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ORIGINAL PAPER

Excimer Fluorescence as a Tool for Monitoring Protein Domain Dynamics Applied to Actin Conformation Changes Based on Circulary Polarized Fluorescence Spectroscopy

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Abstract Fluorescence-detected circular dichroism (FDCD) was introduced into the study of protein conformation changes. Actin was used as a model protein which undergoes dynamic conformation changes as it polymerizes. Actin labeled with *N*-(1-pyrene)iodoacetamide (PIA) showed monomer fluorescence peak at 386 and 410 nm, and excimer fluorescence peak at around 480 nm. Excimer was formed by PIA-dimers labeled to different sites of amino acid residues. New information concerned with actin structural changes were monitored by fluorescence emission spectra excited with left- and right-circulary polarized light at 355 nm. FDCD intensities were shown as the difference in the fluorescence emission ΔF, where $\Delta F = (F_L - F_R)/(F_L + F_R)$ denoting F_L and F_R as emissions obtained by excitation with left- and right-circulary polarized light. When solvent conditions of PIA-actin were changed by addition of NaCl, TFE, or ATP, ΔF showed sensitive responses to these compounds. From the analysis of ΔF_M and ΔF_E which represent the peaks of ΔF at the monomer- and excimer-emission band, the information concerned with the actin intrastructural changes were obtained. This method based on monitoring the excimer fluorescence with FDCD could be used for other proteins to extract finer structural changes that cannot be detected by the normal fluorescence spectroscopy.

Keywords Twist · Excimer fluorescence · Actin · Trifluoroethanol · FDCD

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Introduction

As a new method for protein conformation study using fluorescence spectroscopy, here we propose an introduction of excimer-emission monitoring based on FDCD to study the dynamic structural changes of protein in solution. A new method for protein conformation study using FDCD has been shown to be sensitive and selective, as we can study at a low analyte concentration and minimize non fluorescent contribution [1]. As protein labeled with fluorophores is monitored by FDCD, the signal gives the information concerned with protein structural changes based on fluorophores polarization. Especially, when an excimer is formed, the signal of FDCD from the excimer fluorescence is supposed to extract new information about the configuration of labeled sites. Here, fluorescent probes PIA were labeled to a model protein actin. PIA were bound to one of the subdomains. Actin is now attracting attentions of researchers again. As a recent study recognized that without understanding of its structural changes in the active states of muscle contraction, the molecular mechanism would not be understood. Development of a method to measure the intrastructural changes of a molecule will be especially needed for understanding the contraction mechanism. The analysis of crystals by X-ray or electron diffraction were effective for understanding the static structure of molecules. However, for elucidation of dynamic structural changes, development of other

