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Comparison of Terbium(III) Luminescence Enhancement in Mutants of EF Hand Calcium Binding Proteins*

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The luminescent isomorphous Ca^{2+} analogue, Tb^{3+}, can be bound in the 12-amino acid metal binding sites of proteins of the EF hand family, and its luminescence can be enhanced by energy transfer from a nearby aromatic amino acid. Tb^{3+} can be used as a sensitive luminescent probe of the structure and function of these proteins. The effect of changing the molecular environment around Tb^{3+} on its luminescence was studied using native Cod III parvalbumin and site-directed mutants of both oncomodulin and calmodulin. Titrations of these proteins showed stoichiometries of fill corresponding to the number of Ca^{2+} binding loops present. Tryptophan in binding loop position 7 best enhanced Tb^{3+} luminescence in the oncomodulin mutant Y57W, as well as VU-9 (F99W) and VU-32 (T26W) calmodulin. Excitation spectra of Y57F, F102W, Y65W oncomodulin, and Cod III parvalbumin revealed that the principal Tb^{3+} luminescence donor residues were phenylalanine or tyrosine located in position 7 of a loop, despite the presence of other nearby donors, including tryptophan. Spectra also revealed conformational differences between the Ca^{2+}- and Tb^{3+}-bound forms. An alternate binding loop, based on Tb^{3+} binding to model peptides, was inserted into the CD loop of oncomodulin by cassette mutagenesis. The order of fill of Tb^{3+} in this protein reversed, with the mutated loop binding Tb^{3+} first. This indicates a much higher affinity for the consensus-based mutant loop. The mutant loop inserted into oncomodulin had 32 times more Tb^{3+} luminescence than the identical synthetic peptide, despite having the same donor tryptophan and metal binding ligands. In this paper, a ranking of sensitivity of luminescence of bound Tb^{3+} is made among this subset of calcium binding proteins. This ranking is interpreted in light of the structural differences affecting Tb^{3+} luminescence enhancement intensity. The mechanism of energy transfer from an aromatic amino acid to Tb^{3+} is consistent with a short-range process involving the donor triplet state as described by Dexter (Dexter, D. L. (1953) *J. Chem. Phys.* 21, 836). This cautions against the use of the Förster equation in approximating distances in these systems.

Calcium binding proteins of the calmodulin family bind cellular Ca^{2+} at physiologically sensitive levels (Marban *et al.*, 1980). These proteins can undergo conformational changes leading to molecular events, such as enzyme modulation, as well as cellular events, such as motility and contractile processes (for reviews, see Cheung, 1983; Gerday *et al.*, 1988). The homologous unit of activity in the calmodulin superfamily of proteins is a loop metal binding structure known as the "EF hand" (Kretsinger and Knockolds, 1973; Strynadka and James, 1989). It consists of an α helix, a loop of 12 amino acids that can chelate Ca^{2+} or Mg^{2+}, and another α helix orthogonal to the first helix.

Calcium does not possess any optical spectroscopic properties which allow its direct use as a structural probe. On the other hand, the lanthanides terbium(III) (Tb^{3+}) and europium(III) (Eu^{3+}) have been used extensively as isomorphous luminescent analogues of Ca^{2+} for probing structure/function relations of calcium binding proteins (for reviews, see Horrocks, 1982; Dockter, 1983; O'Hara, 1987). Di- and trivalent members of the lanthanide series will substitute for Ca^{2+} because they have similar binding characteristics and ligand specificities (Brittain *et al.*, 1976; Horrocks *et al.*, 1977; Evans, 1983).

Tb^{3+} luminescence upon binding to an EF hand loop can be enhanced by energy transfer (ET)[1] from a fluorescent donor, either an aromatic amino acid or an extrinsic conjugated chromophore. Excitation in the ultraviolet spectral region of the aromatic amino acids phenylalanine (Phe; F), tyrosine (Tyr; Y), and tryptophan (Trp; W) can lead to the transfer of energy to a nearby Tb^{3+}, followed by luminescence of Tb^{3+}. The Tb^{3+} luminescence arises from transitions between the $^5D_4$ to $^7F_6$ states (490 nm) and the $^5D_4$ to $^7F_5$ states (545 nm) (Bhaumik and El-Sayed, 1965). Variables affecting the quantum yield of Tb^{3+} luminescence in calcium binding protein chelates include spectral overlap of donor emission with Tb^{3+} absorption, donor-Tb^{3+} distance, and the efficiency of the chelator to exclude water from the coordination sphere of Tb^{3+}.

