Thiol-Reactive Luminescent Chelates of Terbium and Europium

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Received September 17, 1998; Revised Manuscript Received November 18, 1998

Thiol-reactive lanthanide complexes have been synthesized that are luminescent when bound to terbium and/or europium. The complexes consist of a diethylenetriaminepentaacetate (DTPA) chelate covalently joined through one amide bond to a chromophore, carbostyril 124, and via a second amide bond to a maleimide, bromoacetamide, or pyridyldithio moiety. Site-specific attachment and characterization of the complexes attached to DNA-activating protein NtrC, to various sites on myosin, or to DNA are presented. The compounds coordinate a surprisingly large number of ligation sites of terbium when a hydrazide spacer is used between the chelate and thiol-reactive moiety, although this extra ligation can cause quenching when europium is used. Synthesis is a simple two- or three-step reaction, and purification is straightforward. The compounds should be useful as nonisotopic replacements, as long-lifetime probes in imaging, and as donors in luminescence resonance energy transfer. They are examples of a wide class of chelates that can be made conjugatable via readily available hetero- or homo-bifunctional linkers.

INTRODUCTION

Luminescent lanthanide chelates are of interest in time-resolved fluorescence assays (reviewed in refs *1* and *2*) as long-lifetime (millisecond) probes to increase contrast in fluorescence imaging when autofluorescence is a problem (*3*, *4*) and as donors in resonance energy transfer experiments (*5*−*12*). The chelate of diethylenetriaminepentaacetic acid covalently joined to a chromophore, 7-amino-4-methyl-2(1*H*)-quinolinone (also named carbostyril 124 or cs124), makes an excellent donor in energy transfer experiments (*7*, *8*, *10*) and can be conjugated to amine-containing macromolecules via either the anhydride or isothiocyanate forms (*13*). The conversion of one or even two DTPA[1] carboxyl groups to amides does not affect the coordination (*14*), and a sufficiently high binding constant is maintained for most purposes. Pyridyldithio forms of DTPA and triethylenetetraaminehexanoic acid, which are luminescent with europium, have been made previously by placing the reactive moiety on the chromophore (*15*). Other thiol-reactive chelates have also been developed (*16*).

Here we present the synthesis of maleimide, bromoacetamide, and pyridyldithio forms of DTPA−cs124 for reaction with thiols. We have recently used the maleimide form in energy transfer experiments on the muscle protein myosin (*17*). The synthesis is a simple two- or three-step reaction in which the dianhydride of DTPA is first reacted with the 7-amino of cs124 and then with a hetero-bifunctional compound containing an amino on one end and a thiol-reactive moiety on the other end. Alternatively, the second anhydride can be reacted with a diamino compound, which is then further reacted with an NHS−thiol reactive cross-linker. When the linker contains a hydrazide, under favorable conditions we find the carbonyl can ligate the lanthanide, resulting in a higher terbium quantum yield.

EXPERIMENTAL METHODS

Chemicals and Materials. The following were purchased from Aldrich: diethylenetriaminepentaacetic acid dianhydride (caDPTA); 7-amino-4-methyl-2(1*H*)-quinolinone (also named carbostyril 124 or cs124); anhydrous dimethyl sulfoxide, dimethylformamide, and hydrazine (Sure/Seal bottles); triethylamine (dried by activated molecular sieves before use); ethylenediamine (redistilled); C18-silica on glass-backed TLC plates (150 μm layer thickness); 3-maleimidopropionic acid *N*-hydroxylsuccinimide ester; acetonitrile (99.93%, HPLC grade). Glacial acetic acid and ammonium hydroxide were purchased from Fisher Scientific. *N*-Hyroxysuccinimidyl bromoacetate was purchased from Molecular Biosciences (Boulder, CO). Glutathione (reduced form) was purchased from Sigma. 3-(2-Pyridyldithiol)propionyl hydrazide (PDPH) was purchased from Pierce. ϵ-Maleimidocaproic acid hydrazide−HCl (EMCH) was from Bioaffinity Systems (Roscoe, IL). cDNA for the cys73 mutant of myosin regulatory light chain (RLC) was the generous gift of Dr. Susan Lowey. NtrC (mutant D54C) was the generous gift of Dr. Michael Nohaile. Distilled and deionized water (18 MΩ cm^{-1}) was used throughout. All glassware was washed with a mixed acid solution and thoroughly rinsed with deionized, distilled water (*18*). All plastic labware was purchased from Bio-Rad (metal-free). All chemicals were of the purest grade available.