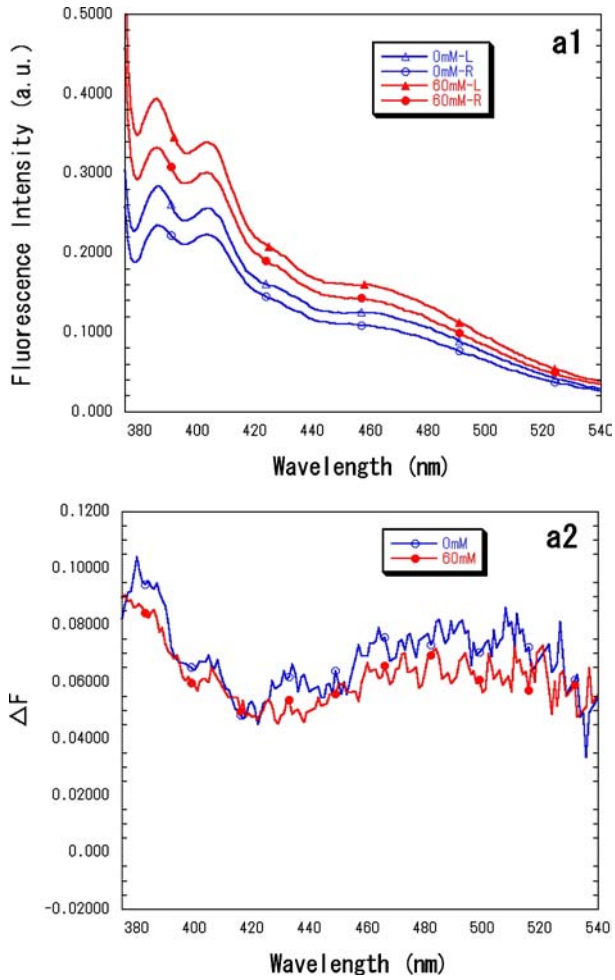


Fig. 1 Fluorescence emission spectra of PIA-actin in A-buffer excited at 355 nm. PIA-actin concentration was 0.46 mg/ml. (a1) Excited with left-circulary polarized light (△, ▲) in the absence (open mark) and presence of 60 mM NaCl (closed mark), and right-circulary polarized light (○, ●) in the absence and presence of 60 mM NaCl. (a2) ΔF spectra in the absence (○) and presence (●) of 60 mM NaCl

methods is needed. Actin binds ATP in the cleft of domains. Our previous observation showed that ATP induced filament formation of actin in a low-salt buffer [2]. This suggested that ATP induced the intrastructural change of actin. In addition to this, our observation of actin microcrystals under high-voltage electron microscope (HVEM) showed a diffraction suggesting the reflection of α-helices tilted from the filament axis. Then, we began to develop a method to measure dynamic structural changes of a molecule in solution. Here, we have combined the observations of fluorphore polarization with excimer fluorescence. As excimer fluorescence appears as interactions of two fluorphores, it has a space-sensitive character. NaCl, TFE, and ATP were often applied to actin to induce its conformation changes. Applying them to PIA-actin in the low-salt buffer, ΔF was monitored. In response to their additions, ΔF exhibited the characteristic behaviors. Changes in ΔF could be ascribed to changes in the structures of labeled subdomains [3]. Present method will be applicable to other proteins which needs to monitor conformation dynamics for the understanding of functional mechanisms.

Experimental

Materials

Trifluoroethanol (TFE) was obtained from Nakarai Chemical Co. Actin was extracted from rabbit skeletal muscle and

