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Synthesis of a highly luminescent terbium chelate and its application to actin

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We synthesized a highly luminescent terbium chelate which consists of DTPA as a chelating group, phalloin as an actin-binding ligand and an aromatic ring as a sensitizer of Tb emission. The synthesis was based on the property of DTPA dianhydride that has the capacity to react with two amines, while retaining its ability to chelate Tb^{3+} (the resulting DTPA possesses three carboxyl groups). We screened 17 kinds of aromatic compounds for their ability to sensitize Tb emission with near UV excitation. All of the compounds tested had both a 6-membered ring and one or two amino group(s). Among them, cytosine was the best sensitizer. From the peak–peak ratio, the fluorescence intensity of a solution of Tb-DTPA-cytosine-phalloin-labeled actin was about one-fourth of an equimolar solution of tetramethylrhodamine. The excitation peak was about 302 nm. Emission from a single muscle fiber stained with this new Tb chelate was so intense that we could accumulate sufficient photon counts in a short time. Also, actin bundles stained with the Tb chelate were able to be visualized under a fluorescence microscope. The stability constant of the chelator was $10^{22.46}$ M^{-1} for Tb^{3+}. This value was very similar to that of DTPA, which possessed five carboxyl groups. The new Tb chelate had additional expediencies. It was electrically neutral, which made it quite suitable for studying electrostatic circumstances around the terbium site on actin. We present data on this issue. Tb^{3+} itself is optically isotropic. However, cytosine of the Tb chelate has a polarized absorption transition dipole moment. Therefore, in conjunction with the long-lived excited state, the synthesized Tb chelate has potential of serving to probe the slow rotational relaxation of actin filaments. We can simply replace phalloin with other agents to label not only actin but also various proteins. Such Tb chelates will be a quite useful tool for a variety of biological studies.

Introduction

Terbium, a rare-earth lanthanide ion, shows unique sharp line luminescence that arises from transitions of the $^5D_4$ state to the ^7F_J states [1]. Moreover, the effective ionic radius of terbium is almost the same as that of Ca^{2+} [2]. So, terbium has been widely employed for investigating calcium binding sites of many calcium-binding proteins [3]. The luminescence of free terbium in water solution is weak because of its weak absorption and strong quenching by water OH groups [4]. However, it is often enhanced upon binding to proteins by the reduced quenching mechanism and sometimes by excitation energy transfer from nearby aromatic amino acid residues. The latter mechanism results in much more effective excitation. In addition, terbium has a long excited state lifetime (in the millisecond range). This unique feature has been applied to other types of biological study. The long-lived luminescence can exclude interference of short-lived background luminescence, which allows one to detect substrates such as antigens with high sensitivity. The sensitized luminescence of terbium (also of europium) has been employed for time-resolved fluoroimmunoassay, taking over the position of radioimmunoassay. Just as in the case of usual fluorophores, the excitation energy of terbium can be transferred to suitable chromophores by dipole–dipole interaction. When we use energy transfer to estimate the distances between donor and acceptor that are essentially fixed at specific sites on an object, terbium has an advantage that the orientation factor, k^2, becomes less ambiguous because of the isotropic dipole moment. In a different situation where either acceptors or donors are freely diffusing in solution, the rate of energy transfer is significantly enhanced by translational diffusion of donors and ac-

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Abbreviations: ET, energy transfer; Z_a, electric charges.

ceptors during the excited state of terbium. With the millisecond range of the excited state lifetime, energy transfer occurs in the rapid-diffusion limit [5,6]. This type of energy transfer is highly sensitive to the distance of the closest approach of diffusing donors and acceptors [5], and moreover, to electrostatic interaction between them [7]. The former feature has been utilized to reveal the depth of chromophores in proteins and membrane systems [8,9]. The latter has been optimized to probe electrostatic circumstances in the vicinity of donor-attached or acceptor-attached sites on macromolecules [7,10,11].

As seen above, terbium is a quite informative tool for biological studies. Terbium has been usually used in the chelated form except when it is used to study calcium-binding sites of proteins. Chelation shields terbium from water OH groups, giving rise to an increase in its quantum yield as well as in the excited state lifetime. A reactive group can be introduced to a chelator in order to attach its Tb chelate specifically to a site on protein. As already mentioned, terbium alone absorbs light so weakly that a strong excitation source like a laser is often required. However, a chelator with an aromatic ligand can sensitize the terbium emission, which makes terbium very useful. Meares et al. have synthesized such chelators, which are now famous as Meares' reagents (i.e., bromoacetamido-benzyl-EDTA, isothiocyanobenzyl-EDTA, etc.) [6,12–14]. The benzyl group absorbs ultraviolet light around 260 nm. The UV excitation is, however, sometimes interfered with by UV-absorbing molecules contained in biological samples. Also, UV-irradiation often damages biological macromolecules. If possible, it is best to have a chelator which bears a sensitizing molecule that absorbs light in the range longer than 300 nm. So, we decided to screen several compounds for their ability to sensitize terbium luminescence. In this screening we employed DTPA dianhydride, which converts to an chelator after hydrolysis [15,16]. It can also react with amines and retain its chelating function even after both the anhydride groups have reacted with amines. This feature of DTPA dianhydride facilitates synthesis of chelators that have both a sensitizing ligand and a site-selective ligand or a chemically reactive group. We employed phalloin as the site-selective ligand to study actin. Screening of several candidates as the sensitizing ligands revealed that cytosine was the best one because it sensitized the terbium emission with the largest extent and importantly the excitation maximum was obtained around 302 nm. The luminescence from the resulting chelate, Tb-DTPA-cytosine-phalloin (Tb-DTPA-CP), was so intense that bundles of actin filaments stained with this Tb chelate were visualized under a fluorescence microscope. In addition, Tb-DTPA-CP had zero electric charge as net. This attribute is favorable for studying electric circumstances around its attached site on actin by measuring efficiencies of the diffusion-enhanced energy transfer to freely diffusing charged acceptors in the solution. We actually observed that efficiency of energy transfer (ET) from excited free Tb-DTPA-CP to diffusing rhodamine B derivatives was independent of the electric charges (Z_a) of the acceptors. ET of actin-bound Tb-DTPA-CP was, on the other hand, enhanced very sharply with increasing Z_a, indicating that the phalloin-binding site of actin resides in minus electric potential.