Purification. Reverse-phase high-performance liquid chromatography was performed at room temperature on a Waters model 600 system with a Dynamax 60 Å C_{18} column (10 or 25 mm i.d. × 250 mm, Rainin, at 3 or 8

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[1]Abbreviations: DTPA, diethylenetriaminepentaacetic acid; caDTPA, dianhydride of DTPA; cs124 or carbostyril 124, 7-amino-4-methyl-2(1*H*)-quinolinone; DMSO, dimethyl sulfoxide; DMF, dimethylformamide; EDA, ethylenediamine; EMCH, ϵ-maleimidocaproic hydrazide; EMPH, ϵ-maleimidoproprionic hydrazide; PDPH, 3-(2-pyridyldithiol)propionic hydrazide; HMM, heavy meromyosin; RLC, regulatory light chain of myosin; NtrC, nitrogen regulatory protein C; RP-TLC, reverse-phase thin-layer chromatography.

10.1021/bc980113w CCC: $18.00
Published on Web 02/05/1999

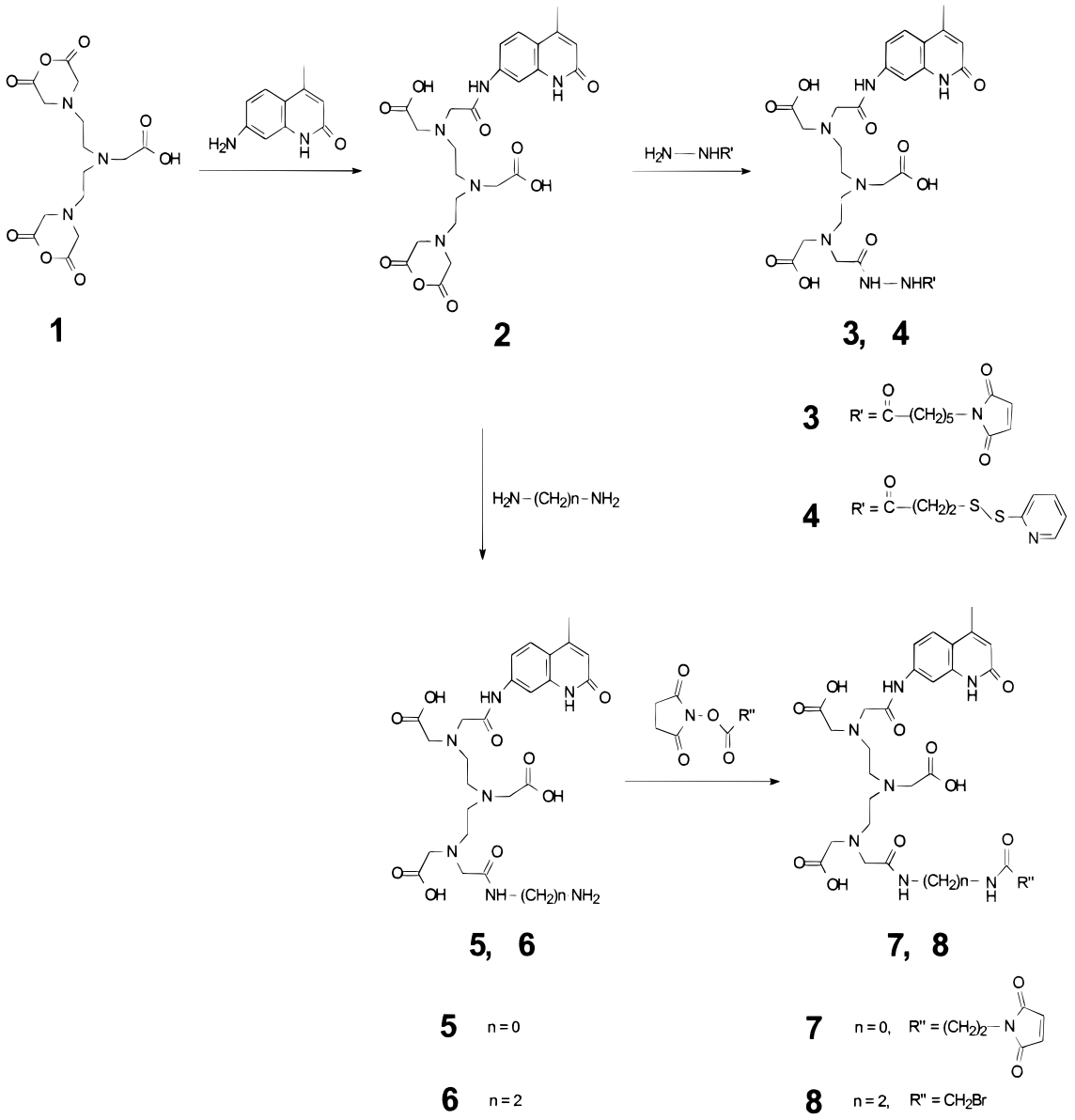

Figure 1. Synthetic reaction scheme for thiol-reactive DTPA−cs124 chelates.

mL/min, respectively) using a linear gradient (solvent A = 0.1 M triethylammonium acetate, pH 6.5; solvent B = acetonitrile). Fractions were vacuum-dried at room temperature, and the powder was stored at −80 °C.

Spectroscopy. Time-resolved and gated luminescence measurements were made on a laboratory-built spectrometer described previously (*8*, *10*), employing a 5 ns excitation pulse at 337 nm followed by time-resolved detection of lanthanide emission (*8*, *10*).

Synthesis (Figure 1). *DTPA−cs124−EMPH (**7**).* caDTPA (**1**) (50 mg, 141 μmol) was dissolved in 0.5 mL of anhydrous DMSO. Triethylamine (20 μL, 141 μmol) dried over molecular sieves was added to the caDTPA−DMSO solution. A solution of cs124 (19 mg, 109 μmol, 0.77 equiv in 0.5 mL of anhydrous DMSO) was added