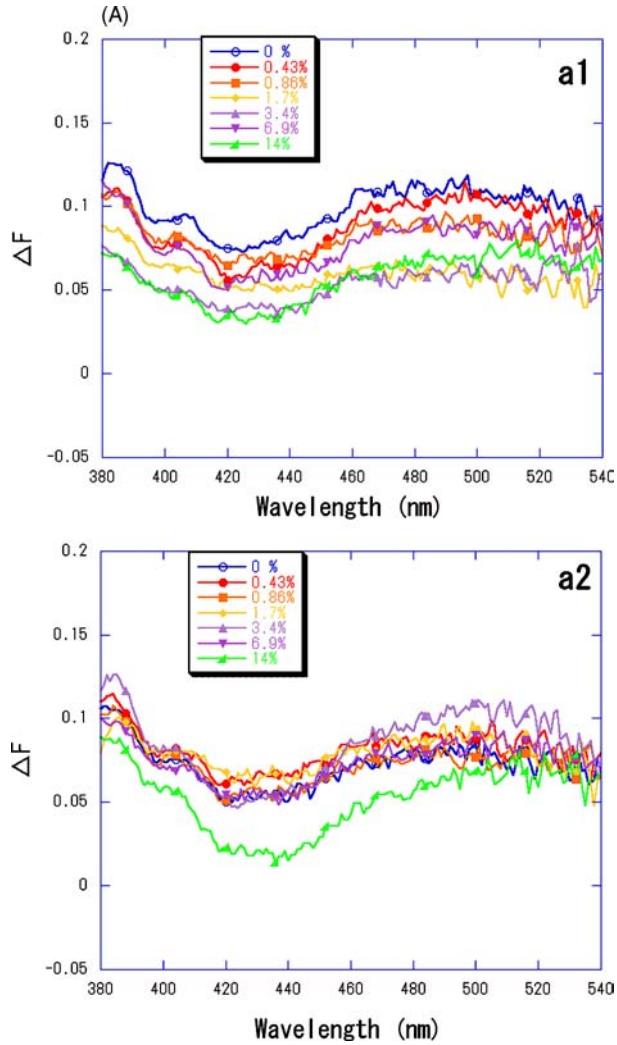


Fig. 2 (A) TFE effect from 0 to 14% on ΔF spectra of PIA-actin (0.46 mg/ml) in A-buffer. (a1) in the absence of NaCl. (a2) in the presence of 60 mM NaCl. (B) Analyses of ΔF spectra shown in Fig. 2A. (a1) Plots of ΔF_E and ΔF_M against TFE in the absence of NaCl. (a2) Plots of ΔF_E and ΔF_M against TFE in the presence of 60 mMNaCl. (b1) Plots of wavelengths of the peaks of ΔF_E and ΔF_M against TFE in the absence of NaCl. (b2) Plots of wavelengths of the peaks of ΔF_E and ΔF_M against TFE in the presence of 60 mM NaCl. (c1) Plots of $\Delta F_E/\Delta F_M$ in the absence of NaCl. (c2) Plots of $\Delta F_E/\Delta F_M$ against TFE in the presence of 60 mM NaCl.