Conjugation of an aromatic ligand to Tb chelate amplifies usefulness of Tb not only because of the brighter luminescence from Tb, but also because of a newly added attribute, i.e., optical anisotropy. Tb itself is optically isotropic in both the excitation and emission sides, but aromatic-ligand-attached Tb chelates have a polarized absorption transition dipole moment. We raise the possibility that such Tb chelates can be applied to measurements of slow rotational relaxation. This aspect has to our knowledge not been mentioned earlier.

Materials and Methods

Synthesis of δ-aminophalloin

δ-Aminophalloin was synthesized according to Wieland et al. [17]. First, tosylphalloidin was prepared. Tosyl chloride (12 mg, 10 equiv.) was added to the solution of phalloidin (50 mg, 0.063 mmol) in pyridine (1 ml) at 0°C and the solution was stirred for 5 h at room temperature. The solution was concentrated in vacuo and tosylphalloidin was purified by reverse phase HPLC. Yield, 25 mg (41.9%). Tosylphalloidin was treated with 2.5 M NH_3 in methanol at room temperature for 6 h. The solution was diluted with excess water and lyophilized. Finally δ-aminophalloin was purified by reverse-phase HPLC. The purified δ-aminophalloin was stored as solid at −35°C.

Preparation of acceptors

To prepare acceptors with various electric charges, the following amino acids or their derivatives were reacted with rhodamine B isothiocyanate; Glu-Glu, Glu, Gly, Gly-methyl ester, Arg-methyl ester, Lys-Lys-methyl ester. The calboxymethylated amino acids and dipeptide were obtained as follows. Briefly, 0.25 ml of thionyl chloride was slowly added to 1 ml of anhydrous methyl alcohol cooled at −10°C. 10 min later, 1 mmol of amino acids or dipeptides were added in solid to this solution. The mixtures were stirred for 2 days at room temperature. They were then concentrated under reduced pressure, washed several times with methanol and finally freed of solvent in vacuo. Reaction of rhodamine B isothiocyanate (0.2 mM) with amino acids or their derivatives (2 mM) was carried out in 30–50%

methanol in dark for 10 h at room temperature. The reaction products were purified by either silica-gel chromatography or DEAE chromatography.

Protein preparations

Myosin was prepared from rabbit back and leg skeletal muscles and stored in 0.3 M KCl, 50% (v/v) glycerol at −20°C. Chymotriptic S-1 was obtained by a standard method. S-1 (A1) and S-1 (A2) were purified using preparative DEAE HPLC. The eluted S-1 was concentrated by Centriflo (Amicon) ultrafiltration. The concentrated S-1 was lyophilized in 0.15 M sucrose, 30 mM ammonium acetate and 0.5 mM dithiothreitol at pH 7.0. The lyophilized S-1 was stored at −35°C. Acetone powder of rabbit skeletal muscle was prepared as described [18]. Actin was purified by the method of Spudich and Watt [19]. Labeling of F-actin with Tb-DTPA-CP was carried out just by mixing equimolar amounts of F-actin and Tb-DTPA-CP at 0°C in a solution containing 5 mM Tes (pH 7.0), 0.2 mM $CaCl_2$ and 1 mM $MgCl_2$. The bundles of the labeled F-actin were obtained by adding 5 mM $MgCl_2$ and S-1 to the F-actin solution [20]. The molar ratio of S-1 to actin was 1 : 6.

ATPase activity measurements

ATPase activities of S-1 alone (2 μM S-1 (A1)) and acto-S 1 (0.1 μM S-1 (A1) plus 2 μM actin) were assayed in 10 mM Tris–HCl (pH 8.0), 5 mM $MgCl_2$ and 3 mM ATP at 25°C. The amount of P_i liberated was determined by the method of Fiske and SubbaRow with a slight modification.

Preparation and staining of single muscle fibers and myofibrils

Bundles of rabbit psoas muscle were glycerinated for periods ranging from a week to several months in a solution containing 80 mM KCl, 2 mM $MgCl_2$, 2 mM EGTA, 5 mM K_2HPO_4 (pM 7.0), 50% (v/v) glycerol at −20°C. The glycerinated fiber bundles consisting of 10 to 20 fibers were dissected and washed several times with ice-cold rigor solution (80 mM KCl, 20 mM Tes (pM 7.0), 1 mM EGTA, 0.5 mM leupeptin). They were then labeled with 0.1 mM Tb-DTPA-CP for 30 min in the rigor solution at 4°C. The free Tb-DTPA-CP was removed by washing the fibers with the rigor solution for 1 h at 0°C with changing the solution several times. A single fiber was dissected from the stained fiber bundles and the both ends were fixed horizontally to a stainless rectangle by a fast-drying glue. The rectangle was put in a 1 × 1 cm quartz fluorescent cuvette. The horizontally fixed fiber ran diagonally in the cuvette. Myofibrils were obtained from the stained fiber bundles as described previously [21]. Briefly, the stained fibers were cut with scissors into small pieces and homogenized in a hand-made mixer which was similar to a Sorvall Omni-mixer with 10 ml of the rigor solution on ice. Myofibrils were centrifuged and gently resuspended in the rigor solution.