dropwise. The reaction was carried out at room temperature for 30 min, and then 10.7 μL of anhydrous hydrazine (282 μmol, 2 equiv) was added to above the reaction mixture. Fifteen minutes later, one-third of the solution was added to solid 3-maleimidopropionic acid *N*-hydroxysuccinimide ester (25 mg, 2 equiv) at room temperature and the reaction quenched after 30 min by adding 7 mL of aqueous triethylammonium acetate (TEAA) (0.1 M, pH 6.5). The remaining two-thirds of the solution was stored at −80 °C for future reaction. Alternatively, the intermediate product DTPA−cs124−hydrazide (**5**) was first purified by HPLC, redissolved in DMSO, and reacted with *N*-hydroxysuccinimide ester as above. This eliminated contaminating DTPA−$(cs124)_2$ in the final product (see directly below).

The reactions were monitored by C18 RP-TLC. When acetonitrile/TEAA (2:8, v/v) was used as RP-TLC developing system, the R_f values of DTPA−cs124, DTPA−cs124−hydrazine, and DTPA−cs124−hydrazine−maleimide were 0.67, 0.60, and 0.40, respectively. The final product was purified by HPLC using a 40-min linear gradient, from 10 to 20% solvent B. Three major peaks with 12.2, 17.6, and 20.2 min retention times were collected and characterized as DTPA−cs124, DTPA−$(cs124)_2$, and DTPA−cs124−hydrazine−maleimide (DTPA−cs124−EMPH), respectively. [HPLC purification of the intermediate product, DTPA−cs124−hydrazine, when done, eliminated the $(DTPA-cs124)_2$ peak from the final reaction.] FAB-MS (glycerol matrix) of the 20.2 min retention time peak shows mass (MH^+) of 715.2686 (found); 715.2687 (calculated). The product was dried on

a Speed-vac and stored either at −80 °C in solid form or in TEAA buffer at ∼30 mM DTPA−cs124−EMPH.