The amino acid responsible for ET to bound Tb^{3+} can be determined from the excitation spectra when the Tb^{3+} emission is monitored. Pathways involving Phe can be ruled out when excitation is above 280 nm. Martin and Richardson (1979) suggested that Tb^{3+} luminescence enhancement arising from Trp ET would be greater than that from Phe or Tyr, because of the better spectral overlap of Trp fluorescence and Tb^{3+} absorbance in the region 300–400 nm. They also suggested that R_0 (distance of 50% probability of ET) would be at 5 to 10 Å between the donor and Tb^{3+}. Kleinerman (1969a,

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[1] The abbreviations used: ET, energy transfer; OM, rat hepatoma oncomodulin; brOM, bacterial recombinant oncomodulin; PV, parvalbumin; Φ, quantum yield; CaM, calmodulin; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EHPG, *N*,*N*′-ethylenebis(2-(2-hydroxyphenyl)glycine), known also as EDTA-*b*-HPA; apo, apoprotein; holo, holoprotein.

1969b) stated that between 5 and 10 Å, donor → Tb^{3+} pairs may undergo a variety of types of energy transfer, both Förster (1965) and Dexter (1953), in order to account for the shorter interaction range than observed with common organic ET pairs. Recent work with model peptides has suggested that short range (6 Å or less) Dexter energy transfer dominates Tb^{3+} luminescence enhancement and that position 7 of the 12-member binding loop is the optimum location for a Tb^{3+} ET donor. (MacManus *et al.*, 1990). Low quantum yields of chelated lanthanides are not necessarily the result of inefficient energy transfer, but are often caused by other quenching processes (Kleinerman, 1969a). For example, a direct correlation between the number of coordinating water molecules and the luminescence decay time of directly excited Tb^{3+} has been shown by Salama and Richardson (1980).

There have been many studies utilizing lanthanide binding to the EF hand protein family to obtain structural information on the effects of metal binding. Initial studies of parvalbumins (PV) showed that Phe in position 7 in the CD site metal binding loop enhanced Tb^{3+} luminescence by ET (Sowadski *et al.*, 1978; Nelson *et al.*, 1979; Rhee *et al.*, 1981; Miller *et al.*, 1980; Henzl *et al.*, 1986). Other parvalbumins with Tyr-48 (Eberspach *et al.*, 1988) or Trp-102 (Horrocks and Collier, 1981; Breen *et al.*, 1985a, 1985b) were also shown to be capable of enhancing Tb^{3+} luminescence by means of ET. The distance between these residues and the bound Tb^{3+} was stated to be consistent with the Förster ET model. X-ray structures of Tb^{3+}-loaded PV demonstrated accessibility of the Tb^{3+} to oxygen electron donors of the solvent external to the protein (Sowadski *et al.*, 1978). Studies of Eu^{3+} binding to OM and its mutants have been presented showing structural effects and metal binding affinities (Henzl *et al.*, 1986; Williams *et al.*, 1987; Henzl and Birnbaum, 1988; Hapak *et al.*, 1989; Treviño *et al.*, 1990, 1991; Palmisano *et al.*, 1990). In addition, other Ca^{2+} binding proteins and mutants have been investigated using Tb^{3+} luminescence enhancement.

The primary goal of this work is to develop an understanding of Tb^{3+} luminescence processes in this family of proteins using a subset of native and mutant proteins including oncomodulin, parvalbumin, and calmodulin. This set of proteins allows the examination of the structural basis of energy transfer between the fluorescent amino acids and Tb^{3+}. Tb^{3+} luminescence excitation spectra are able to reveal new insights into relationships between aromatic amino acids as only those responsible for Tb^{3+} ET are evident. Despite the wide variety of papers describing Tb^{3+} luminescence in biological systems, none offer a rank of sensitivity. Hence we present a ranking of sensitivity of Tb^{3+} luminescence bound to this subset of calcium binding proteins. This ranking can be interpreted in the light of the structural factors affecting luminescence sensitivity. Namely, in the case where two donor aromatic amino acids are the same, structural differences of donor-acceptor separation and chelation efficiency are revealed.

MATERIALS AND METHODS

Mutant proteins were prepared as previously reported (MacManus *et al.*, 1989; Hutnik *et al.*, 1990, 1991). For fluorescence spectra and titrations, lyophilized, purified proteins (MacManus *et al.*, 1989) were dissolved in a pH 6.5 buffer of 10 mM PIPES (Sigma), 100 mM KCl, prepared from Chelex 100-treated, distilled, deionized water. The proteins were diluted to a final *A* of 0.10 at 285 nm, as measured using a Varian DMS 200 UV spectrophotometer.