Time-resolved fluorescence measurements

For time-resolved fluorescence measurements, the excitation source used was a bulb type xenon flashtube (EG&G, FX-801U) triggered by TTL signals from a multichannel scaler (NAIG, E564 Process Memory). The pulsed light was first passed through a UV-cut off filter (Toshiba, UV-29, $\lambda(\frac{1}{2}T_{max}) = 290$ nm, $T_{max} = 90\%$), then passed through a UV-transmitting black glass filter (Sigma Koki, UTVAF-340, $\lambda_{max} = 340$ nm, $\Delta\lambda_{1/2} = 47$ nm, $T_{max} = 80\%$) and focused with two symmetric-convex fused-silica lenses onto the sample in a 1 × 1 cm fused-silica quartz cuvette. The resulting excitation pulse at the sample was 5 μs wide, illuminating a 1 × 3 mm rectangle. The fluorescence emission at right angles was passed through a triacetyl cellulose sharp-cut filter (Fuji Film, SC-52, $\lambda(\frac{1}{2}T_{max}) = 523$ nm, $T_{max} = 92\%$), passed through an all-dielectric band-pass filter (Vacuum Optical Corp. of Japan, DIF-BPF-4, $\lambda_{max} = 547.2$ nm, $\Delta\lambda_{1/2} = 23$ nm, $T_{max} = 81\%$) and focused with a symmetric-convex fused-silica lens onto a H 1161 photomultiplier (Hamamatsu Photonics). The anode signal was preamplified with a model 2005 preamplifier (Camberra), amplified with a E511A linear amplifier (NAIG) and discriminated with a E512A single-channel analyzer (NAIG). The resulting pulses were then counted synchronously with trigger by using a multichannel scaler which was connected to a personal computer (Epson, 286VG). The stored data were transferred to a UNIX work station (Sony, NEWS NA-564 system) and the fluorescence lifetime was then determined with a least-square fit of the data (from which the background data had been subtracted) to a single-exponential decay, where the first 20–30 points of the total 512 points of photon counting data were omitted from the analysis.

Estimate of the stability constant of DTPA-CP for Tb^{3+}

Since the affinity of DTPA-CP for Tb^{3+} was assumed to be very strong, we employed a competition method to estimate the stability constant. DTPA was used as a competitive chelator. The stability constant of DTPA for Tb^{3+} has been known to be $10^{22.71}$ M^{-1} (from Dojindo Laboratories). Setting K_{ML}^{DTPA} and $KM_{ML}^{DTPA\text{-}CP}$ as the stability constants of DTPA and DTPA-CP, respectively, $[Tb^{3+}]_{1/2}$ as the added terbium concentration at which half of the DTPA-CP is occupied with Tb^{3+}, we find that;

$$(1)\quad [Tb^{3+}]_{1/2} = 1/K_{ML}^{DTPA\text{-}CP} + \tfrac{1}{2}[DTPA\text{-}CP]_0 + \Delta[Tb^{3+}]_0$$

where $\Delta[Tb^{3+}]_0$ is given by

$$(2)\quad \Delta[Tb^{3+}]_0 = \{K_{ML}^{DTPA}/(K_{ML}^{DTPA} + K_{ML}^{DTPA\text{-}CP})\} \times [DTPA]_0,$$

and $[DTPA\text{-}CP]_0$ and $[DTPA]_0$ represent total concentrations of DTPA-CP and DTPA, respectively. $\Delta[Tb^{3+}]_0$ represents an amount by which $[Tb^{3+}]_{1/2}$ shifts to the right in the presence of DTPA. We titrated DTPA-CP with Tb^{3+} in the presence of various concentrations of DTPA. Since the fluorescence of Tb-DTPA and free Tb^{3+} was negligibly small, the concentration of Tb-DTPA-CP was able to be determined by measuring its fluorescence at 547 nm when excited at 305 nm. $[Tb^{3+}]_{1/2}$ was obtained from the titration curves and $\Delta[Tb^{3+}]_0$ was plotted vs. $[DTPA]_0$. The slope of this plotting gives $K_{ML}^{DTPA}/(K_{ML}^{DTPA} + K_{ML}^{DTPA\text{-}CP})$.

Steady-state absorption and fluorescence measurements

Absorption spectra of rhodamine derivatives and various aromatic compounds were recorded on a Shimazu UV-260 spectrophotometer. Excitation and emission spectra of various Tb chlates and actin-bound Tb-DTPA-CP were recorded on a Hitachi F-3010 fluorescence spectrophotometer.

Precaution

Surfaces of glass tubes, quartz cuvettes and pipette tips usually carry electric charges. So, to keep the charged acceptors from being adsorbed onto these surfaces, surfaces of all glassware and plasticware, with which acceptor solutions were handled, were freshly coated with silicon using Sigma-Coat (Sigma) and commercially-siliconized pipette tips were used.

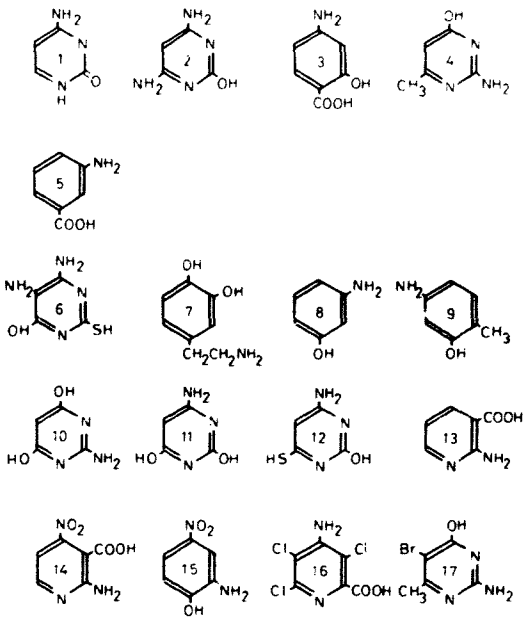

Fig. 1. Chemical structures of 17 kinds of amine-containing aromatic compounds: (1) cytosine; (2) 4,6-diamino-2-hydroxypyrimidine; (3) amino salicylate; (4) 2-amino-4-hydroxy-6-methylpyrimidine; (5) *m*-aminobenzoate; (6) 4,5-diamino-6-hydroxy-2-mercaptopyrimidine; (7) dopamine; (8) *m*-aminophenol; (9) 5-amino-2-methylphenol; (10) 2-amino-4,6-dihydroxypyrimidine; (11) 4-amino-2,6-dihydroxypyrimidine(6-aminouracil); (12) 4-amino-6-hydroxy-2-mercaptopyrimidine; (13) 2-aminonicotinic acid; (14) 4-nitroanthranilic acid; (15) 2-amino-4-nitrophenol; (16) 4-amino-3,5,6-trichloropicolinic acid; (17) 2-amino-5-bromo-6-methyl-4-pyrimidinol.

