Spectral Fluctuation of a Single Fluorophore Conjugated to a Protein Molecule

Tetsuichi Wazawa,* Yoshiharu Ishii,*† Takashi Funatsu,† and Toshio Yanagida*†‡

*Single Molecule Processes Project, International Cooperative Research Project, †Yanagida Biomotron Project, Exploratory Research for Advanced Technology, Japan Science and Technology Corporation, 2-4-14 Senba-higashi, Mino, and ‡Department of Physiology and Biosignaling, Osaka University School of Medicine, 2-2 Yamada-oka, Suita, Osaka, Japan

ABSTRACT We have measured the fluorescence spectra of a single fluorophore attached to a single protein molecule in aqueous solution using a total internal reflection fluorescence microscope. The most reactive cysteine residue of myosin subfragment-1 (S1) was labeled with tetramethylrhodamine. The spectral shift induced by a change in solvent from aqueous buffer to methanol in both single-molecule and bulk measurements were similar, indicating that, even at the single molecule level, the fluorescence spectrum is sensitive to microenvironmental changes of fluorophores. The time dependence of the fluorescence spectra of fluorophores attached to S1 molecules solely showed a fluctuation with time over a time scale of seconds. Because the fluorescence spectra of the same fluorophores directly conjugated to a glass surface remained constant, the spectral fluctuation observed for the fluorophores attached to S1 is most likely due to slow spontaneous conformational changes in the S1 molecule. Thus, single-molecule fluorescence spectroscopy appears to be a powerful tool to study the dynamic behavior of single biomolecules.

INTRODUCTION

Understanding the changes that occur in protein conformation is critical in elucidating the molecular mechanism for biological function of the protein. In solution, protein conformations are determined based upon the ensemble-averaged data of a large number of molecules. If every protein molecule is in a unique conformational state, the averaged data can describe the steady-state conformation. However, proteins are in an equilibrium between multiple conformational states or populate different chemical states of cyclic chain reactions, such as the hydrolysis of adenosine 5′-triphosphate by myosin. For these proteins, their behavior is inferred using a model. Single-molecule detection methods will overcome these problems by directly detecting the changes in conformation that take place among different states.

Recently, imaging of single-protein molecules using fluorescence microscopy has made remarkable progress after development of single-fluorophore imaging methods, which were used for specimen on an air-dried surface (Trautman et al., 1994). Single fluorophores in aqueous solution have been imaged using a total internal reflection fluorescence microscope, in which the background fluorescence is greatly reduced (Funatsu et al., 1995); when a laser beam is totally reflected in the interface between the glass surface and solution, illumination is limited to only the vicinity near the glass surface. In addition, measurements in aqueous solution allow us to observe the behavior of protein molecules at work. By monitoring the trajectory of single fluorophores attached to single-protein molecules, the sliding motion of single-motor protein molecules was observed (Vale et al., 1996) and single-molecule diffusion was detected (Schmidt et al., 1996; Dickson et al., 1996; Xu and Yeung, 1997). Chemical reaction exerted by single-enzyme molecules was detected (Funatsu et al., 1995; Tokunaga et al., 1997; Ishijima et al., 1998; Lu et al., 1998). Fluorescence intensity fluctuations have been observed in single molecules of green fluorescent protein (Dickson et al., 1997), and interpreted as conformational transitions of the protein. Combining single-molecule imaging and fluorescence polarization techniques, molecular motion of an actin and myosin were recorded (Sase et al., 1997; Warshaw et al., 1998).

Fluorescence spectroscopy, a method to detect changes in the microenvironment of fluorophores, has been combined with single-molecule imaging in a crystalline at liquid-helium temperature using confocal microscopy (Jelezko et al., 1996), and at interfaces at room temperature with near field microscopy (Trautman et al., 1994; Ha et al., 1996), far field microscopy (Macklin et al., 1996; Lu and Xie, 1997), two-photon excitation microscopy (Sánchez et al., 1997), and confocal microscopy (Weston et al., 1998). Fluorescence spectra of single molecules in aqueous solution at room temperature have also been reported (Lu et al., 1998).

To study the conformational changes of proteins at the single-molecule level, we constructed a microscope, based

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Send reprint requests to Dr. Yoshiharu Ishii, Single Molecule Processes Project, ICORP, JST, 2-4-14 Senba-higashi, Mino, Osaka 562-0035, Japan. Tel.: +81-727-28-7003; Fax: +81-727-28-7033. E-mail: ishii@yanagida.jst.go.jp.

Dr. Funatsu's present address is Department of Physics, School of Science and Engineering, Waseda University, 3-4-1 Ohkubo, Shinjuku, Tokyo 169-8555, Japan.

Abbreviations used: S1, myosin subfragment-1; DMF, *N*,*N*-dimethylformamide; DTT, dithiothreitol; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; TMR, tetramethylrhodamine; TMR-S1, TMR-labeled S1; TMR-silane, TMR linked to a silica glass surface by γ-aminopropyltriethoxysilane.

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upon a total internal reflection fluorescence microscope, by which fluorescence spectra can be measured from single fluorophores attached to a protein in aqueous solution. S1 was labeled with TMR, and the images and fluorescence spectra of the individual molecules were monitored. The changes of the spectral position of single fluorophores attached to the proteins in methanol compared to an aqueous buffer were similar to bulk measurements, indicating that the fluorescence spectrum is sensitive to the microenvironment, even at the single-molecule level. The fluorescence spectrum of single TMR-S1 molecules showed slow spectral fluctuation with time, but that of single fluorophores directly attached to glass surface fluctuated minimally. The spectral fluctuation observed for the fluorophore attached to a protein most likely reflects spontaneous conformational fluctuation of the protein, which cannot be detected in bulk measurements.