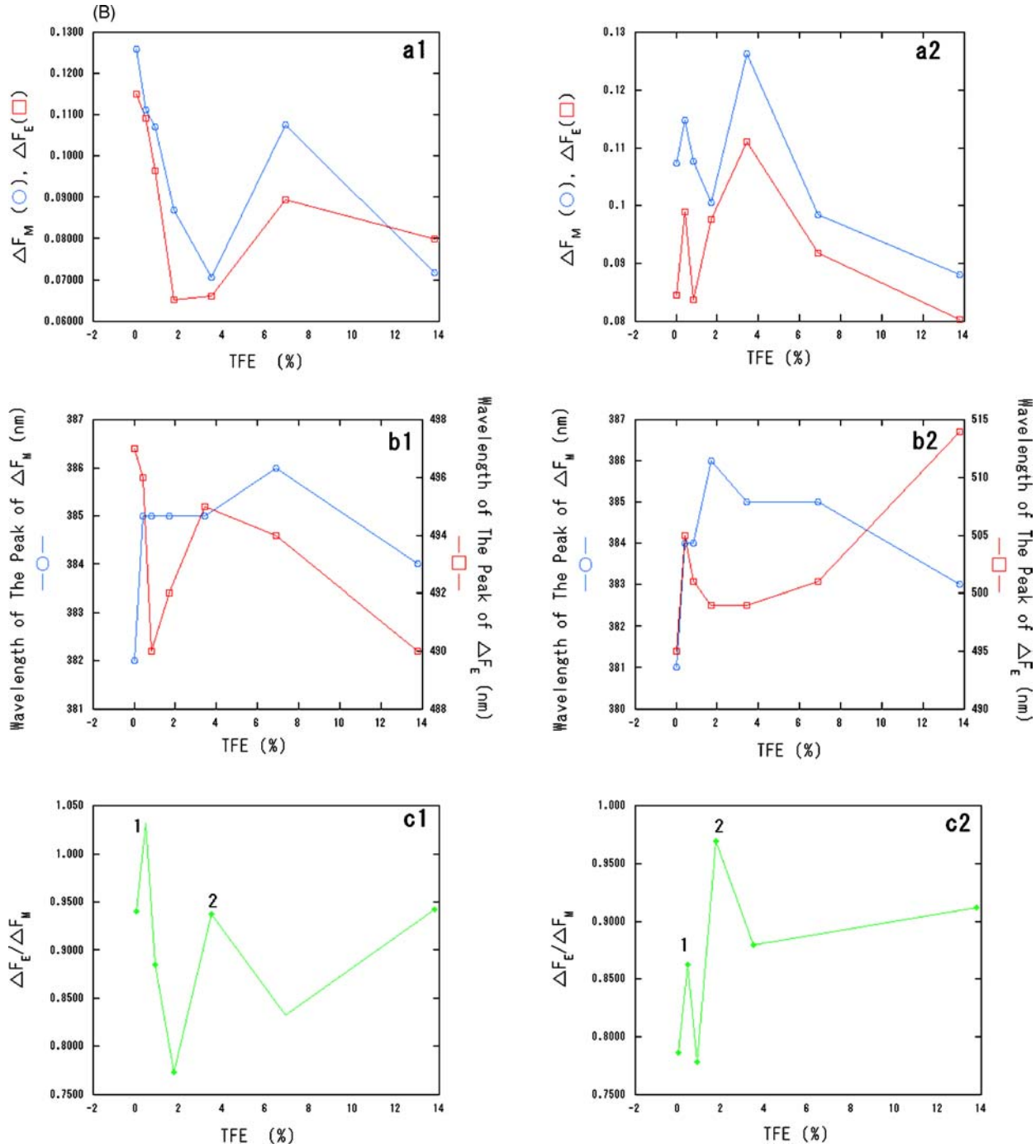


Fig. 2 Continued

purified as described previously [4]. Labeling of actin in a low-salt A-buffer (0.1 mM ATP, 0.1 mM $CaCl_2$, 2 mM Tris–HCl pH 8.0 and 1 mM NaN_3) was performed with fluorescent probes *N*-(1-pyrene)iodoacetamide (PIA) (Molecular Probes). Labeling ratio of PIA to actin was 1.98. PIA was labeled at cys10 and cys374 [5]. ATP bound on PIA-actin was removed by anion exchange resin Dowex 1×8 (100–200 mesh) which was added in the ratio 1:100 (Dowex 1×8:PIA-actin) (w/w), to A-buffer at 4 °C. The resin was removed from a solution by filtration.

FDCD measurements

FDCD measurements were performed with F-2000 spectrophotometer, 10 nm bandwidth for excitation and emission

(Hitachi Co.). HNP′B film (3M Co., USA) was used as a polarizer and for the excitation of samples by circularly polarized light, a retardation plate (Shigma Koki Co.) was set at the excitation side. These attachments were aligned as reported [6]. The linear polarizer was set in the vertical direction. The retardation plate of a quarter wavelength (1 mm thick at 355 nm) was set at the angle of 45° from the vertical direction. Left- and right-circularly polarized light for excitation was selected by rotating the retardation plate by 90°. To avoid photoselection of PIA-actin, another polarization film was set at the emission side with the orientation of the critical angle of 54.7° from the vertical direction [7]. The baselines for FDCD experiments were assigned using pyrene as a non-chiral fluorophore in the visual region [1]. Temperature was set at 23°C by circulation of water through the jacketed cell holder, in which 4 mm pathlength cell was set with a use of adaptor. Spectra were collected with photomultiplier at 400 V.