The thiol reactivity of the DTPA−cs124−EMPH was tested by adding an excess of solid glutathione (reduced form) with a solution of DTPA−cs124−EMPH in 0.1 M TEAA (pH 6.5) at room temperature for 1 h and then checked on RP-TLC and MS. The RP-TLC showed the DTPA−cs124−EMPH peak disappeared and a more rapidly moving peak appeared; high-resolution FAB-MS (glycerol as matrix) of the reaction mixture showed 1022.35; 1022.35 (calcd). These results indicate complete reaction of starting material into product.

*DTPA−cs124−EMCH (**3**).* To a solution of caDTPA (27.6 mg, 77 μmol) in 1 mL of DMSO was added dropwise cs124 (13.5 mg, 77 μmol) in 200 μL of DMSO, and the mixture was vortexed immediately. Two or four hours later, solid EMCH (27.6 mg,105 μmol) was added, and the mixture was vortexed until the EMCH had dissolved. The reaction proceeded overnight and was then frozen at −70 °C until purification. Immediately before HPLC purification, 9× volume of 0.5 M triethylammonium acetate, pH 6.5, was added to quench the reaction. The product was purified by reverse-phase HPLC using a 30 min linear gradient from 10 to 45% solvent B at 3 mL/min. The product was collected as a well-resolved peak at 22 min and immediately frozen and lyophilized to dryness. Yield was ∼25%, estimated by HPLC profile. FAB-MS: *m/e* 757 (glycerol matrix).

*DTPA−cs124−EDA−Bromoacetamide (**8**).* DTPA−cs124−ethylenediamine (**6**) (DTPA−cs124−EDA) was synthesized as reported previously (*13*). To a solution of DTPA−cs124−EDA (5.9 mg, 10 μmol, containing about an equal amount of DTPA−cs124) in 1 mL of TEAA (0.1 M, pH 8.5) was added *N*-hydroxysuccinimidyl bromoacetate (NHS-Br, 10 mg, 3 equiv). With vigorous stirring, the white solid of NHS-Br was dissolved gradually in the reaction solution over a few minutes at room temperature. The reaction was complete in 30 min. The R_f value of DTPA−cs124−EDA−bromoacetyl (DTPA−cs124−EDA−Br) was 0.45 [C18 RP-TLC in acetonitrile/TEAA (2:8, v/v)]. The newly formed compound showed full reactivity with glutathione (reduced form), monitored by RP-TLC. The final product was purified by HPLC (45-min linear gradient, from 10 to 25% solvent B at 8 mL/min), and the peaks at 18.8 and 25.2 min retention times were collected and characterized as DTPA−cs124 and DTPA−cs124−EDA−Br. High-resolution FAB-MS (glycerol as matrix) of the 25.2 min retention time peak shows 712.1942 (found); 712.1942 (calcd)

*PDPH−DTPA−cs124 (**4**).* Cs124 (10.5 mg, 60 μmol) was dissolved in 0.3 mL of dry DMSO and added dropwise to a 0.9 mL solution of caDTPA (21 mg, 61 μmol) in dry DMSO and allowed to react at room temperature for 45 min. Solid PDPH (10.7 mg, 47 μmol) was added to 1 mL of the above solution and allowed to react for >2.5 h at room temperature. Solid caDTPA (1.1 mg, 3 μmol) was occasionally added in an attempt to bind any residual free cs124 or PDPH. The reaction was quenched by addition of an equal volume of 400 mM sodium bicarbonate, pH 7.0. Purification was by HPLC (gradient: 0−14% solvent B from 4 to 14 min, 14−20% solvent B from 14 to 84 min, at 8 mL/min). The product at 51 min was dried on a Speed-vac, redissolved in water, passed through a C18 Sep-Pak (Waters), eluting with 50:50 acetonitrile/water and dried to give a white powder. The product was identified by FAB mass spectrometry, UV−vis, and lanthanide luminescence. Reaction with 2.4× glutathione (20 mM sodium bicarbonate, pH 7.0) resulted in increased absorbance in the 310−380 nm range due to release of the absorbing pyridyl group [$\epsilon_{343} = 8.1 \times 10^3\ M^{-1}\ cm^{-1}$ (*19*)], indicating nearly quantitative reaction with the PDPH moiety. FAB-MS, *m/e* 758.