Results and Discussion

The chemical structures of 17 kinds of amine-containing aromatic compound are depicted in Fig. 1. These compounds were screened for their ability to sensitize the Tb emission when they were bonded to Tb-DTPA. These compounds were arbitrarily chosen except that all of them contained either OH (or keto) or COOH, that might play a role in chelating Tb^{3+}. The each compound (20 μmol) was added to the solution of DTPA dianhydride (20 μmol) in dry DMSO (1 ml) and the solutions were stirred for 3 h at room temperature. Without any purification, aliquots of the reacted solutions were diluted with 20 mM Tris–HCl solution (pH 8.0) to the final concentration of 5 μM. 5 μM of $TbCl_3$ was added to the diluted chelator solutions and the terbium fluorescence at 547 nm was monitored with the excitation wavelength being scanned. The first five compounds in Fig. 1 showed a sensitizing effect on the Tb emission. The others had essentially no effect. As shown in Fig. 2, among the compounds tested cytosine gave the largest sensitizing effect. *para*-Amino salycilate also sensitized the Tb emission, as has been reported before [22], but the effect was about 30% relative to cytosine. As shown in Fig. 3, the excitation spectrum of Tb-DTPA-cytosine had a large peak at 302 nm and a minor peak at 247 nm and the large excitation band extended up to 350 nm. Cytosine can take two tautomers, i.e., ketonic and enolic forms. In aqueous solution the ketonic form is dominant. Since salycilate takes the enolic form and sensitizes the Tb emission, it does not seem important for the sensitizing effect that cytosine exists in the ketonic form. A similar compound, 4-amino-2,6-dihydroxypyridimine, which has an additional hydroxy group, did not show a sensitizing effect at all. So, it is difficult to predict what chemical structure is essential for the sensitizing effect. In the mechanism of direct energy transfer from the excited aromatic ligands to the adjacent Tb, the energy levels of the excited states must be more important than the chemical structures. But it is still difficult to predict this from the absorption spectra of aromatic compounds, since reaction with DTPA dianhydride often changes the absorption spectra and it is also difficult to infer the changes beforehand.

As we found that cytosine was the best sensitizer, we decided to attach both cytosine and and aminophalloin to DTPA. The presumed structure of Tb-DTPA-CP is shown in Fig. 4. Cytosine (2.2 mg, 20 μmol) and

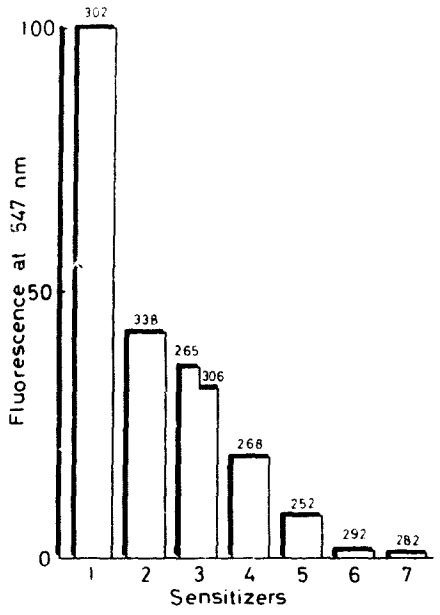

Fig. 2. Sensitizing effect of various compounds upon the Tb emission. These compounds were conjugated with Tb-DTPA and the fluorescence intensities of these Tb chelate solutions (5 μM in 20 mM Tris–HCl (pH 8.0)) were observed at 547 nm. Since the highest sensitizing effect was obtained with cytosine, its fluorescence intensity was normalized to be 100. The fluorescence intensities of the others were given relative to that of Tb-DTPA-cytosine. Each number on the bars shows the peak excitation wavelength. The numbers from 1–7 representing sensitizer compounds were the same as those given in Fig. 1.

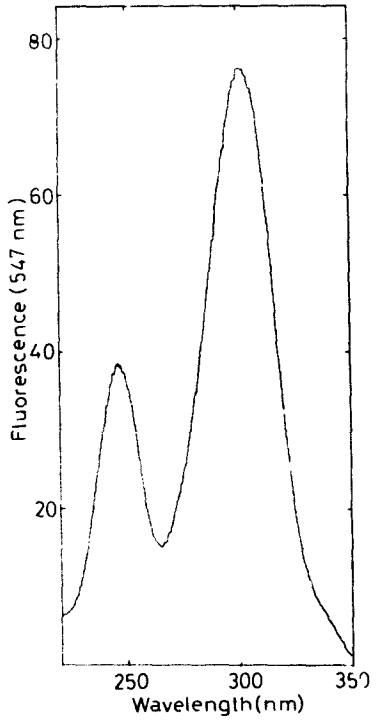

Fig. 3. The excitation spectrum of a solution of Tb-DTPA-cytosine. The Tb emission of 5 μM Tb-DTPA-cytosine in 20 mM Tris–HCl (pH 8.0) was monitored at 547 nm.