MATERIALS AND METHODS

Instrumentation

A spectroscopic device was incorporated into a total internal reflection fluorescence microscope (Funatsu et al., 1995) (Fig. 1). The linearly polarized 514.5-nm line output of a CW argon laser (Model 2017-06S; Spectra Physics, Mountain View, CA) was modulated to circularly polarized light by a quarter-wave plate and attenuated by a neutral density filter. After passing through a focusing lens and a cubic prism, the laser beam was totally reflected at the incident angle of 69° to the norm at the silica glass slide–sample solution interface. An evanescent field was produced with a penetration depth of ~130 nm. The illumination area was 250 × 110 μm^2 ($1/e^2$ value) at the specimen plane. The fluorescence emission from the specimen was collected with an oil-immersion microscope objective (1.40 NA, 60×, PlanApo; Nikon, Tokyo, Japan) and switched for either imaging or spectroscopy by a removable mirror.

For imaging, the fluorescence was filtered by a barrier filter (570DF30; Omega Optical, Brattleboro, VT) and then focused by a relay lens onto the faceplate of an image intensifier (Model VS4-1845; Video Scope International, Sterling, VA) coupled to an SIT camera (C2400-08; Hamamatsu Photonics, Shizuoka, Japan). The images were recorded on a video cassette recorder.

For spectroscopy, the fluorescence passed through a notch filter (HNPF-514.5-1.0; Kaiser Optical Systems, Ann Arbor, MI) and focused on a pinhole by an achromatic lens. Only fluorescence that passed through the pinhole was brought into a spectrograph and camera system. The diameter of the pinhole was 150 μm, which corresponded to 1.5 μm at the specimen, because the images at the pinhole were magnified by 100×. The fluorescence was dispersed by wavelength with a spectrograph (reciprocal linear dispersion, 13.0 nm/mm; Model M10-TP; Spectral Instruments, Tokyo, Japan) and the spectral image was projected onto the focal plane of a back-illuminated cooled CCD camera (CCD, 1024 × 256 pixels, each 27-μm square; CCD15-11-0-232; Wright Instruments, London, U.K.). The dispersion-axis bin size of the cooled CCD camera was kept to 6 (pixels) so that the apparent dispersion per bin was 13.0 (nm/mm) × 27 (μm/pixel) × 6 (pixels/bin) = 2.11 (nm/bin). The spectrum detected with the cooled CCD camera was stored and analyzed by a personal computer. To minimize photobleaching, the laser beam was blocked by a mechanical shutter synchronized with an electric shutter of the cooled CCD camera.

Protein preparation

Myosin was extracted and purified from rabbit skeletal muscle (Harada et al., 1990). S1 was obtained by an enzymatic digestion of myosin by papain (Lowey et al., 1969).

The most reactive thiol group (SH1) of S1 was specifically labeled with tetramethylrhodamine-5-maleimide (T-6027; Molecular Probes, Eugene, OR) (Yamashita et al., 1974). S1 was mixed with tetramethylrhodamine-5-maleimide at the molar ratio of 1:1.5 in 50 mM NaCl and 50 mM HEPES (pH 7.0) at 0°C. After one-hour incubation, the reaction was terminated by adding 10-fold molar excess DTT over the dye. The sample was gel-filtrated, dialyzed, and centrifuged to remove unreacted dyes and aggregates of S1. The degree of labeling was 1.0, as calculated by the absorption using the molar extinction coefficients of 92,000 $M^{-1}cm^{-1}$ at 555 nm and 21,000 $M^{-1}cm^{-1}$ at 280 nm for TMR, and 110,000 $M^{-1}cm^{-1}$ at 280 nm for S1.

SH1 of myosin was labeled with tetramethylrhodamine-5-maleimide (T-6027; Molecular Probes) by a similar procedure. Myosin was mixed with tetramethylrhodamine-5-maleimide at the molar ratio of 1:1.7 in 0.6 M KCl and 20 mM HEPES (pH 7.0) at 0°C. After one-hour incubation, the mixture was diluted with the ninefold excess volume of an ice-cooled solution containing 1 mM DTT. After centrifugation, the precipitate was dissolved in 0.6 M KCl and 20 mM HEPES (pH 7.0), followed by dialysis against the same buffer. The degree of labeling was 1.1, as determined using the molar extinction coefficient of 250,000 $M^{-1}cm^{-1}$ at 280 nm for myosin. Myosin filaments were obtained by dilution with a low ionic

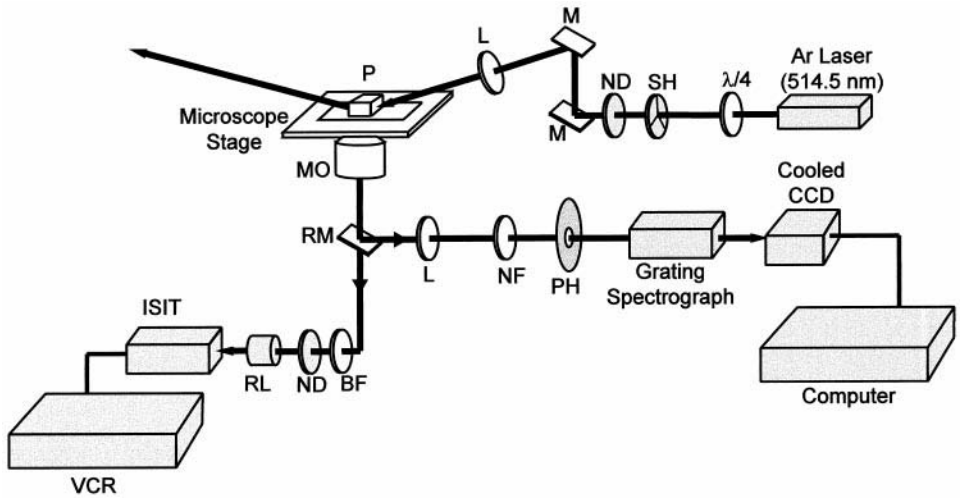

FIGURE 1 Schematic drawing of a fluorescence microscope for imaging and spectroscopy of single molecules in aqueous solution. A spectroscopic system was incorporated into the total internal reflection fluorescence microscope. The fluorescence emission from the samples collected by a microscope objective (MO) is switched for either imaging or spectroscopy by a removable mirror (RM). λ/4, quarter-wave plate; BF, barrier filter; ISIT, image-intensifier-coupled SIT camera; L, lens; M, mirror; ND, neutral density filter; NF, notch filter; P, prism; PH, pinhole; RL, relay lens; SH, electric shutter; VCR, video cassette recorder.

strength buffer containing 50 mM KCl, 10 mM HEPES (pH 7.0) and 1 mM $MgCl_2$ (Koretz et al., 1982).

