyl-terminal region, in the light of the results of the present study, might be too small to influence the overall flexibility of the small domain. As an alternative explanation, it is possible that the effect of the more rigid structure in the local environment of Cys-374 residue in Mg-G-actin is counterbalanced by the larger flexibility of the remaining part of the protein matrix between Lys-61 and Cys-374. The

identical slope of f' versus temperature in the two forms of actin assumes an exact balance over the whole temperature range, which makes this latter case very unlikely to occur.

Based upon these data, one can conclude that, unless the protomer flexibility behaves differently in F-actin, the difference of bending flexibility of the filaments of Ca-actin and Mg-actin is not related to the different flexibility of the small domain in the two forms of actin. Of course, conformational differences occurring upon Ca^{2+}-Mg^{2+} exchange (e.g., Carlier, 1990; Estes et al, 1992; Kim et al., 1995; Moraczewska et al., 1996; Nyitrai et al., 1997) that are not detected by our method with the applied labeling might contribute to the proposed difference in the protomer conformation (Orlova and Egelman, 1993), which apparently is the key to explain the flexibility difference of filaments.

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