DTPA dianhydride (7.1 mg, 20 μmol) were mixed in 1 ml of dry DMSO and the solution was stirred for 5 min at room temperature. Aliquots of the solution (63.5 μl) were added to δ-aminophalloin (1 mg, 1.27 μmol). The solution was vortexed for 5 min and was allowed to stand for 3 h at room temperature. The solution was then diluted about 30-fold with water and was applied to a reverse-phase HPLC column. Elution solvents were 10 mM acetate (pH 3.4; solvent A) and CH_3CN (solvent B). The gradient was 0% B (0–5 min) and 0–100% B (5–100 min) and the flow rate was 2.0 ml/min. The elution profile was monitored at 315 nm (Fig. 5). The eluates were immediately frozen by liquid N_2 and then lyophilized. The each lyophilized fraction was dissolved in 1 ml of ethanol and its optical absorbance was measured at 310 nm. The molar extincion coefficient of DTPA-CP was tentatively assumed to be $1 \cdot 10^4$ cm^{-1} (This value was estimated from the molar extinction coefficients of non-purified DTPA-Cytosine (roughly $5 \cdot 10^3$ cm^{-1}) and of phalloidin (roughly $5 \cdot 10^3$ cm^{-1}). The concentration of the each fraction was determined by using this number. The samples (1 μM) were complexes with equimolar amounts of Tb in 20 mM Tris–HCl (pH 8.0). Their fluorescence intensity was measured at 547 nm with excitation at 300 nm. The 1st and 6th fractions of Fig. 5 showed intense fluorescence and the 2nd and 7th fractions showed about 10% of the fluorescence of the 1st and 6th fractions, respectively. The large fraction, 8, did not emit fluorescence. So, fractions, 1, 2, 6, 7 were further analyzed for their ability to bind to F-actin. Among them, only the 6th and 7th fractions bound to F-actin. Hereafter we used only the 6th fraction. The amount of DTPA in the 6th fraction was determined by measuring the fluorescence

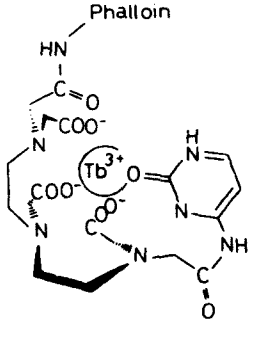

Fig. 4. Presumed structure of Tb-DTPA-CP. The keto group of cytosine participates in the coordination, since the stability constant of DTPA-CP for Tb^{3+} was almost the same as that of DTPA. This structure was made in light of the reported structure of Tb-DTPA-4-aminosalicylate [22].

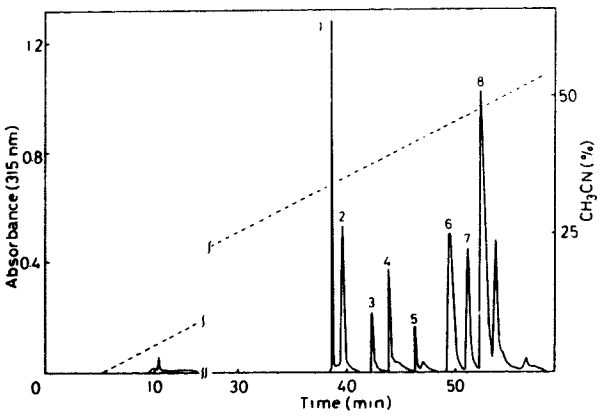

Fig. 5. Reverse-phase HPLC elution profile of the reaction mixture between cytosine, amino-phalloin and DTPA dianhydride. The broken lines show CH_3CN gradient. See the text for more details.

intensity of the mixtures with various amounts of Tb. The amount of phalloin contained in the fraction was determined by measuring the fluorescence intensity of the terbium complex in the presence of various concentrations of F-actin. As shown in Fig. 6, the fluorescence intensity increased with increasing the amounts of F-actin and then saturated, whereas addition of F-actin to free Tb^{3+} did not cause any enhancement of the Tb^{3+} emission. DTPA and phalloin existed in the 6th fraction with 1:1 molar ratio. So, the fraction 6 was identified to be DTPA-CP. The molar extinction coefficient of DTPA-CP in ethanol was exactly determined to be $1.19 \cdot 10^{-4}$ at 310 nm. Finally, this fraction was again lyophilized and stored at −35°C. We did not store it in the form of the Tb chelate, since we noticed that fluorescence intensity of the Tb chelate gradually diminished under this strange condition.

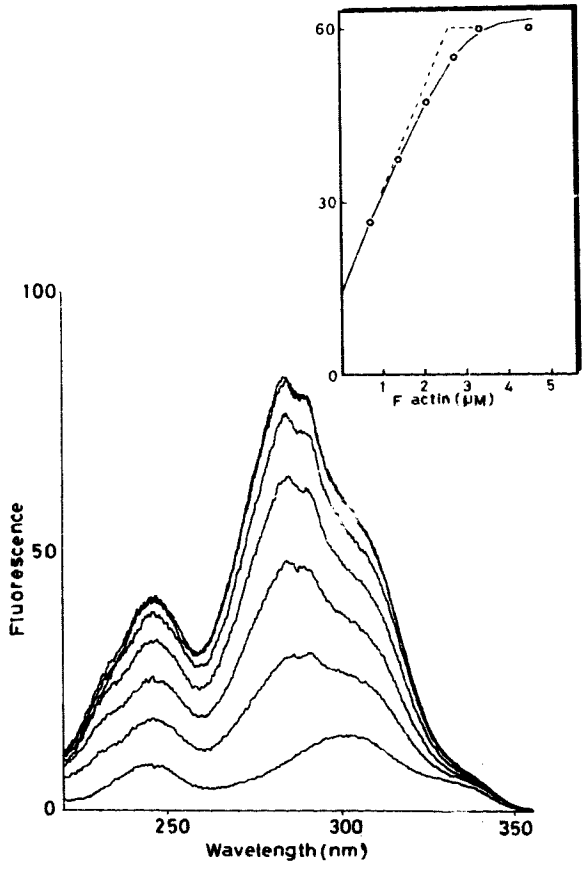

Fig. 6. The excitation spectra of Tb-DTPA-CP alone and Tb-DTPA-CP plus various amounts of F-actin. The Tb emission was monitored at 547 nm. The samples contained 2.74 μM Tb-DTPA-CP and various amounts of F-actin in solutions containing 20 mM Tris–HCl (pH 7.8), 20 mM KCl, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$ at 25°C. The inset shows the relation of the fluorescence intensity with excitation at 300 nm vs. the concentrations of F-actin. Upon compensating the fluorescence intensity for the dilution effect, the fluorescence intensity, when excited at 300 nm, gained a 4.5-fold increase by 1:1 complex formation with F-actin. The apparent dissociation constant of Tb-DTPA-CP for F-actin was about 60 nM.