Crystals growth and HVEM observation

Using the method reported previously [8], the buffer for crystallization of actin was searched. Microcrystals of actin in A-buffer were grown in capillaries (2.0 mm in diameter × 100 mm). The capillaries were dipped in 1 ml A-buffer supplemented with 10 mM ATP. Subsequently, 3 ml of aceton were added to this solution and kept overnight at 4°C. Next day, Microcrystals grown in the capillaries were used for diffraction under Hitachi, H-1250M HVEM operated at 1000 kV. Microcrystals were mounted on the carbon coated 200 mesh grids.

Results and discussion

Effect of NaCl on FDCD of PIA-Actin

Figure 1 shows fluorescence emissions of PIA-actin excited with left- and right-circulary polarized light. As 60 mM NaCl was added to PIA-actin in A-buffer, the fluorescence intensities of PIA-actin increased. This is known as the polymerization of PIA-actin in a low salt buffer. ΔF showed the peaks at around 386 and 500 nm (Fig. 1, a2), which corresponded to the peaks of pyrene monomer and excimer emission. These peaks were expressed as ΔF_M and ΔF_E, respectively. When NaCl was added to the solutions, compared to the values before addition of salt, ΔF_M and ΔF_E decreased. Then, the polymerization of PIA-actin induced a decrease in ΔF. This seems to reflect the increase in rigidity [9] of PIA-actin due to the increase in constraint by polymerization.