Fluorescence spectra and titrations were performed using a SLM 8000C spectrofluorimeter with a Neslab Endocal refrigerated bath at 20 °C. Emission spectra were taken with excitation and emission band passes of 4 nm. The excitation wavelength was chosen at 285 nm to minimize the overlap of second order diffraction (570 nm) with the Tb^{3+} emission at 545 nm. Any ET involving Phe can also be ruled out with 285 nm excitation. Emission spectra were corrected for the blank contribution and the instrument response and normalized to an *A* of 0.100 at 285 nm in a quartz cell of 1 cm pathlength. Excitation spectra were recorded at the fluorescence maximum of the intrinsic fluorophores Tyr (310 nm) and Trp (350 nm) and at the luminescent maxima of Tb^{3+} (545 nm). Excitation and emission spectra were automatically corrected for lamp intensity variations. Excitation spectra were taken with an excitation band pass of 4 nm and an emission band pass of 8 nm. A cutoff filter in the emission beam (Corning 3603-75C) was used to eliminate second order wavelength interference. Excitation spectra were corrected for the blank and normalized to 1.0 at the common excitation wavelength 285 nm.

Apoprotein was prepared by trichloroacetic acid precipitation (Haiech *et al.*, 1981; Hutnik *et al.*, 1991). A 1 M terbium stock was prepared from $TbCl_3$ (Aldrich) in distilled deionized water and kept at low pH by the addition of HCl. This stock was calibrated using an EDTA microtitration (Lyle and Rahman, 1963; Pribil, 1967; Williams *et al.*, 1987) using xylenol orange (Aldrich) as an indicator. Titration data were obtained by the addition of aliquots of $TbCl_3$ with continuous stirring (MacManus *et al.*, 1990).

Quantum yields of proteins with Tyr and Phe were calculated relative to the standard L-tyrosine (Aldrich) ($\Phi = 0.135$ (Chen, 1967)), freshly prepared in the deoxygenated pH 6.50 PIPES buffer at 20 °C and diluted to an *A* of 0.10 at 285 nm. Quantum yield of proteins with Trp were calculated relative to the standard *N*-acetyl-L-tryptophanamide (Aldrich) ($\Phi = 0.14$ (Eisinger, 1969)) in the same manner. A stock solution of quinine sulfate in 0.05 M sulfuric acid ($\Phi = 0.55$ (Chen, 1967)) was used to obtain the quantum yield of each protein-Tb^{3+} complex at the 490 nm emission of Tb^{3+}. No standards were suitable for overlapping with the 545 nm emission of Tb^{3+}, thus a solution of the complex of EHPG (Aldrich) and Tb^{3+} (from a 1:1 molal stock) was used. A relative yield of 1 was assigned to its 545 nm luminescence solely for the purpose of ranking 545 nm emission from these proteins (MacManus *et al.*, 1990). Spectra of each of the quantum yield standards were taken before each protein experiment. Protein concentrations were obtained with the bicinchoninic acid (BCA) protein assay (Pierce) using brOM as a standard and by amino acid analysis.

RESULTS AND DISCUSSION

Oncomodulin—OM has 2 tyrosines capable of enhancing Tb^{3+} luminescence. The fluorescence spectra and Tb^{3+} titrations of native rat hepatoma oncomodulin (OM) and the bacterial recombinant native oncomodulin (brOM) were identical under these conditions as they were for Hutnik *et al.* (1991). The addition of Tb^{3+} to Ca^{2+}·brOM caused quenching of the tyrosine fluorescence at 310 nm, and the characteristic Tb^{3+}- enhanced emissions at 490 and 545 nm were observed.

Fig. 1*A* shows the excitation spectra of the apo·, Ca^{2+}·, and Tb^{3+}·brOM complexes, normalized at 285 nm. Excitation spectra at 310 nm emission was similar to the absorption spectra of brOM that lead to Tyr-57 and Tyr-65 fluorescence. These include both the direct absorbance of Tyr and absorbance of Phe which transfers energy to Tyr. The Ca^{2+}·brOM 310 nm excitation spectra shows evidence of prominent Phe vibronic structure on the high energy side of the spectrum, which must originate from Phe→Tyr-65[2] and/or Phe→Tyr-57 ET. The apo·brOM excitation spectra shows 15% less Phe vibronic fine structure at 260 nm, suggesting reduced Phe→Tyr-65 and/or Phe→Tyr-57 ET. In the presence of Tb^{3+}, there is 17% less Phe vibronic fine structure when compared with the Ca^{2+}·brOM spectra at 260 nm, suggesting less Phe→Tyr-65 and/or Phe→Tyr-57 ET. The excitation spectra of the Tb^{3+} 545 nm emission gives the absorbance of the Tb^{3+}·brOM complex that leads to Tb^{3+} luminescence. However, this spectrum shows the largest Phe contribution in the high energy

[2] The notation "A→B" is read as follows: A or B represents the IUPAC-IUB-established one-letter amino acid code or elemental symbol. A is the species which is absorbing the principal excitation beam. B is the species which accepts energy transfer from A, resulting in the excitation of B. The arrow represents the direction of energy transfer.