Fluorophore-silane conjugation

TMR was attached to glass through a silane coupler. To obtain TMR-conjugated silane (TMR-silane), 2 mM γ-aminopropyltriethoxysilane (KBE903; Shin-Etsu Chemicals, Tokyo, Japan) was mixed with 2 mM 6-(tetramethylrhodamine-5(and-6)-carboxyamido)hexanoic acid succinimidyl ester (TAMRA-X-OSu) (T-6105; Molecular Probes) at the molar ratio of 1:1 in DMF (Infinity Pure Grade; Wako Pure Chemicals, Osaka, Japan). The mixture was incubated at room temperature for 3 hours. TAMRA-X-OSu reacts with the amino group of γ-aminopropyltriethoxysilane.

The TMR-silane solution was diluted by 10^4-fold with methanol and placed on a clean silica glass slide and the sample was covered with a coverslip. The ethoxysilyl group of TMR-silane was allowed to react with silica glass at room temperature for 1 min, and then the medium was washed with methanol to remove unreacted material and replaced with aqueous solution containing 100 mM NaCl, 20 mM HEPES (pH 7.0), and an oxygen scavenger, which contains 4.5 mg/ml glucose, 36 μg/ml catalase (C-10; Sigma, St. Louis, MO), 216 μg/ml glucose oxidase (G-2133; Sigma) and 0.5% 2-mercaptoethanol (Harada et al., 1990).

Specimen preparation

For single-molecule microscope measurements, 700 pM TMR-S1 was diluted by 100-fold with a buffer (100 mM NaCl and 20 mM HEPES, pH 7.0) plus the oxygen scavenger. Ten microliters of the diluted sample was placed on a silica glass slide and covered with a coverslip. For the experiments in methanol, the buffer solution was replaced by a pure methanol (Infinity Pure Grade; Wako Pure Chemicals) without the oxygen scavenger.

For ensemble spectrum measurements, 10 μl of 70 nM TMR-S1 was placed on a silica glass slide and covered with a coverslip. The protein molecules were adsorbed onto the glass surface and then the medium was replaced with aqueous buffer with the oxygen scavenger. The number of TMR-S1 molecules contributing to the spectrum was estimated to be ~1000. The other ensemble spectrum was obtained from a suspension of TMR-S1 in a cuvette. The cuvette was placed on the microscope stage. The spectrum was taken with the same detection system. These ensemble and single-molecule spectra, therefore, can be directly compared without correcting for the wavelength-dependent sensitivity of the detection system.

Fluorescence imaging

Fluorescence images were taken by an SIT camera coupled with an image intensifier and recorded by a video cassette recorder for analysis. Fluorescence intensity was obtained by digitizing the image during image analysis. For single-molecule measurement, only data that showed single-step photobleaching (Funatsu et al., 1995) were used.

Fluorescence spectrum of single molecules

A series of fluorescence spectra was taken every 0.61 sec with the collection time of 0.5 sec. The spectral position was quantitatively determined by calculating spectral mean λ_m defined as,

$$\frac{1}{\lambda_m} = \left(\sum_i \frac{I(\lambda_i)}{\lambda_i}\right) \Big/ \left(\sum_i I(\lambda_i)\right),$$

where λ_i is a wavelength at a position corresponding to the ith bin of the CCD camera, and $I(\lambda)$ is a fluorescence intensity at a wavelength of λ (the summations were performed for wavelengths from 540 to 660 nm).

Simulation of spectral fluctuation due to measurement noises

For spectrum measurements, the fluorescence intensity fluctuates due to the fluctuation of the number of photons and the readout noise of a CCD camera. The effect of these noises on the fluctuation of spectral mean was estimated by Monte Carlo simulation. The fluctuation of the numbers of photons was given by Poisson distribution function, and the distribution of the readout noise of the CCD camera was given by Gaussian distribution function. For data analysis, we used RNPOI and RNNOA subroutines (IMSL STAT/LIBRARY; Visual Numerics Inc., Houston, TX) under Visual Fortran (Digital Equipment Corporation, Nashua, NH). We took the mean spectrum as the average of the first 13 spectra (see Fig. 4) and the readout noise of the CCD camera as 5.0 (e^-).

RESULTS

Fluorescence images for single TMR-S1 molecules and TMR-myosin filaments

After TMR-S1 was adsorbed onto the surface of a slide glass, the fluorescence images were obtained with the total internal reflection fluorescence microscope. Single TMR-S1 molecules were observed as fluorescence spots (Fig. 2 *a*). As the concentration of TMR-S1 increased, the number of the fluorescent spots increased whereas the fluorescence intensities of the individual spots remained constant, consistent with the fluorescence spots arising from single TMR-S1 molecules. A typical time course of the fluorescence intensity of an individual spot is shown in Fig. 2 *b*. After the irradiation of the laser beam for a certain period of time, the fluorescence intensity dropped to baseline in a single-step manner due to photobleaching, confirming that each single spot (Fig. 2 *a*) is arising from a single fluorophore (Funatsu et al., 1995).