Addition of Metal. $TbCl_3$ or $EuCl_3$ was added to the chelate in a 0.9:1 mole ratio at >0.1 mM concentration at pH 6−7, usually in 0.1 M TEAA, pH 6.5, buffer and allowed to bind on ice for ∼15 min. The complex was either stored at −80 °C or used immediately for reaction with biomolecules.

Coupling to Biomolecules. Coupling of Tb−DTPA−cs124−EMPH with the regulatory light chain of myosin, a 20 kDa calmodulin-like protein has been described (*17*), although we now routinely omit the reductant TCEP. We have also coupled it to a cysteine mutant of RLC (cys73) (*20*) at 25 μM protein with a 5-fold excess of chelate and to a single-cysteine mutant (D54C) of NtrC protein, a 20 kDa protein for which the atomic structure has been solved by NMR (*21*). Tb−(Eu−)DTPA−cs124−EDA−Br was coupled to heavy meromyosin (HMM), a 400 kDa fragment of myosin (∼14 μM protein), at a ratio varying from 2:1 to 10:1, and the reaction was carried out on ice overnight in 20 mM Mops, pH 7.0/5 mM $MgCl_2$. The excess lanthanide chelates were removed by passing the reaction mixture through a Sephadex G-25 size exclusion column. Tb−EMCH−DTPA−cs124 was reacted with a DNA (5′-$HSCH_2CH_2NHPO_3$−CCT-AGC-CAG-TGG), containing a free thiol at the 5′ end [introduced by coupling of cystamine via a slight modification of the method of Chu and Orgel (*22*)], and HPLC purified.

RESULTS AND DISCUSSION

Figure 1 shows the synthetic pathway for the various thiol-reactive chelates. HPLC, mass spectra, reaction with small or large thiol-containing molecules, and lanthanide luminescence all prove the chelates' structures and reactivities. The products were generated either in a two-step (one-pot) reaction, where DTPA dianhydride was first reacted with cs124 and then to an amine-thiol cross-linker (such as EMCH or PDPH), or via a three-step (one- or two-pot) procedure where a DTPA−cs124−diamine compound was generated and the free amine converted into a thiol-reactive entity via reaction with an NHS-thiol cross-linker. The former has greater simplicity but was not always possible. In particular, we made DTPA−cs124−EMPH both ways, but with the two-step reaction, HPLC retention of the DTPA−cs124−EMPH was similar to that of the byproduct DTPA−$(cs124)_2$, and more significantly, dramatically different yields were found, possibly due to variability in the purity of the starting EMPH material.

General Overview. The excited-state lifetime and emission spectra of Tb^{3+} and Eu^{3+} in the thiol-reactive chelates are shown in Table 1 and Figures 2 and 3. Figure 3 shows that both lanthanides have the usual sharply spiked emission spectrum. Table 1 and Figure 2 show that the lifetimes of both lanthanides are, as expected, in the millisecond range. The exact lifetimes depends on the lanthanide, chelate, and linker between maleimide and chelate and what they are attached to. For lanthanides, one of the main determinants of lifetime is the number of waters bound to the lanthanide (*23*). DTPA or DTPA−cs124 coordinates eight of nine possible sites, and the remaining site is filled by a water molecule, which reduces the excited-state lifetime through vibrational quenching. Surprisingly, for the chelate derivatives reported here, this last site can be filled by the carbonyl oxygen of the hydrazide (but not ethylenediamine) linker (see also Coordination Sites). For terbium, this is advan-