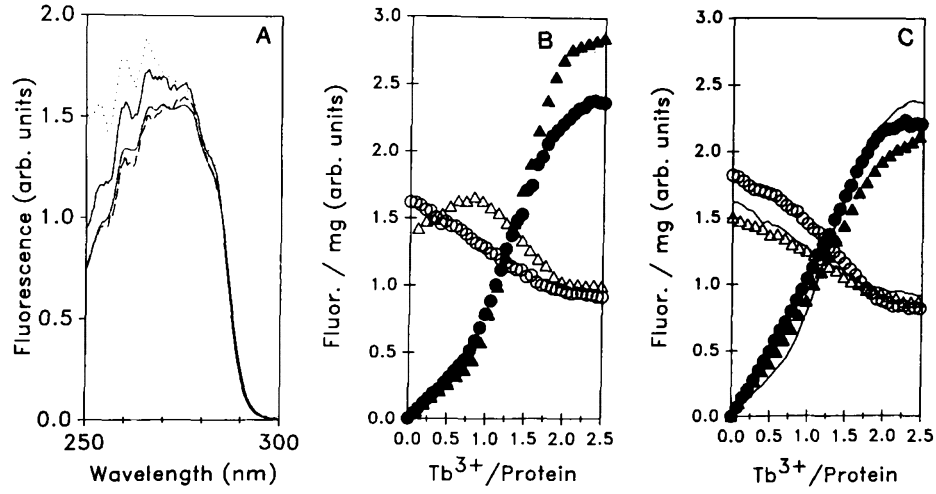

FIG. 1. **Excitation spectra and Tb^{3+} titrations of oncomodulin and mutants of the CD loop.** Samples were treated as described under "Materials and Methods." *Panel A*, excitation spectra of brOM normalized at 285 nm: ——, Ca^{2+}, 310 nm emission; - - -, apo, 310 nm emission; — · —, $+Tb^{3+}$, 310 nm emission; · · · ·, $+Tb^{3+}$, 545 nm emission. In *panels B* and *C*, *filled symbols* are data at 545 nm emission, *open symbols* at 310 nm emission. *B*, Tb^{3+} titration of holo· (○, ●) and apo·brOM (▲, △). *C*, Tb^{3+} titration of D59E (○, ●) and L58I (▲, △) brOM mutants. —— represents data from brOM titration.

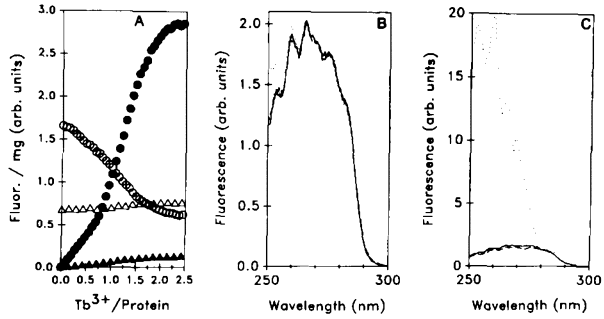

FIG. 2. **Excitation spectra and Tb^{3+} titrations of Y57F and Y65F oncomodulin.** *A, filled symbols* are data at 545 nm emission, *open symbols* at 310 nm emission. Titration of holo·Y65F (○, ●) and holo·Y57F (▲, △). *Panels B* and *C* are excitation specra of Y65F and Y57F oncomodulin, respectively. Excitation spectra are normalized at 285 nm. ——, Ca^{2+}, 310 nm emission; - - -, $+Tb^{3+}$, 310 nm emission; · · · ·, $+Tb^{3+}$, 545 nm emission.

region from 260–275 nm, 14% more than holo·brOM at 260 nm. The excitation spectra of the mutant Y65F (herein, Fig. 2*B*) at 310 nm has a similar shape, suggesting that some Tb^{3+} luminescence enhancement must occur by Phe→Tyr-57→ Tb^{3+} ET, but a particular Phe is not implicated.