The fluorescence properties of individual molecules were compared with those of the ensemble of the molecules after they were integrated over molecules. Ensemble fluorescence measurements were made with a TMR-myosin filament (~1 μm long) in which a large number of TMR-S1 portions are arrayed through the aggregation (filament) of the rest portion (rod) of myosin. TMR-myosin filaments were observed as fluorescence spots with much greater intensity as compared with single TMR-S1 molecules (Fig. 3 *a*). After correcting for the sensitivities of the image intensifier and SIT camera, the neutral density filter, and the excitation laser power, the fluorescence intensity was about 180-fold greater than that of the single TMR-S1 molecules, suggesting that a single TMR-myosin filament has approximately 180 fluorophores. The data agree with the expected number of S1 head contained in 1 μm myosin filament (Perry, 1996). The fluorescence intensity of a TMR-myosin filament decreased gradually with time due to photobleach-

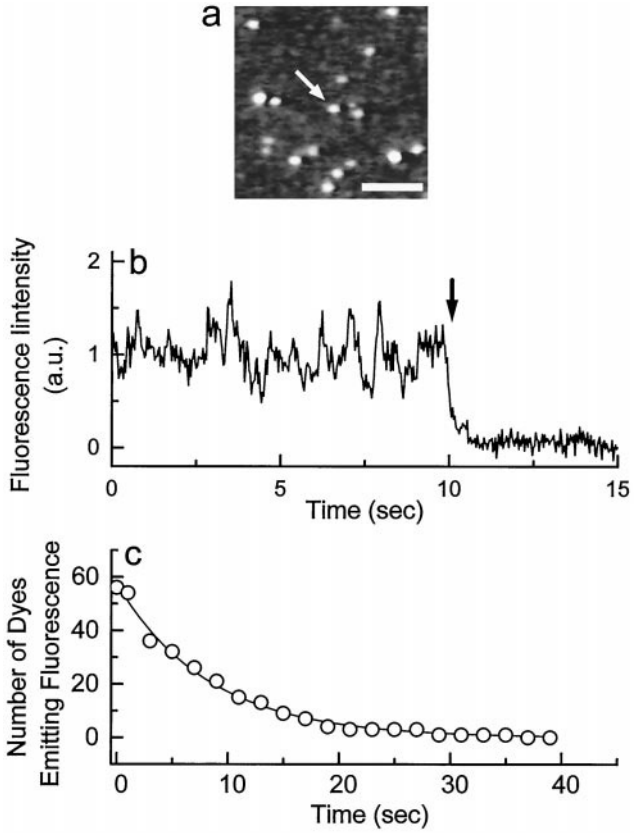

FIGURE 2 Fluorescence images of single TMR-S1 molecules in aqueous solution. (*a*) Fluorescence images. Single spots correspond to single TMR-S1 molecules (see text for details). The specimen was illuminated by an argon laser at 514.5 nm with the excitation power of 14 mW at the specimen plane. For emission, a bandpass filter of transmission wavelengths between 555 and 585 nm was used. The images were integrated over 16 video frames or 0.53 sec. Bar, 5 μm. (*b*) The time course of the fluorescence intensity from a single spot pointed by the arrow in (*a*). The laser power was 14 mW. The fluorescence dropped to baseline due to photobleaching at the time indicated by an arrow. (*c*) The exposure time until photobleaching was measured for individual molecules, and the data were summarized by counting the number of molecules remaining fluorescent and plotting it as a function of time. The decay is fitted to a single exponential function with a rate constant of 0.12 sec^{-1} (*solid curve*).

ing of the fluorophores (Fig. 3 *b*). The decay was fit with a single exponential curve with a pseudo first-order rate constant of 0.034 sec^{-1}.

This rate for ensemble fluorescent decay is predicted from our measurement of individual single fluorophores. Photobleaching of individual fluorophores was a stochastic process, and the number of the fluorophores that remain fluorescent was fitted by a single exponential curve with the pseudo first-order rate constant of 0.12 sec^{-1} (Fig. 2 *c*). Correcting for laser power (5 mW for ensemble measurements and 14 mW for single-molecule measurement) and assuming that the photobleaching rate is proportional to the laser power, at a laser power equivalent to the ensemble measurements, we calculate a rate constant of 0.033 sec^{-1}.

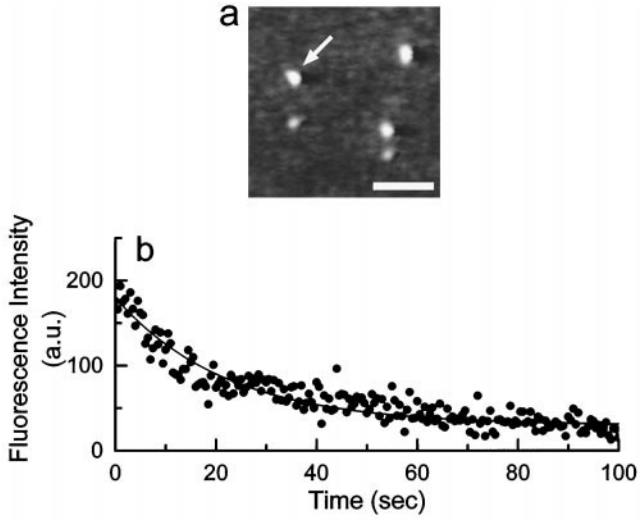

FIGURE 3 Fluorescence images of TMR-myosin filaments, aggregates of TMR-S1. (*a*) Fluorescence images. Bar, 5 μm. (*b*) Time course of the fluorescence intensity of a single spot pointed by the arrow in (*a*). The experiment was done in the same way as in Fig. 2 except for the laser power (5 mW) and the sensitivity of the detection system. The data were fitted to a single exponential decay (*solid curve*) with a rate constant of 0.034 sec^{-1}.

This value is in good agreement with the rate constant of the decay for TMR-myosin filaments.