Table 1. Lifetimes of Thiol-Reactive Europium and Terbium Complexes in H_2O and D_2O, Their Ratio, and the Number of Coordinated Waters in the Lanthanide Primary Sphere[a]

lanthanide complexes[a]	τ_{H_2O} (ms)	τ_{D_2O} (ms)	τ_{H_2O}/τ_{D_2O}	no. of waters
Tb−(Eu−)DTPA−cs124[b]	1.55 (0.62)	2.63 (2.42)	0.59 (0.26)	1.1 (1.26)
Tb−(Eu−)DTPA−cs124−AH[c]	1.91 (Q)	2.31 (Q)	0.83	0.37
Tb−(Eu−)DTPA−cs124−EDA−bromoacetyl[d]	1.51 (0.60)	2.70 (2.37)	0.56 (0.28)	1.22 (1.32)
Tb−(Eu−)DTPA−cs124−EMPH[e]	1.51 (0.55)	2.53 (2.05)	0.60 (0.27)	1.13 (1.40)
Tb−DTPA−cs124−EMPH−R[f]	18% 0.46 82% 1.80			
Eu−DTPA−cs124−EMPH−R'[g]	0.62			
Tb−DTPA−cs124−EMCH−R[h]	1.60	2.1	0.76	0.6
Tb−(Eu−)DTPA−cs124−PDPH[i]	1.82 (1.32)	2.22 (2.32)		0.41 (0.34)

[a] The measurements for the free reactive chelates were taken in 20 mM MOPS/5 mM $MgCl_2$, pH 7.0, at room temperature. Numbers in parentheses are for Eu^{3+}; those not in parentheses are for Tb^{3+}. [b] Data from ref *24*. [c] DTPA−cs124−AH = DTPA−cs124−acetic hydrazide = $CH_3CONHNH_2$−DTPA−cs124. Synthesis followed the general protocol outlined in Figure 1: reaction of caDTPA with cs124 followed by addition of an excess of $CH_3CONHNH_2$ and HPLC purification. [d] Tb^{3+} lifetime decreases *slightly* when conjugated to proteins (see Figure 1). [e] Free chelate. Tb lifetimes are major component. There was a minor short-lifetime (<0.5 ms) component as well. [f] Conjugated to NtrC in buffer defined above (footnote *a*) such that there is <1 Tb chelate per protein. [g] Conjugated to glutothione. Lifetime indicates ligation of carbonyl hydrazide from linker is not occurring. Conjugated to DNA it is highly quenched due to extra ligation (data not shown). [h] Conjugated to single-stranded 12-mer DNA in 20 mM $MgCl_2$/50 mM NaCl/10 mM Tris, pH 8.0. [i] Unconjugated compound showed very large concentration-dependent quenching. To achieve the reported Tb lifetimes required titrations down to 5 nM. Tb lifetime was biexponential, with 75% of population corresponding to long-lifetime reported; shorter lifetime was ≈0.5 ms. Eu lifetimes could not be accurately determined at these concentrations. The results for Eu are reported in the presence of glutothione, which displaced the pyridyl group of PDPH, produced a large (>200-fold) increase in signal, and largely eliminated the concentration quenching.

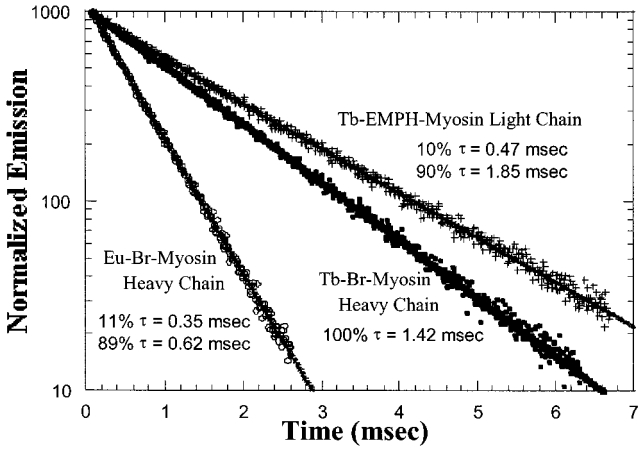

Figure 2. Lifetime of Tb^{3+} and Eu^{3+} bound to seven or eight attached to myosin fragments.