The titration of brOM with Tb^{3+} (Fig. 1*B*) contrasted with those reported by Henzl *et al.* (1986), who showed a titration curve but with a smaller number of data points. Here, with more points, both the apo· and Ca^{2+}·brOM titrations showed a strong biphasic response upon the addition of Tb^{3+}. Changes in the curve corresponded to 1 and 2 eq of Tb^{3+} filling the EF and CD sites sequentially. The titration of the apo·brOM was different from the titration of the Ca^{2+}·brOM. The titration of apo·brOM resulted in 23% more Tb^{3+} luminescence compared to that of Ca^{2+}·brOM after the addition of 2 eq of Tb^{3+}. Ca^{2+} competition with Tb^{3+} in the CD site could cause this difference in Tb^{3+} luminescence.

While the Tb^{3+} emission in the Ca^{2+}·brOM titration was strongly biphasic, the decrease in Tyr fluorescence at 310 nm was not. In the case of apo·brOM, the 310 nm fluorescence first increased by 17% during the filling of the first site, then decreased by 39% upon filling the second site. The increase in Tb^{3+} luminescence can be attributed to ET from the aromatic donor, suggesting Tyr should be quenched in the Tb^{3+} titration. However, this result was consistent with results of a Ca^{2+} titration of apo·brOM and apo·Y65F (Hutnik *et al.*, 1991), where the Tyr-57 fluorescence increased as the first site (EF) was filled. The enhancement of Tyr fluorescence when the empty EF site fills with Tb^{3+} is obviously greater than the quenching of Tyr due to ET to Tb^{3+}. This result can only be accounted for by a conformational change as the empty EF site fills, putting Tyr-57 in a more fluorescent conformation. Titration of the holo·brOM does not show this change, presumably because Ca^{2+} is present and Tyr-57 is already in the conformation caused by metal binding.

Nonaromatic Mutations in Oncomodulin's CD Loop—Using site-directed mutagenesis, we have examined the effects of changing the molecular environment of the metal binding CD loop of oncomodulin. For example, the OM mutant D59E[3] has been shown to make the CD site of OM more parvalbumin-like (MacManus *et al.*, 1989; Hapak *et al.*, 1989; Palmisano *et al.*, 1990; Golden *et al.*, 1989). Therefore, the Tb^{3+} luminescence enhancement of this mutant was investigated. The OM mutant L58I was prepared in order to investigate the contribution to the CD site Ca^{2+} specificity by Ile-58 (Williams *et al.*, 1987) as this position is involved in 2 intersite hydrogen bonds (Ahmed *et al.*, 1990).

The ability of the D59E and L58I mutants to bind Tb^{3+} and enhance its luminescence is shown in Fig. 1*C*. Upon addition of Tb^{3+}, both exhibited a decrease in tyrosine fluorescence at 310 nm and an increase in luminescence at 490 and 545 nm. The Tb^{3+} titrations of these proteins (Fig. 1*C*) were different from those of Ca^{2+}·brOM in that the D59E Tb^{3+} luminescence titration at 545 nm was less biphasic than brOM. Hapak *et al.* (1989) have shown that Eu^{3+} fills the EF site of D59E first and the CD site second. Quenching of Tyr fluorescence at 310 nm in D59E was biphasic. Tyr was quenched more efficiently as the loop in which it is situated, the CD loop, was filled. This indicated that D59E was filled in the same sequential manner with Tb^{3+} as with Eu^{3+}. The titration of Tb^{3+} luminescence shows 27% more luminescence from 1 eq of Tb^{3+} in D59E than in the corresponding loop in brOM, despite having the same ET donors. If the D59E substitution gave better coordination efficiency for the CD site, then the difference between its titration and that of brOM would be expected to be entirely in the CD site Tb^{3+} luminescence, between 1 and 2 eq. There was 3% less Tb^{3+} luminescence at 2:1 from D59E than from brOM.

The decrease in Tyr fluorescence at 310 nm of L58I was similar to that of brOM, being slightly biphasic, indicating

[3] The notation "letter-number-letter" is read as follows: the first letter represents the IUPAC-IUB-established one-letter amino acid code of the native residue, the number identifies the position of the native residue, and hence the position of the point mutation, and the second letter is the code for the amino acid which is being substituted by mutagenesis in place of the native residue.

sequential binding. Like D59E, the Tb^{3+} luminescence enhancement is greater in the EF loop of L58I (13% at 1:1) compared to brOM, but it is also 12% less in the CD loop at 2:1. This could be explained by a shift of position of the donor Tyr-57 toward the EF loop. Treviño *et al.* (1991) have reported that the CD site Ca^{2+} affinity of L58I is reduced. A reduction in Tb^{3+} chelation with competing Ca^{2+} could also account for the differences in Tb^{3+} luminescence compared to brOM. The changes in the titrations of both D59E and L58I suggest that CD site residue changes may affect the EF site. In both mutants, the data may be rationalized by suggesting that Tyr-57 is displaced closer to the EF site and further from the CD site. It is not possible to identify which of these is displaced relative to brOM.