Fluorescence emission spectra of single TMR-S1 molecules

To obtain the fluorescence emission spectra of a single TMR-S1 molecule, the fluorescence within a circle of 1.5-μm diameter at the specimen plane was passed through a pinhole (see Materials and Methods). The fluorescence was then dispersed by wavelength with a spectrograph, and the image of the spectrum was taken with a cooled CCD camera. Fluorescence spectra were recorded every 0.61 sec with a 0.50-sec integration of signal. A series of fluorescence spectra is shown in Fig. 4. After several seconds, because of photobleaching in a single step, the spectrum suddenly disappeared.

The fluorescence spectra of single TMR-S1 molecules were compared with two ensemble fluorescence spectra (Fig. 5). One ensemble spectrum was obtained with a large number of TMR-S1 molecules adsorbed on a glass surface. Concentrated TMR-S1 solution ($\sim$70 nM; 10^4-fold higher concentration than for the single-molecule experiments) was applied onto a slide glass. Judging from the fluorescence intensity, the fluorescence emission from more than 1,000 TMR-S1 molecules adsorbed within the circle of 1.5 μm in diameter on the glass was passed through the pinhole for spectroscopy. The spectrum thus obtained was similar to that of a single molecule. The spectral position, denoted as spectral mean, was quantitatively evaluated as a weighted

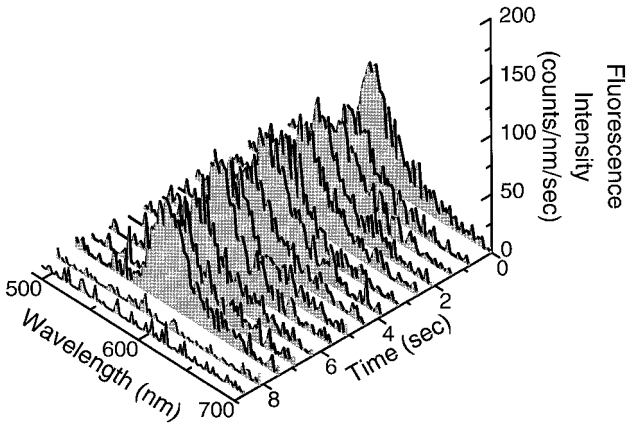

FIGURE 4 Change in fluorescence spectrum of a single TMR-S1 molecule with time. Spectra were taken sequentially every 0.61 sec with the collection time of 0.5 sec for each spectrum at a laser power of 45 mW. The fluorescence dropped to baseline due to photobleaching around 8 sec.

average wavelength of the fluorescence spectrum (see Materials and Methods). The results are summarized in Table 1. The average spectral mean for single molecules was similar to that of the ensemble spectrum. Another ensemble spectrum was obtained from a suspension of TMR-S1 in a cuvette. The cuvette was placed on the microscope stage and the spectrum was taken with the same detection system, thereby allowing the fluorescence spectrum to be compared with that of a single molecule without correcting for the difference in the wavelength-dependent sensitivity of the detection system. The fluorescence spectrum from the suspension in solution in a cuvette was not different when compared to that of the ensemble spectrum of TMR-S1 molecules bound to a glass surface, indicating that the effect of adsorption of proteins onto the glass surface on the fluorescence spectrum was negligible (Table 1).

To test if the fluorescence spectrum is sensitive to the microenvironment of the fluorophore at the single-molecule

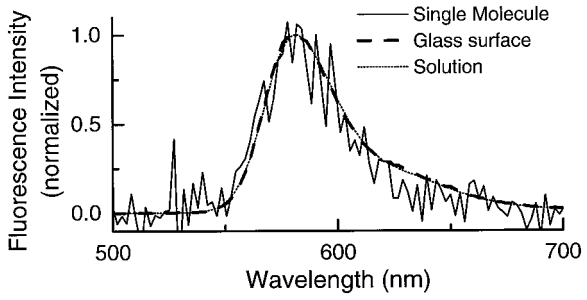

FIGURE 5 Comparison of fluorescence spectrum of a single TMR-S1 molecule with ensemble spectra. The solid curve is a typical fluorescence spectrum of a single TMR-S1 molecule. The dashed curve is an ensemble spectrum of TMR-S1 molecules adsorbed onto the surface of the slide glass under microscope, and the dotted curve is an ensemble spectrum of TMR-S1 in aqueous solution in a cuvette with the same detection system. The laser power for both ensemble measurements was 2 mW.

TABLE 1 Spectral means (nm) for ensemble and single-molecule spectra of TMR-S1

Solvent	Ensemble Spectrum (Glass Surface)*	Ensemble Spectrum (Cuvette)†	Single-Molecule Spectrum
Aqueous solution	593.2	593.0	589.2‡
Methanol	587.7	588.1	585.3§

*The values were calculated from ensemble spectra of a large number of molecules adsorbed on a glass surface. Measurement errors for the spectral means were negligible, within ±0.1 nm.
†The values were calculated from ensemble spectra of a large number of molecules suspended in a cuvette (standard error, <0.1 nm).
‡The value was calculated from an average spectrum over 44 TMR-S1 molecules (standard error, 0.7 nm).
§The value was calculated from an average spectrum over 35 TMR-S1 molecules (standard error, 1.1 nm).

level, the fluorescence spectra of single TMR-S1 molecules were measured in two different solvents: aqueous buffer and methanol. For the ensemble spectra, the spectral mean was 5.5 nm shorter in methanol than in an aqueous buffer solution (Fig. 6 *a* and Table 1). A similar spectral change was observed for single TMR-S1 molecules. Methanol