The stability constant of DTPA-CP for Tb^{3+} ($K_{ML}^{DTPA-CP}$) was determined by a competion method (see Materials and Methods). The total concentration of DTPA-CP was kept constant (0.245 μM). Various amounts of Tb^{3+} (0–1 μM in the final concentration) were added to solutions containing DTPA-CP, 0–0.5 μM DTPA and 20 mM Tes (pH 7.0) and the solutions were allowed to stand for 3 h at room temperature. The fluorescence of each sample was then measured. $\Delta[Tb^{3+}]_0$ was plotted vs. $[DTPA]_0$ (Fig. 7). As expected, the plot gave a straight line. From the slope and the known stability constant of DTPA for Tb^{3+} ($K_{ML}^{DTPA} = 10^{22.71}$ M^{-1}), $K_{ML}^{DTPA-CP}$ was estimated to be $10^{22.46}$ M^{-1}. We further confirmed this large stability constant using a different competitive chelator, CyDTA, whose stability constant for Tb^{3+} is $10^{19.3}$ M^{-1} (from Dojindo Laboratories). Up to 10 μM of CyDTA did not affect the titration curve of DTPA-CP. So, the stability constant was little reduced by attaching cytosine and phalloin to DTPA. This was rather surprising since the number of carboxyl groups was reduced from five to three by the attachment. The keto group of cytosine, therefore, must participate in the cordination.

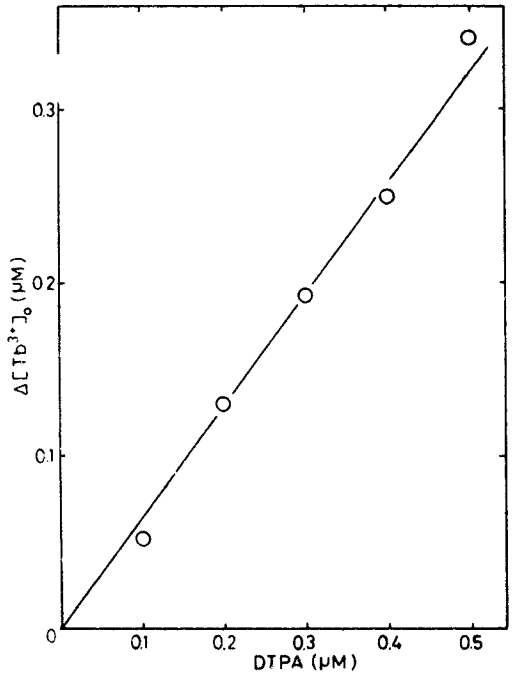

Fig. 7. Estimation of the stability constant of DTPA-CP for Tb^{3+}. DTPA-CP (0.245 μM) was titrated with Tb^{3+} in the presence of various concentrations of DTPA (0–0.5 μM), by measuring the fluorescence intensity of Tb-DTPA-CP at 547 nm with excitation at 305 nm. Only this complex fluoresced in substance. $\Delta[Tb^{3+}]_0$ reprsents the amount by which the titration curves shift to the right at [Tb-DTPA-CP] = 1/2[DTPA-CP]$_0$ due to the competitive effect of DTPA. See Materials and Methods for more details.

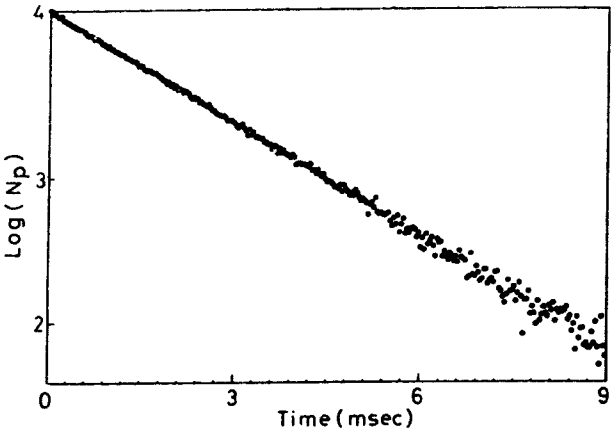

Fig. 8. Time-resolved fluorescence emitted from a single muscle fiber whose thin filaments were stained with Tb-DTPA-CP. The logarithm of the fluorescence intensity is plotted as a function of time after a 5 μs exciting light pulse. The fluorescence was measured in a buffer solution containing 80 mM KCl, 20 mM Tes (pH 7.0), 2 mM $MgCl_2$, 0.2 mM $CaCl_2$. The intensity of the excitation pulsed light was attenuated, otherwise we could not attain single-photon-counting mode because of the too intense emission. This decay clearly shows a single-exponential character. The lifetime was estimated to be 1.58 ms.

With peak to peak comparison, the intensity of terbium emission (at 547 nm with excitation at 300 nm) from a solution of 1:1 complex of F-actin and Tb-DTPA-CP was 25.8% of that from an equimolar solution of tetramethyl-rhodamine that has been often used to visualize single actin filaments under a fluorescence microscope. (This intensity was about 10^5-times greater than that of free Tb.) So, we tried to visualize single actin filaments by staining actin with Tb-DTPA-CP, but we were unsuccessful, in part because of the background luminescence caused by irradiation with 300 nm wavelength of incident light (the objective lens used was an Olympus DApo 100 × UV). Actin bundles were, however, clearly visualized. We also stained single muscle fibers with Tb-DTPA-CP and obtained myofibrils from the stained fibers. The myofibrils were observed under a fluorescence microscope. The images showed a fluorescence pattern similar to those stained with tetramethylrhodamine-phalloidin, i.e., only the Z-bands and I-bands fluoresced. We measured time-resolved fluorescence photon counts from a single fiber (Fig. 8). The emission showed a single exponential decay with the decay constant of 1.58 ms. In our optical system for the photon counting we needed only 1000 repetition cycles (the corresponding time required for the data collection was about 15 s) of pulse excitation and photon counts acquisition to obtain data accumulation sufficient for determination of the decay time. When fibers were stained with Tb-DTPA-phalloin,

bundles consisting of 10–20 single fibers and much more repetition cycles were necessary.