Phe Replacement of Oncomodulin Tyr-57 and Tyr-65—Upon excitation above 280 nm, the energy transfer from tyrosine is limited to Tyr-57 in Y65F and Tyr-65 in Y57F. With these mutants, the contribution of the individual tyrosines on the ET process could be studied. Of the mutants Y65F and Y57F, the titrations (Fig. 2*A*) showed that most of the enhanced emission of Tb^{3+} was due to ET from Tyr-57. The addition of Tb^{3+} to Y65F results in 22% more Tb^{3+} luminescence than with brOM, with the larger change occurring in the filling of the second (CD) site. This increase comes *despite* the loss of ET donation from Tyr-65. The replacement of the D-helix Tyr-65 with Phe either changes the position of Tyr-57 or makes the loop chelate more efficiently, causing this increase. This location is at a well characterized kink in the D-helix of OM and PV structures (Strynadka and James, 1989). Replacing Tyr, a helix-breaker, with Phe, a helix-former (Fasman, 1987), could modify the structure of this kink and affect the CD site causing an increase in Tb^{3+} luminescence.

The titration of Y57F at 285 nm shows Tb^{3+} luminescence from Tyr-65 to Tb^{3+} only amounts to 5.5% that of the total luminescence from brOM, hence little energy transfer occurs between Tyr-65 and Tb^{3+} in either site. By contrast, the titration of Y57F when excited at 260 nm is shown in Fig. 5*C*, where a clearly biphasic response of Tb^{3+} is seen. Hence the Phe-57 ET donation is sufficient to show the sequential filling of Tb^{3+}.

The fluorescence from the Ca^{2+}·Y57F at 310 nm (Fig. 2*A*) was less than half that of Y65F, indicating that Tyr-65 is highly quenched (Hutnik *et al.*, 1991). This increased slightly as the titration proceeds, indicating Tyr-65 is in a slightly more fluorescent conformation in the Tb^{3+}-bound Y57F than the Ca^{2+}-bound form. The excitation spectra of the Y65F mutant (Fig. 2*B*) were similar to the Tb^{3+} excitation spectrum of brOM at 545 nm (Fig. 1*A*) and showed no change in the Phe contribution to the 310 nm emission of Y65F after the addition of Tb^{3+}. This indicated that the Phe→Tyr-57 energy transfer is the same in the Ca^{2+} or Tb^{3+} forms. The 545 nm excitation spectrum is very similar to the 310 nm excitation spectrum, but shows 4% more Phe contribution at 260 nm. This 4% would represent the contribution of direct Phe→ Tb^{3+} energy transfer.

The normalized excitation spectra of the Ca^{2+}·Y57F mutant (Fig. 2*C*) fluorescence at 310 nm has a predominant Tyr shape, with some Phe fine structure. The 545 nm excitation spectra, however, shows the predominant contribution of ET from Phe-57 to the luminescence enhancement of Tb^{3+}. MacManus *et al.* (1990) found that a Phe donor in position 7 of a calcium binding peptide loop would dominate the excitation spectra, despite other nearby Tyr residues. The excitation spectra from 310 nm emission after Tb^{3+} addition is 10% less at 260 nm, clearly evident on the spectra with an expanded scale. This could be interpreted as less Phe→Tyr-65 energy transfer. But the normalization of the spectra at 285 nm can make an increase in Tyr fluorescence look like a decrease in Phe→Tyr ET. The titration end point at 285 nm excitation shows a 13% increase in Tyr-65 fluorescence. This suggests that the change seen in the brOM 310 nm excitation spectra after Tb^{3+} addition (Fig. 1*A*) is also due to an increase in Tyr-65 fluorescence. The result from these two mutants shows that the principal ET donor of OM for Tb^{3+} luminescence is Tyr-57 (CD loop position 7). They also show that Tb^{3+} binding induces a conformation that differs somewhat from that of the Ca^{2+} form.

Tryptophan in Oncomodulin CD and EF Loops—If Förster energy tranfer invovling the singlet state of the donor applies, a Trp ET donor is expected to increase the sensitivity of the Tb^{3+} luminescence over that of Tyr. This would be owing to an improved spectral overlap of Trp fluorescence and Tb^{3+} absorbance, as well as a higher extinction coefficient of Trp (Martin and Richardson, 1979). The effect of introducing Trp in binding loop position 7 in each of the CD and EF sites of OM was examined with the mutants Y57W and K96W. Trp has not been found at loop position 7 in a natural EF hand Ca^{2+} binding protein studied to date (Marsden *et al.*, 1990). In the case of K96W, the mutation provided an EF site Trp in addition to the CD site Tyr. Differences in the excitation and emission spectra of these 2 Trps, Y57W and K96W, suggest they are in different environments (Hutnik *et al.*, 1991). Based on the crystal structure of OM, Trp-57 would be within 3–4 Å of Lys-96.