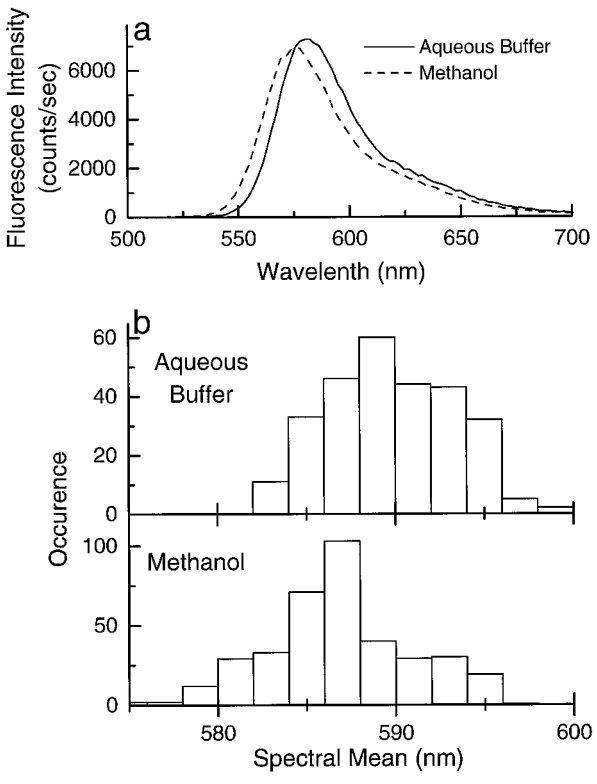

FIGURE 6 The effect of methanol on the fluorescence spectrum. (*a*) Ensemble fluorescence spectra of TMR-S1. The solid curve is a spectrum obtained in an aqueous buffer solution and the dashed curve in methanol. (*b*) Single molecule measurement of TMR-S1. Single molecule spectrum was measured and the spectral mean was calculated for each spectrum. The data of the spectral mean in aqueous buffer solution (*top*) and in methanol (*bottom*) are summarized in the histograms.

shifted the averaged spectral mean to a shorter wavelength (Fig. 6 *b*). Thus, the fluorescence of single molecules showed similar responses to changes in the microenvironment.

Spectral fluctuation of single TMR-S1 molecules

The fluorescence intensity of a single TMR-S1 molecule fluctuated with time (Fig. 4). The total fluorescence intensity was calculated by integrating the intensity over wavelength for each spectrum and the time course was plotted in Fig. 7 *a*. The total fluorescence intensity fluctuated similarly to that observed in single-molecule imaging (Fig. 2 *b*).

To determine the etiology for the spectral changes, the spectral mean was calculated for each spectrum and plotted as a function of time (Fig. 7 *b*). The spectral mean of the TMR-S1 molecule varied between 588 and 595 nm. This range, obtained from the time dependence of a single molecule, was similar to the distribution range over molecules (Fig. 6 *b*). Generally, the fluorescence intensity was greater when the spectrum was at a shorter wavelength (Fig. 7, *a* and *b*). Shown in the inset of Fig. 7 *b* are two typical spectra at the longer and shorter spectral means. These two spectra are distinguishable, indicating that the changes in spectral mean reflect real spectral changes. The spectral mean correlated with the ratio in the fluorescence intensities between the shorter wavelength side (540.0–589.6 nm) and the longer wavelength side (589.6–660.4 nm) (data not shown), together indicating that the spectral changes are not an artifact of the measurement but a measure of a real spectral shift.

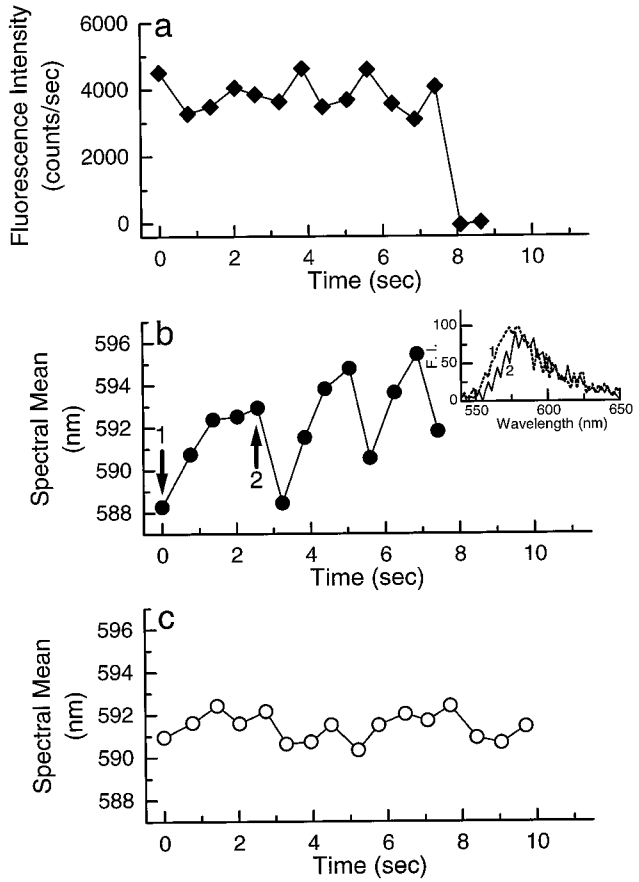

FIGURE 7 Fluorescence spectrum fluctuation of a single TMR-S1 molecule. (*a*) Total fluorescence intensity. The total fluorescence intensity was obtained by integrating fluorescence intensity for wavelengths from 540 to 660 nm for each spectrum and plotting as a function of time. (*b*) Fluorescence spectral mean. The spectral mean was calculated for each spectrum and plotted as a function of time. In the inset, two fluorescence spectra having different spectral means (at the time indicated by the arrows 1 and 2 for *dotted* and *solid curves*, respectively) are plotted to show that changes in the spectral mean reflect real spectral changes. (*c*) Control was obtained with TMR directly attached to the glass surface (TMR-silane) without proteins.