To check whether Tb attached to DTPA-CP is inert to F-actin, the ATPase activity of Tb-DTPA-CP-acto-S-1 was compared to that of non-labeled acto-S-1. 2 μM of non-labeled F-actin activated the S-1 ATPase about 20-fold (the basic activity of S-1 was 6.31 mol P_i/mol S-1 per min, while the acto-S-1 ATPase activity was 121 mol P_i/mol S-1 per min). Tb-DTPA-CP-F-actin activated the S-1 ATPase with essentially the same extent. Judging from the Tb fluorescence of Tb-DTPA-CP-F-actin, the bound Tb was not exchanged with Mg^{2+} (5 mM) that was present in the assay medium. We can thus conclude that the chelated Tb is inert to actin.

The net electric charge of Tb-DTPA-CP is zero. This was confirmed by measuring efficiencies of energy transfer from the excited free Tb-DTPA-CP to rhodamine B derivatives that carry various electric charges (Fig. 9). As a reference, we also showed the data with Tb-DTPA-phalloin which had a unit negative charge. The energy transfer efficiencies (ET) with Tb-DTPA-CP was independent of the number of electric charges (Z_a) of the acceptors, while ET with Tb-DTPA-phalloin was monotonously enhanced with increasing Z_a from -3 to $+2$. These data strongly indicate that efficiency of the diffusion-enhanced energy transfer responded sharply to the electrostatic interaction between the donors and acceptors. This nature of the diffusion-enhanced energy transfer allows us to study electrostatic circumstances around donor-attached sites on macromolecules. We studied electric circumstances around the phalloin-binding site of actin by attaching Tb-DTPA-CP to F-actin. The absorption spectra, a determinant of the energy transfer efficiency, of the rhodamine B derivatives did not depend upon the amino acids or dipeptides that were attached. So, differences in ET observed with the same concentration of the different rhodamine B derivatives should be caused solely by differences in the electric charges of the acceptors. As shown in Fig. 9, ET with F-actin-bound Tb-DTPA-CP was enhanced with increasing Z_a from -3 to $+2$, clearly indicating that the phalloin-binding site of actin resided in minus electric potential. The ET at $Z_a = 0$ was much less than that of free Tb-DTPA-CP. This is probably due in part to that Tb-DTPA-CP being slightly buried in a crevice of F-actin which results in longer closest aproach between the donor and acceptors and in part to F-actin screening the bound donor from 'winds' of acceptor molecules diffusing from the protein wall side. The phalloidin-binding site on actin has been identified by chemical cross-linking [23]. The cross-linked amino acid residues are Glu-117, Met-119 and Met-355. The main cross-linked sites are Glu-117 and Met-119, whereas cross-linking at Met-355 is rather minor. Glu-117 and Met-119

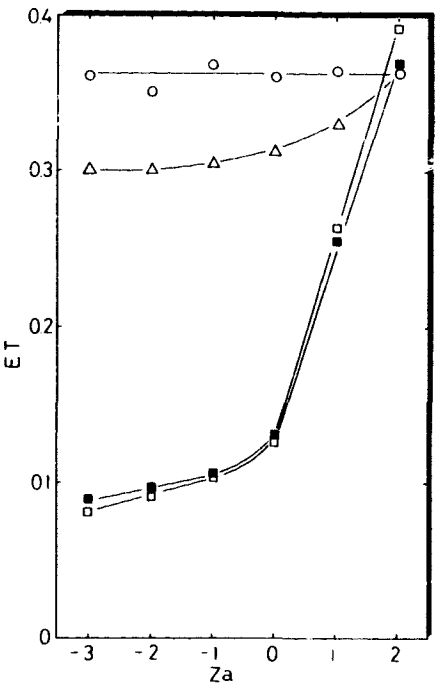

Fig. 9. Energy transfer from excited Tb-DTPA-CP to rhodamine B derivatives. The fluorescence was measured in 20 mM Tes (pH 7.0), 2 mM $MgCl_2$. Z_a represents the amounts of net charges of the acceptors (see Materials and Methods). The optical absorbance at 556 nm of all of the acceptors was fixed at 0.05. ET shows efficiency of the energy transfer estimated from $1-\tau/\tau_0$, where τ_0 and τ are the excited state lifetimes in the absence and presence of acceptors, respectively. τ_0s of free and actin-bound Tb-DTPA-CP were 1.27 ms and 1.58 ms, respectively. τ_0 of free Tb-DTPA-phalloin was 1.79 ms. ○, 0.2 μM Tb-DTPA-CP alone; □, 0.2 μM Tb-DTPA-CP plus 0.2 μM F-actin; ■, 0.2 μM Tb-DTPA-CP plus 0.2 μM 1:1 complex of acto-S-1; △, 5 μM Tb-DTPA-phalloin alone.

are located on a large α-helix starting from Glu-107 and ending with Glu-125 according to the atomic structure of actin presented by Kabsh et al. [24]. The large α-helix (Glu-107–Glu-125) has zero electric charge in the net amount, but is close to a massive α-helix (Trp-79–Asn-92) which possesses four negative charges and one positive charge. The excitation spectra of Fig. 6 showed that the excitation band around 285 nm was more enhanced than the other bands when Tb-DTPA-CP was complexed with F-actin. This indicated that there occurred energy transfer from tryptophan residue(s) to the terbium, suggesting that Tb-DTPA-CP was in the vicinity of tryptophan residue(s) of actin. The massive α-helix mentioned above contains Trp-79 and Trp-86. It therefore seems most likely that the observed minus electric potential at the Tb-DTPA-CP binding site arises from the negative charges of the massive α-helix (Trp-79–Asn-92).