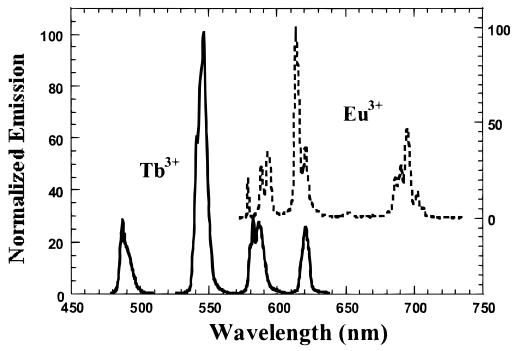

Figure 3. Normalized Tb and Eu emission spectra in DTPA−cs124−EDA−Br. Tb^{3+} and Eu^{3+} display their characteristic sharply spiked spectra. Free chelate dissolved in 20 mM Mops, pH 7.0/5 mM $MgCl_2$ at a concentration of 0.5 μM. Resolution is 0.3 nm, excitation was at 40 Hz of 3.4 μJ/pulse at 337 nm for Eu, 2.0 μJ/pulse for Tb; integration time is 1 s for Tb and 10 s for Eu. Note that signal is normalized. Tb emission is considerably brighter (roughly 20×) than Eu emission. Eu spectrum is displaced for clarity of presentation.

tageous. However, for europium, with one exception (PDPH bound to glutathione), this ligation causes very large quenching, presumably through vibrational deactivation. Single-exponential lifetimes (as opposed to biexponential) are also advantageous, and the bromoacetamide chelate produces this, whereas the maleimide can be either single exponential or biexponential, depending on the biomolecule attachment site and solvent conditions. In all cases, coupling to thiol-containing biomolecules could be monitored by comparing the absorbance of the cs124 when coupled to DTPA (12000 M^{-1} cm^{-1}) at 337 nm to that of the biomolecule (typically 260 nm for DNA, 280 nm for proteins). [The absorption spectra has been reported (*24*).] For DTPA−cs124−PDPH, coupling could also be monitored by the release of the pyridylthio group. Reaction with the RLC was also monitored by MALDI mass spectroscopy.

Specific Thiol-Reactive Forms. *Maleimide.* The maleimide form has the advantage of quick and efficient reaction with thiols. Tb−DTPA−cs124−EMPH was previously attached to the regulatory light chain of myosin and used successfully as a donor in resonance energy transfer experiments in myosin (*17*) (see Figure 2). To further characterize this reaction, we have now performed mass spectrometry (MALDI) on the chelate bound to a single-cysteine (cys73) mutant of RLC. Two peaks of molecular weight of 20002.9 and 19285.2 in approximately 4:1 ratio were found (data not shown). The former is labeled RLC, and the latter is unlabeled RLC. Note that the difference in mass is 717.7, in excellent agreement with the mass of EMPH−DTPA−cs124. The mass spectrometery data also show that labeling is efficient (∼80% yield based on relative peak heights) and, as expected for a maleimide, is likely highly specific for thiols as only one chelate per protein is bound. Previous energy transfer measurements using this chelate-labeled protein further indicate that the label is bound specifically to cysteine-108 (*17*). The excited-state lifetime of Tb in this reaction was biexponential, with a minor, short-lifetime component and with a major long-lifetime component of ∼1.8 ms at room temperature (2.0 ms at 4 °C) (Table 1 and Figure 2). The brightness of the terbium−maleimide when bound to RLC was equivalent to or slightly brighter than that of free Tb−DTPA−cs124 (the unreactive, no linker parent compound), which we have previously shown to have a 2 pM detection limit.

In addition, the EMPH or EMCH chelate was reacted with thiolated DNA, with NtrC, and with the heavy chain of myosin at cysteine-707. When attached to the 5′ end of thiolated DNA, Tb−DTPA−cs124−EMPH went from biexponential to single exponential at higher salt. With

NtrC it was also biexponential, although in both cases the short-lifetime component was a minority (10−20%). Finally, because the maleimide forms were made using a hydrazide linker, the number of waters bound to the terbium was decreased in most cases (discussed further below), which results in longer lifetime (>1.8 versus 1.5 ms without hydrazide; Table 1), higher quantum yield, and brighter emission compared to those without the extra ligation. Europium in the EMPH linker was highly quenched under conditions where extra ligation was present, such as bound to the 5′ end of thiolated DNA, but displayed its usual 0.6 ms unquenched lifetime in H_2O-based buffer when extra ligation did not occur (see Table 1, footnote *g*).