TFE effects on FDCD in the absence and presence of NaCl

A cosolvent TFE is known to induce α-helical structure to the peptide having the *N*-terminal sequence of actin [10]. Changing the volume fractions of TFE from 0 to 14%, ΔF was examined. Spectra of ΔF in the absence and presence of NaCl are shown in Fig. 2A, a1 and a2. The peaks of ΔF_E and ΔF_M of each curve were plotted as a function of TFE (Fig. 2B, a1 and a2). Both in the absence and presence of salt, ΔF_E and ΔF_M changed in parallel with varying concentrations of TFE. In the absence of salt, ΔF_E and ΔF_M showed a simple decreasing curve until about 2% TFE, then, started to increase and reached the peaks at around 6.9% TFE. However, in the presence of salt, besides main peak at 3.5%, a small peak was observed at 0.43%. In addition, the concentration of the main peak was 3% lower than that in the absence of salt. Consequently, TFE effect on ΔF depends on the polymerization of PIA-actin. In the following, the wavelengths corresponding to the peaks of ΔF_E and ΔF_M were shown as a function of TFE concentrations (Fig. 2B, b1 and b2). In the absence of salt, the wavelengths of the peak of ΔF_E showed the blue shifts at 0–0.9 of TFE and 3.5–14% TFE. However, in the presence of salt, the blue shifts of the peak of ΔF_E were only observed at 0.43–1.7% (Fig. 2B, b2). It has been reported that unstable excimer emission in which the fluorophores were in the twisted orientations, showed the blue shift of its band compared to the stable excimer emission band [11]. However, the red shift of absorption was caused by the enhancement of the fluorophores' interactions (overlap)

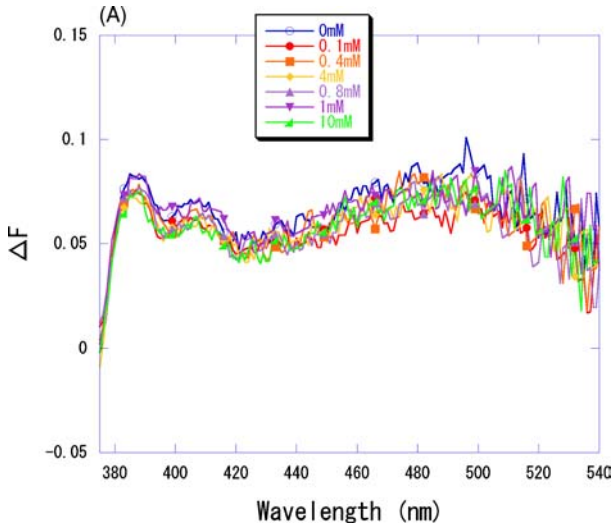
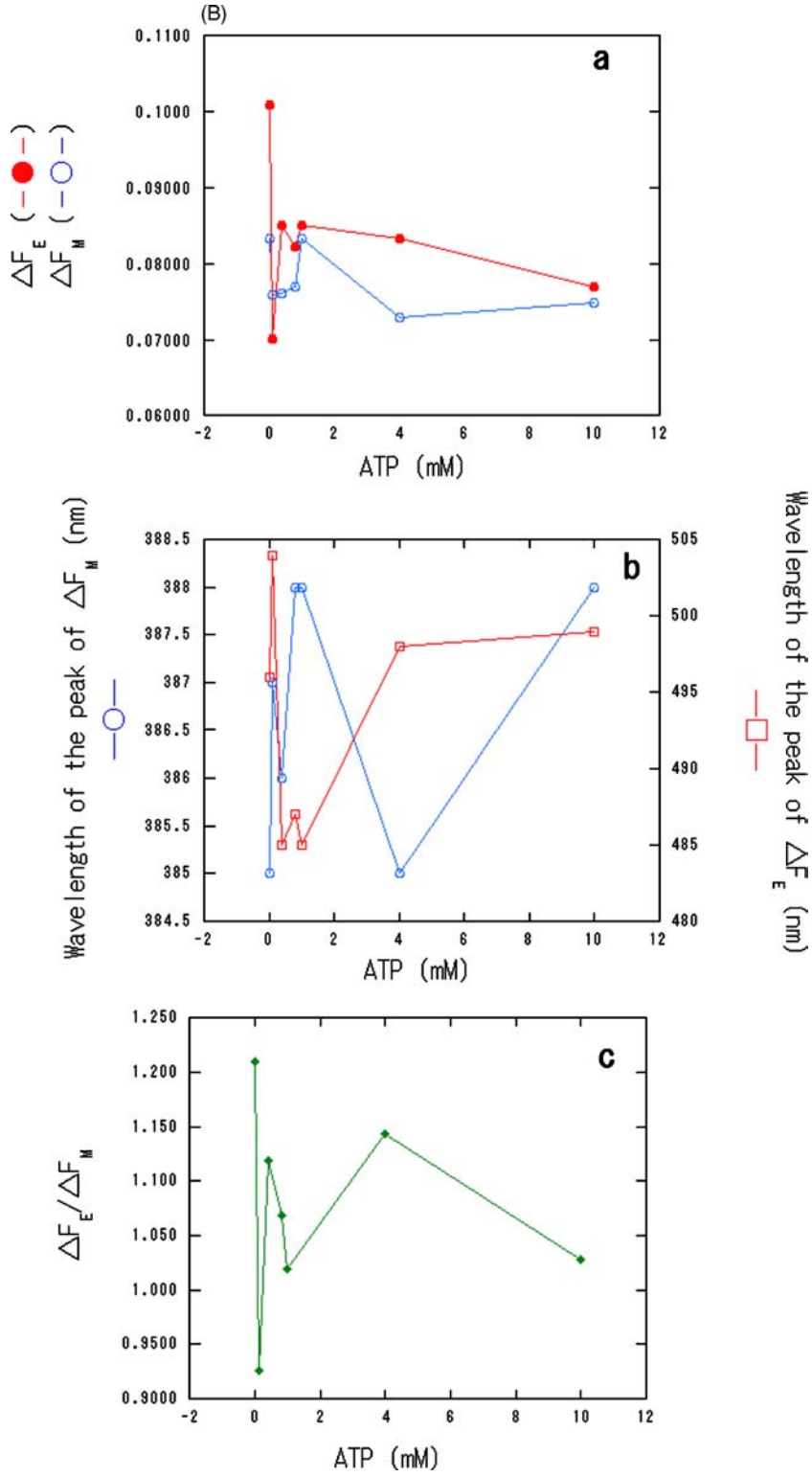