The titrations of these two proteins with Tb^{3+} (Fig. 3*A*) indicate they enhance Tb^{3+} luminescence more than OM mutants with tyrosine ET donors. Y57W showed a distinct biphasic response to the addition of Tb^{3+}. The shape was similar to that of brOM, in that filling the first site provided less Tb^{3+} luminescence (13% of total) than the filling of the second site. The titration of apo·Y57W showed an increase in Tb^{3+} emission over Ca^{2+}·Y57W, similar to the increase seen in apo·brOM over Ca^{2+}·brOM. Again, this can be attributed to CD site competition with Ca^{2+} in the holoprotein.

In contrast, the K96W Tb^{3+} titration showed a greater increase in Tb^{3+} luminescence (69% of total) when the first site was filled. This was different from any of the other single residue OM mutants. Trp, in the EF loop position 7, should donate energy more efficiently to the Tb^{3+} occupying the same loop. Hence, the titration of this OM mutant confirms the fact that Tb^{3+} fills the EF site first and the CD site second,

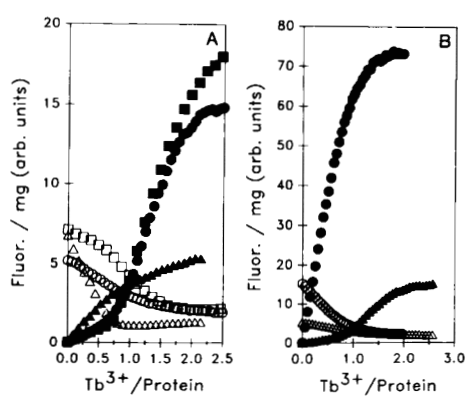

FIG. 3. **Tb^{3+} titrations of oncomodulin mutants with Trp in site 7 of a metal binding loop.** In *panels A* and *B*, *filled symbols* are data at 545 nm emission, *open symbols* at 350 nm emission. *A*, titration of apo·Y57W (□, ■), holo·Y57W (○, ●), and holo·K96W (▲, △). *B*, titration of holo·CDOM33 (○, ●) and holo·Y57W (▲, △) brOM mutants.

similar to Eu^{3+} (Hapak *et al.*, 1989).

The levels of Tb^{3+} luminescence enhancement of these two mutants were notably different. Although K96W has two ET donors (Tyr-57 and Trp-96) in the vicinity of the Tb^{3+}, the Tb^{3+} luminescence is one-third that of Y57W. This substitution of Lys-96 by Trp results in a different structural relationship with the two binding loops, allowing deactivation of the excited state to occur. Furthermore, the Tyr-57 undergoes ET to a low fluorescent Trp-96 (Hutnik *et al.*, 1991) which would compete with ET to Tb^{3+}.

The decrease in Trp fluorescence demonstrates the fluorescence behavior of the ET as the titration proceeds. The Ca^{2+}-Y57W 350 nm fluorescence decreased by two-thirds when the first site bound Tb^{3+}, and by one-third when the second site fills. But this was not proportional to the increased Tb^{3+} luminescence. Filling the EF (first) site with Tb^{3+} must cause a conformational change such that the fluorescence of Trp-57 is quenched in addition to that due to Tb^{3+} ET alone. The apo-Y57W response showed an even greater decrease of Trp-57 fluorescence upon filling the EF site. A similar quenching was seen by Hutnik *et al.* (1991) on titration of apo-Y57W with Ca^{2+}. The K96W mutant Trp fluorescence is markedly quenched as the EF site filled with Tb^{3+}, but it is not possible to determine if quenching in this site is greater than that due to ET alone. After the Trp fluorescence reaches its lowest point at a 1:1 ratio, there is a reproducible small increase at 2:1. This suggests a conformational change in Trp-96 as the CD site fills with Tb^{3+}, which makes Trp-96 more fluorescent despite ET to Tb^{3+}.