The control experiment with single TMR molecules directly attached to a glass surface without proteins showed only small spectral fluctuation (Fig. 7 *c*). TMR was linked to a silica glass surface by γ-aminopropyltriethoxysilane. The spectral mean varied from molecule to molecule (data not shown). It is likely that the condition of the glass surface is not homogeneous, resulting in a variety of environments. However, the spectral mean changed with time to a much lesser extent compared to that of TMR-S1.

The difference in spectral fluctuation between TMR-S1 and TMR-silane was confirmed statistically. The mean square of variations in spectral mean was greater for TMR-S1 than for TMR-silane after averaging over molecules (Table 2). These numbers include contribution of the experimental noise. To determine the effect of experimental noises, a simulation was performed by generating random noise due to the fluctuation of the number of photons and the readout noise of the detection system (see Materials and Methods). The mean square of variation due to these experimental noises was 2.6 nm^2. The excess magnitude of the spectral fluctuation of TMR-S1 after subtracting the experimental noise ($8.4 - 2.6 = 5.8$ nm^2) was larger than that of TMR-silane ($4.0 - 2.6 = 1.4$ nm^2) by a factor of 4. In fact, the difference of the values between TMR-S1 and TMR-silane was statistically significant as compared with the experimental distribution (Table 2). Thus, it was concluded that the spectral fluctuation for TMR-S1 is a genuine effect observed when the fluorescence dye is attached to a protein.

The time-scales of the spectral fluctuation were also investigated. The autocorrelation functions of spectral mean for TMR-S1 and TMR-silane were calculated. The time constants obtained from the average autocorrelation functions were less than the sampling interval per one spectrum (0.61 sec), so we could not determine the time scales of the spectral fluctuation accurately.

The results obtained here were not affected by a 5-fold increase in the excitation laser power.

TABLE 2 The magnitudes of fluctuation of spectral means for TMR-S1 and TMR-silane in aqueous solution

	Magnitude of Fluctuation (nm^2)*
TMR-S1	8.4 ($\pm$0.22, S.E.†)‡
TMR-Silane	4.0 ($\pm$0.32, S.E.†)§
Contribution of fluctuation of the number of photons and readout noise of detection system	2.6¶

*The magnitude of fluctuation of spectral mean ($\langle|\Delta\lambda_m|^2\rangle$) was calculated as the mean square sum of spectral-mean differences between two spectra adjacent in time for each fluorophore,

$$\langle|\Delta\lambda_m|^2\rangle = \frac{\sum_{i=1}^{N}\sum_{j=1}^{M_i}(\lambda_i(t_{j+1}) - \lambda_i(t_j))^2}{\sum_{i=1}^{N} M_i},$$

where $\lambda_i(t)$ is a spectral mean (nm) at time t for the spectra taken from a fluorophore i; M_i is the number of spectra taken from the fluorophore i during fluorescence emission; N is the number of fluorophores encountered here.

†The standard errors were calculated as

$$\sqrt{\frac{\sum_{i=1}^{N}(|\Delta\lambda_m|_i^2 - \langle|\Delta\lambda_m|^2\rangle)^2}{N}} \Big/ \sqrt{N},$$

where $|\Delta\lambda_m|_i^2$ denotes the average magnitude of the fluctuation in spectral mean for a TMR-S1 molecule i, i.e.,

$$|\Delta\lambda_m|_i^2 = \frac{\sum_{j=1}^{M_i}(\lambda_i(t_{j+1}) - \lambda_i(t_j))^2}{M_i}.$$

‡The value was determined from 405 spectra of 49 TMR-S1 molecules.

§The value was determined from 487 spectra of 21 TMR-silane conjugates.

¶The value was calculated by Monte Carlo method.

Large blue shift of TMR spectrum

Most of the fluorophores showed single-step photobleaching as described above. However, some of the single fluorophores showed fluorescence changes such as large spectral shifts and blinking. The spectral changes observed were characterized by a large blue shift of the peak (~20 nm) and an increase in fluorescence intensity (Fig. 8). However, this sort of spectral changes was not unique for the fluorophores on the proteins, and similar changes were also observed for those directly conjugated to the glass surface. Moreover, this blue shift was observed in both aqueous solution and methanol, depending on the laser power that excited the fluorophores. The fraction of the fluorophores that showed the blue shift was greater as the excitation laser power increased. Thus, this type of spectral change is likely to be due to some photochemical reactions of TMR itself. Based upon this observation, the fluorophores that showed such spectral changes were excluded from analysis.

DISCUSSION

In this study, we demonstrated that, in aqueous solution, the fluorescence emission spectrum of a single fluorophore

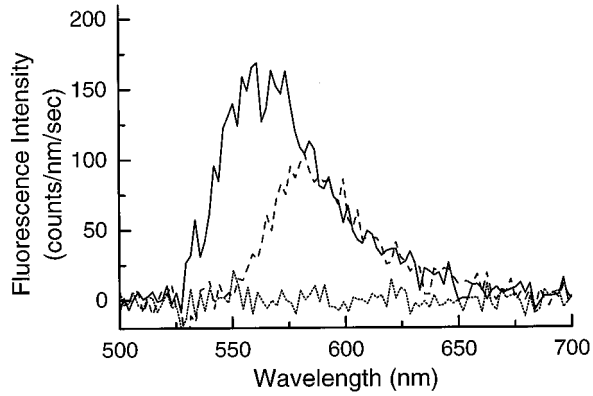

FIGURE 8 Large blue shift of TMR spectrum observed prior to photobleaching. Most fluorophores photobleached in a single step. However, large blue shift was sometimes observed prior to photobleaching (*solid curve*). The spectral mean shifted from 593 to 576 nm with increase in the total fluorescence intensity by about 1.7-fold. *Dashed curve*, the fluorescence spectrum of the fluorophore before the spectral shift; *dotted curve*, the fluorescence spectrum after photobleaching.

attached to a protein molecule can be measured using a total internal reflection fluorescence microscope. Steady-state fluorescence properties of single molecule experiments were similar to those of bulk measurements. With single-molecule measurements, we were able to determine the unique behavior of single-protein molecules, such as spectral fluctuations, which were not apparent in conventional bulk measurements.