Fig. 3 (A) ATP effects on ΔF spectra of PIA-actin in A-buffer (0.29 mg/ml). ATP concentrations were varied from 0 to10 mM. Before additions of ATP to PIA-actin in A-buffer, ATP naturally bound on actin was removed by Dowex1 treatment. (B) Analyses of ΔF spectra shown in (A). (a) Plots of ΔF_E and ΔF_M against ATP. (b) Plots of the bands of ΔF_E and ΔF_M against ATP. (c) Plots of $\Delta F_E/\Delta F_M$ against ATP.

Fig. 3 Continued



[12]. Then as the mirror image of absorption, the red shift of the monomer emission could be attributed to a change in overlap (or slide) of fluorophores. It seems that twist or overlap of fluorophores will induce similar wavelength shifts over the bands of ΔF_E and ΔF_M. Taking this into account, the ratios of ΔF_E to ΔF_M were examined at various concentrations of TFE (Fig. 2B, c1 and c2). Two peaks were observed both in the absence and presence of salt. Comparing the peak positions on the curve in Fig. 2B, c1 to those on the curves in Fig. 2B, b1, the first peak of the curve in Fig. 2B, c1

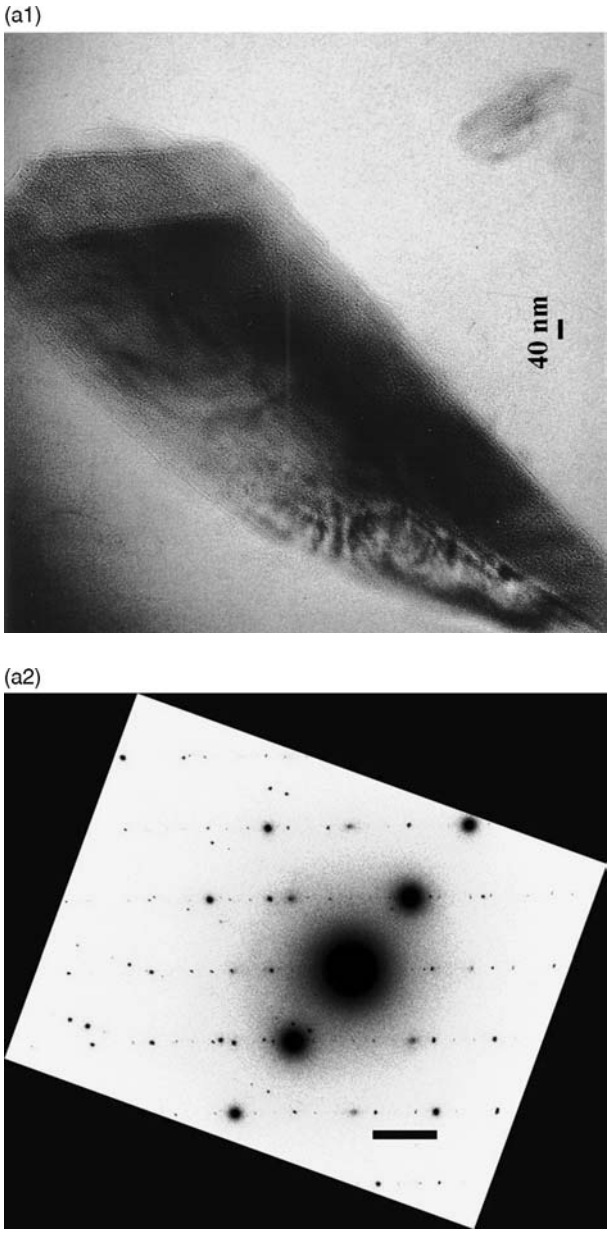


Fig. 4 (a1) Photograph of actin microcrystal observed with HVEM. (a2) Photograph of the electron diffraction of actin microcrystals observed with HVEM. Bar denotes 1/5 Å

coincides with the peak of ΔF_M (Fig. 2B, b1) and the second peak coincides with the peak of ΔF_E. Similarly, in the presence of salt, comparing the curve in Fig. 2B, c2 to the curve in Fig. 2B, b2, the first peak coincides with the peak of ΔF_E (Fig. 2B, b2) and the second peak matches to the peak of ΔF_M.

Consequently, at the first peak the twist effect will be preponderant and the overlap effect at the second peak. Interestingly, the effectiveness of both types of structural changes were reversed by polymerization. Thus, polymerization may regulate the predominance of these effects through the rigidity change in PIA-actin.

ATP effects on FDCD

Actin has a tightly bound ATP on it. In the first step, this ATP was removed by chelating reagent Dowex-1. To this ATP free PIA-actin solution, various amounts of ATP were added (Fig. 3A). As 0.1 mM ATP was added to this solution, ΔF decreased drastically. However, the elevation of ATP to 0.4 mM suppressed the extent of decrease at 0.1 mM. Consequently, another effect of ATP arose at 0.4 mM ATP addition. Next, ΔF_E and ΔF_M obtained from Fig. 3A were plotted as a function of ATP (Fig. 3B, a). Compared to ΔF_M, ΔF_E showed more than three times decrease at 0.1 mM ATP. Such a large decrease was only observed at this concentration. Accompanying to further elevations of ATP concentrations, ΔF_E and ΔF_M reached the peaks at around 1.0 mM, and then turned to decrease. Next, the wavelengths of the bands of ΔF_E and ΔF_M at various concentrations of ATP were examined (Fig. 3B, b). The band of ΔF_E showed blue shifts at 0.1–0.4 mM and red shift beyond 1.0 mM ATP. Whereas, the band of ΔF_M showed red shifts at 0–0.1, 0.4–1.0 and 4.0–10 mM ATP, and blue shifts at 0.1–0.4 and 1.0–4.0 mM ATP. Further, the effectiveness of two types of structural changes at various concentrations of ATP were examined. In Fig. 3B, c, the ratios of $\Delta F_E/\Delta F_M$ were plotted as a function of ATP. The initial sharp decrease in $\Delta F_E/\Delta F_M$ from 1.2 to 0.93 induced at 0.1 mM ATP seems to be attributed to rebinding of the removed ATP. It is because, after addition of ATP, the ratio of $\Delta F_E/\Delta F_M$ became identical to the value of PIA-actin not treated with Dowex-1 (see the initial value in Fig. 2B, c1). As ATP concentrations were increased further beyond 0.1 mM until 10 mM, two peaks appeared at around 0.4 and 4 mM ATP (Fig. 3B, c). Comparing these peaks with those on the curves in Fig. 3B, b, the first peak coincides with the peak of ΔF_M and the second peak coincides with the peak of ΔF_E. Consequently, two types of structural changes were induced alternately at different concentrations of ATP. This kind of alternation may suggest are essential property of actin for the expression of its biological function.