Bromoacetamide. The bromoacetamide form has the distinct advantage of being single exponential with terbium and either single exponential with europium or at worst having a minor (10%) quenched component (Figure 2). The terbium brightness in the unconjugated bromoacetamide chelate (**8**) was equivalent to the unreactive form (Tb−DTPA−cs124 with no linkers). We found, however, the reactivity of bromoacetamide chelates to be significantly less than that of maleimides. It was sufficiently reactive with cysteine-707 on myosin, a site that is known to be highly reactive, but was not sufficiently reactive for labeling the regulatory light chain of myosin.

PDPH. The PDHP−DTPA−cs124 reacts rapidly with thiols to form a reducible disulfide bridge, with the release of pyridylthio. Because the PDPH chelate contains a hydrazide linker, it displays the long lifetime with terbium. Interestingly, this probe appears to be a useful reagent for the detection of glutathione or possibly other small thiol peptides, as it is highly quenched in the absence of glutathione and has a 200-fold intensity increase in its presence.

Coordination State. The number of waters bound to the primary ligation sphere can be calculated by comparing the lifetimes in H_2O and D_2O and using the equation of Horrocks and Sudnik (*23*). The results are shown in Table 1. Recently, Parker et al. have shown that ligated amide N−H bonds can deactivate the excited state of Eu^{3+} with an efficiency equivalent to approximately one-sixth that of a ligated OH bond, so the number of water molecules reported in Table 1 is likely overstated by about this amount (*25*). By comparing the free DTPA−cs124 to DTPA−cs124−acetic hydrazide it can be seen that the number of water molecules decreases for terbium, which we interpret as being due to the ligation of the amide−carbonyl in the hydrazide linkage. This ligation is advantageous, as mentioned above, in that it increases the terbium quantum yield and also results in 1.8-fold higher intensity. The ligation is fairly weak (order of kT) as it is temperature dependent, with lifetime increasing from 1.85 ms at room temperature to ∼2.0 ms at 4 °C for the EMPH derivative. For the DTPA−cs124−EMCH chelate this ligation is even weaker. This weak ligation is not surprising given that it arises from a seven-membered ring formation. Slight changes, such as using an ethylenediamine instead of hydrazine or using DTPA−cs124−triglyceride (*24*) eliminates this extra ligation. Similar subtle changes exist depending on what is bound to at the end of the hydrazine linkage. For example, unconjugated DTPA−cs124 hydrazine−maleimide compounds do not display this extra ligation, whereas we have found that when conjugated to certain proteins, the extra ligation occurs. Surprisingly, this hydrazide ligation tends to efficiently quench the europium excited state, with the exception of Eu bound to DTPA−cs124−PDPH coupled to glutathione (Table 1).

In summary, we have made a number of different thiol-reactive forms of the luminescent lanthanide chelate DTPA−cs124. The maleimide form (**7**) appears to be the most versatile with terbium as it is highly reactive, specific, and bright. A drawback is its biexponential excited-state lifetime when conjugated to some biomolecules. The bromoacetamide derivative overcomes this drawback when it is sufficiently reactive. Overall, the synthetic route is simple and amenable to modification such that a wide variety of different linker lengths can be readily made. These have already been shown to be useful donors in resonance energy transfer experiments in which specific reaction with protein thiols is required.

ACKNOWLEDGMENT

This work was supported initially by NIH Grant AR44420 and later by Amersham Inc. We thank Dr. Michael Nohaile for the gift of NtrC protein and Dr. Susan Lowey for the gift of cDNA for mutant RLCs. We also thank Dr. Ming Xiao for assistance in RLC labeling and Dr. Handong Li for assistance with mass spectroscopy.

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BC980113W