The fluorescence of individual TMR fluorophores attached to S1 molecules exhibited spectral fluctuations (Fig. 7, *a* and *b*). Spectral fluctuation is a characteristic of fluorophores attached to protein molecules. The dye molecule directly attached to the glass surface showed only minimal spectral fluctuation that was not distinguishable from noise. It has been reported that even single dye molecules have a wide distribution of spectral means (Macklin et al., 1996; Ha et al., 1996) and show spectral fluctuations in a polymer–air interface in the time scale of milliseconds to seconds (Lu and Xie, 1997). In aqueous solution, however, the solvent molecules collide with the fluorophore frequently to change the molecular coordinates of the fluorophore (Klessinger and Michl, 1995). Thus, the dye molecules themselves may change the electronic states very quickly and the fluorescence is already averaged sufficiently over various states in the time range of seconds or subseconds.

Thus, the fluorescence spectral fluctuation observed with fluorophores on protein molecules at the time scale of seconds most likely reflects slow conformational transition of proteins via microenvironmental changes. At the single-molecule level, the fluorescence spectrum was proved to be sensitive to the microenvironment; that is, methanol shifted the peak positions of fluorescence spectra in both bulk and single-molecule systems in a similar manner (Fig. 6 and Table 1). Another possible explanation for the slow spectral

fluctuation is change in the interaction of the fluorophores with surrounding amino acid residues. The interaction is determined by configurations of both fluorophores and amino acids nearby, which are related to each other. In most cases, however, these changes in the dye–protein interaction may also be induced by the conformational change of proteins through rearrangement of the amino acid coordinates.

Slow conformational transition may result from multiple conformations that have similar structural energies with high energy barriers between them. The existence of such multiple metastable conformations has been demonstrated for many proteins such as luciferase (Baldwin et al., 1995), serpin plasminogen activator inhibitor 1 (Wang et al., 1996), α-lytic protease (Sohl et al., 1998), subtilisin E (Shinde et al., 1997), and prion (Pan et al., 1993). Recent NMR study has suggested that a cell-signaling protein, ras, undergoes spontaneous transition among the multiple conformations at the time scale of subseconds and seconds in the active state or in the presence of guanosine 5′-triphosphate (Itoh et al., 1997). Single molecule detection methods would make it possible to directly visualize the dynamic changes between multiple conformations of these proteins. The observation of the enzymatic turnovers of single cholesterol oxidase molecules has revealed a slow fluctuation in the rate of the oxidation reaction and thereby in the conformation of the enzyme (Lu et al., 1998). Measurements of fluorescence resonance energy transfer between donor and acceptor at the two sites within a single protein molecule have suggested that their distance fluctuates slowly at the time scale of subseconds to seconds (Ishii et al., 1997).

Theoretically, single-molecule fluorescence should be the same as the bulk measurement when the fluorescences of individual molecules are integrated over molecules. Our results confirmed this premise. Single-step photobleaching of single molecules occurred stochastically, giving rise to an exponential decay of the ensemble fluorescence (Figs. 2 and 3). Steady-state spectral shape is basically the same for both measurements. Methanol shifted the peak positions of fluorescence spectra in both bulk and single-molecule systems in a similar manner (Fig. 6). However, there were small differences in the spectral mean: the average of the spectral mean of individual molecules was a little shorter than the spectral mean in bulk measurement (Table 1). This could occur if the spectrum depends on the excitation power level. In fact, the excitation power in the bulk measurement (2 mW) was much lower, by more than an order of magnitude, compared with that for the single molecule measurement (45 mW). Methanol may eliminate this effect, making an apparent change by methanol smaller in the single-molecule measurements.

The measurements of spectral shape or spectral mean have an experimental advantage over fluorescence intensity. In a single- or dual-channel photometry, fluorescence intensity is measured in a higher temporal resolution with single fluorescent molecules (Ishijima et al., 1998; Warshaw et al., 1998; Weston et al., 1998; Ha et al., 1999). However, the spectral shape depends only modestly on the excitation power level, whereas the fluorescence intensity is proportional to the excitation level. In fact, in a microscope system, the excitation level depends on the depth from the glass surface due to the nature of evanescent field and is not homogeneous over the entire illumination area. In addition, with the simple single- or dual-channel photometry it is difficult to know if large spectral shifts which are involved in chemical changes of the fluorophore itself take place. Thus, the spectral shape is useful for quantitative determination of the microenvironment of the probe.

Large fluorescence spectral changes observed prior to the photobleaching in the single-molecule measurement occur in fluorophores conjugated to both proteins and glass surface (Fig. 8). This may be a photochemical reaction process that we do not know yet. More systematic study is necessary to understand the mechanism. Thus, single-molecule spectroscopy is also a tool to study photochemical reaction that does not occur frequently and is not apparent in ensemble measurements.

The present technique can detect conformational changes of individual protein molecules. This technique could be combined with other fluorescence applications such as fluorescence resonance energy transfer (Ha et al., 1996, 1999; Ishii et al., 1997, 1999). Further improvement in the time resolution of the technique is necessary to relate the protein dynamics to its function. In future, this technique will make it possible to directly couple the structural changes of proteins with their functions, such as force generation, catalysis, and recognition at the single-molecule level. Recently, the coupling of the ATPase reaction and the mechanical events of myosin during interaction with actin was directly determined at the single-molecule level using techniques of single-molecule imaging and nano-manipulation (Ishijima et al., 1998). Combining the present techniques with these ones would allow us to simultaneously detect the chemical and mechanical reactions, and the conformational change of a single myosin molecule at work. Thus, the single-molecule fluorescence spectroscopy will be a powerful tool for studies aimed at the mechanisms of functions underlying protein molecules.

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