Conformation changes observed with variation of excitation wavelengths

As shown above, the peaks of wavelengths of ΔF_E and ΔF_M, and the ratio of $\Delta F_E/\Delta F_M$ changed sensitively corresponding to the intrastructural changes of PIA-actin induced by TFE or ATP. These results were obtained by excitation at

355 nm. Further studies with variation of wavelengths would be interesting to establish the conformation changes at different polarizations. The excitation spectrum of PIA-actin observed at 410 nm emission showed the peak at 347 nm. Here, 325 nm was selected for excitation wavelength. As TFE concentration was increased from 0 to 2%, the ratio of $\Delta F_E/\Delta F_M$ observed at 325 nm excitation showed the increase from 0.715 to 1.61. This is in contrast to the decrease observed at 355 nm excitation (see Fig. 2B, c1). This could be attributed to the energy transfer from aromatic amino acid residues to PIA. The excitation spectrum of PIA-actin observed at 410 nm emission showed a small peak at 296 nm. Tryptophan shows the excitation peak at 291 nm and the emission peak at 340–350 nm [5]. Thus, compared to 355 nm, the excitation at 325 nm is favorable to tryptophan excitation. Consequently, as a result of the conformation change induced by TFE, the energy transfer from tryptophan to PIA was increased, which resulted in ΔF_E increase and following $\Delta F_E/\Delta F_M$ increase. Summarizing these results, 2% TFE induced the conformation change of PIA-actin to bring tryptophan moiety in close proximity to a pyrene moiety. Present result confirms the usefulness of monitoring of ΔF_E and ΔF_M as a measure of conformation changes of PIA-actin. This method could be applied to other proteins also. However, it has some detection limit. Although the concentration ranges applicable to intramolecular and intermolecular excimers are not identical, dilutions lower excimer emissions sensitively. Especially in the case of intermolecular excimer, dilution lowers its formation.

Diffraction of actin

Figure 4, a1 shows the photograph of actin microcrystals obtained by transmission electron microscope. Along the edges of microcrystals, actin filaments were observed. Usually, in a low-salt buffer, actin mini filaments were formed in the presence of excess ATP over actin [3]. Microcrystals were grown in the presence of 10 mM ATP supplemented with acetone, which was introduced according to the report [13]. The diffraction of microcrystals were observed under HVEM, which was useful to get enough reflection intensity from microcrystals. Figure 4, a2 shows the diffraction patterns of actin microcrystals. The reflections of 4.5 Å layer-lines were observed. In the direction of 51° from this layer-line, strong reflections were observed along the filament axis. This result suggests a twist of α-helices. Normally, α-helices are aligned along the filament axis with a spacing of around 5 Å [14]. Taking account of FDCD observation, i.e. twist induced by TFE or ATP, it is provable that the α-helical motif on the subdomain underwent twist as high concentrations of ATP were applied.

Conclusion

FDCD was applied to excimer fluorescence of actin labeled with fluorophores, PIA. The intrastructural changes in labeled subdomains were monitored by introduction of ΔF. Corresponding to CD of monomer and excimer emission, polarization of PIA and relative polarizations of PIAs were observed as ΔF_E and ΔF_M. PIA-actin polymerization induced by NaCl showed a decrease in ΔF. TFE additions to PIA-actin elucidated the presence of two types of structural changes based on the band shifts of ΔF_E and ΔF_M. The application of ATP to PIA-actin induced similar patterns of $\Delta F_E/\Delta F_M$ changes as those observed by TFE application. Microcrystals of actin grown in the presence of high concentration of ATP were observed under HVEM. The diffractions from the microcrystals showed the twist of α-helices around the filament axis. In the context of FDCD and HVEM, it seems that dynamic conformation changes of subdomain observed through the twist of α-helices were induced as high concentration of ATP was added to PIA-actin solution. The present method will be applicable to other proteins that need to measure the dynamic conformation changes of their structures to get insight into their functions from the structural point of view.

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