There is sufficient evidence that the terminal region of actin is a part of the S-1 binding site on actin. The

carboxyl groups of the terminal region can be cross-linked to lysine residues of S-1 using a zero-length cross-linker, EDC [25]. The cross-linked region of S-1 contains five lysine residues in total [26]. Therefore, the negative electric charges at the terminal region of actin must be neutralized almost completely by formation of the actin-S-1 rigor complex. As shown in Fig. 9, binding of S-1 to F-actin caused slight reduction in the ET at $Z_a > 0$, while it slightly enhanced at $Z_a < 0$. At $Z_a = 0$, S-1 binding did not alter the ET. This behavior of ET evidenced that binding of S-1 to actin neutralized only a very little the negative electric potential at the phalloin-binding site on actin. Again, this observation supports our inference that the negative electric potential at the Tb-DTPA-CP arises from minus electric charges on the massive alpha helix (Trp-79–Asn-92), since this helix is far from the terminal region of actin [24,27].

Now we would like to propose the use of sensitized terbium luminescence to probe slow rotational relaxation of macromolecules. Rotational diffusion of macromolecules in solution or within membranes has been studied by measuring fluorescence or phosphorescence depolarization of attached dyes. The measurable time-scale of rotational motion is limited by the excited-state lifetime of the attached optical probes. Room-temperature phosphorescence of eosin and erythrosin has been utilized for studying slower rotational relaxation, since their excited state lifetime is long (about 1 ms) in the absence of oxygen quenching [28,29]. But, the quantum yield is very small. While terbium itself is optically isotropic, Tb-DTPA-CP, as a whole, has a polarized absorption dipole moment, but does not have a polarized emission dipole moment. In spite of the unusual dipole moments, Tb-DTPA-CP may be able to be utilized to study rotational relaxation by measuring a transient recovery of the depleted ground-state of Tb after polarized excitation flash. Tb-DTPA-CP has a long excited-state lifetime (about 1.6 ms), emits intense fluorescence and importantly, its emission is insusceptible to oxgen quenching. These features make the Tb chelate suitable for rotational relaxation measurements with actin. Replacement of phalloin with other ligands allows us to study rotational relaxation of other biological macromolecules.

Acknowledgments

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References

1. Horrocks, W.D., Jr. and Sudnick, D.R. (1979) J. Am. Chem. Soc. 101, 334–340.
2. Brittain, H.G., Richardson, F.S. and Martin, R.B. (1976) J. Am. Chem. Soc. 98, 8255–8260.
3. Tellam, R.L. (1991) Arc. Biochem. Biophys. 288, 185–91.
4. Horrocks, W.D., Jr., Schmidt, G.F., Sudnick, D.R., Kittrell, C. and Bernheim, R.A. (1977) J. Am. Chem. Soc. 99, 2378–2380.
5. Thomas, D.D., Carlsen, W.F. and Stryer, L. (1978) Proc. Natl. Acad. Sci. USA 75, 5746–5750.
6. Stryer, L., Thomas, D.D. and Meares, C.F. (1982) Annu. Rev. Biophys. Bioeng. 11, 203–222.
7. Wensel, T.G. and Meares, C.F. (1983) Biochemistry 22, 6247–6254.
8. Meares, C.F. and Rice, L.S. (1981) Biochemistry 20, 610–617.
9. Thomas, D.D. and Stryer, L. (1982) J. Mol. Biol. 154, 145–57.
10. Wensel, T.G., Chang, C.-H. and Meares, C.F. (1985) Biochemistry 24, 3060–3069.
11. Hirabayashi, Y. and Ando, T. (1992) submitted to Anal. Biochem.
12. Yeh, S.M., Sherman, D.G. and Meares, C.F. (1979) Anal. Biochem. 100, 152–159.
13. Meares, C.F., McCall, M.J., Reardan, D.T., Goodwin, D.A., Diamanti, C.I. and Mctigue, M. (1984) Anal. Biochem. 142, 68–78.
14. Parker, D. (1990) Chem. Soc. Rev. 19, 271–291.
15. Krejcarek, G.E. and Tucker, K.L. (1977) Biochem. Biophys. Res. Commun. 77, 581–585.
16. Hnatowich, D.J., Layne, W.W. and Childs, R.L. (1982) Int. J. Appl. Radiat. Isot. 33, 327–332.
17. Wieland, T., Hollosi, H. and Nassal, M. (1983) Liebigs Ann. Chem. 1983, 1553–1540.
18. Ando, T. (1987) J. Mol. Biol. 195, 351–358
19. Spudich, J.A. and Watt, S. (1971) J. Biol. Chem. 246, 4866–4871.
20. Ando, T. and Scales, D. (1985) J. Biol. Chem. 260, 2321–2327.
21. Borejdo, J., Assulin, O., Ando, T. and Putnam, S. (1982) J. Mol. Biol. 158, 391–414.
22. Oser, A., Collasius, M. and Valet, G. (1990) Anal. Biochem. 191, 295–301.
23. Vandekerckhove, J. Deboben, A., Nassal, M. and Wieland, T. (1985) EMBO J. 4, 2815–2818.
24. Kabsch, W., Mannherz, H.G., Suck, D., Pai, E.F. and Holmes, K.C. (1990) Nature (Lond.) 347, 37–44.
25. Mornet, D., Bertrand, R., Pantel, P., Audemard, E. and Kassab, R. (1981) Nature (Lond.) 292, 301–306.
26. Yamamoto, K. (1989) Biochemistry 28, 5573–5577.
27. Holmes, K.C., Popp, D., Gebhard, W. and Kabsch, W. (1990) Nature (Lond.) 347, 44–49.
28. Garland, P.B. and Moore, C.H. (1979) Biochem. J. 183, 561–572.
29. Kinoshita, K., Jr., Ishiwata, S., Yoshimura, H., Asai, H. and Ikegami, A. (1984) Biochemistry 23, 5963–5975.