Cassette Mutant Involving the CD Loop of Oncomodulin—A cassette mutant was constructed, CDOM33, in which the CD loop of OM was replaced with the sequence DKNADGWIEFEE. This sequence has Trp in position 7 of the binding loop (amino acid position 57) as well as CD site ligands based on a consensus sequence of over 200 calcium binding proteins. This sequence is based on peptide 33 of a study of model peptide loops (MacManus *et al.*, 1990) shown to enhance Tb^{3+} luminescence emission. In general, peptide binding loops have much lower affinities than the corresponding protein loop (Marsden *et al.*, 1990). This peptide was chosen for cassette mutagenesis because it gave the highest Tb^{3+} luminescence enhancement of the set of single Trp peptides in that study. It was also of interest to show how the protein environment of the same loop affected the Tb^{3+} luminescence intensity.

CDOM33 can be compared to Y57W, which has the ET donor in the same position of the metal binding loop. The fluorescence of Trp in Ca^{2+}-CDOM33 protein is 3-fold greater than that in Ca^{2+}-Y57W suggesting major structural differences which result in a decrease in non-radiative deactivation processes. Interestingly, Trp-57 in both proteins is quenched to the same level after Tb^{3+} is added (Fig. 3*B*). The Tb^{3+} emission intensity of CDOM33 was the largest of all proteins in this study. The 545 nm emission at the Tb^{3+} titration end point is 5-fold greater than that of Tb^{3+}-Y57W. Importantly, the largest increase in Tb^{3+} emission was seen as the first site filled (86% of total). Since this increase is assigned to the CD loop where the Trp donor is located, the affinity of the CD site was significantly increased above that of the original EF site. The enhancement of Tb^{3+} luminescence in CDOM33 over Y57W must be due to a tighter coordination of the metal in the new CD site, eliminating quenching and bringing the Trp-57 closer to the bound Tb^{3+}.

Tryptophan Mutants Outside the Binding Loops—Mutants with Trp in other positions, not located in a binding loop, were also examined for their potential to enhance Tb^{3+} luminescence. According to the crystal structure (Ahmed *et al.*, 1990) and previous fluorescence work, OM mutant Y65W has an exposed Trp (Hutnik *et al.*, 1991), and F102W has a buried Trp, in a position analogous to Trp-102 in Cod III PV (Hutnik *et al.*, 1990).

Excitation spectra of the F102W mutant (Fig. 4*A*) shows the previously reported predominant Trp contribution to the emission at 350 nm. In sharp contrast, the excitation spectrum from the Tb^{3+} emission at 545 nm resembles the absorption spectrum of Tyr. No Phe fine structure was seen in the 545 nm excitation, suggesting the direct Phe→Tb^{3+} and the indirect Phe→Tyr-57→Tb^{3+} ET pathways did not occur as in brOM and the previous mutants. This could suggest that the OM Phe-102 is responsible for the phenylalanine enhanced Tb^{3+} luminescence in brOM. However, the blue edge of the 350 nm excitation spectra shows that Trp-102 is acting as a Phe ET acceptor, which would diminish any ET to Tb^{3+} from another Phe residue.

Excitation spectra of Y65W (Fig. 4*B*) show a Trp-dominated 350 nm excitation spectrum, with a small shoulder at 292 nm. The 545 nm Tb^{3+} spectrum has an overwhelming Tyr component, implicating Tyr-57 as the dominant ET donor to Tb^{3+} in Y65W. Phe fine structure between 250 and 280 nm is seen in the Y65W excitation spectra at 545 nm emission (Fig. 4*B*), but not in that of F102W (Fig. 4*A*). This suggests that some Phe→Tb^{3+} or Phe→Tyr-57→Tb^{3+} energy transfer occurs in Y65W, but not in F102W.

The Tb^{3+} titrations (Fig. 4*C*) of these mutants show sequential filling and that the additional Trp outside the binding loops does not greatly enhance Tb^{3+} luminescence output over that of brOM. In fact, the F102W mutant had less Tb^{3+} luminescence than brOM. In F102W, Tyr-57→Trp-102 and Phe→Trp-102 processes compete with Tb^{3+} for ET causing a reduced Tb^{3+} luminescence output in this mutant, compared to brOM. In Y65W, Trp-65 does not reduce the Tb^{3+} luminescence over brOM, suggesting that Phe→Trp-65 is not sufficient to compete with Phe→Tb^{3+} and Tyr-57→Tb^{3+}. The Y65W mutant had a small increase in Tb^{3+} luminescence over brOM, suggesting some contribution by the Trp-65. This is contrary to the excitation spectra at the 545 nm emission. It is possible that the Trp-65 mutation affects the CD loop by a conformational difference, accounting for the small increase in Tb^{3+} luminescence. This would parallel the increase caused by the Phe-65 mutation in Y65F. Substitution of Trp in the kink region of the D helix could modify the structure in this region.

The Trp fluorescence of F102W had a small increase during

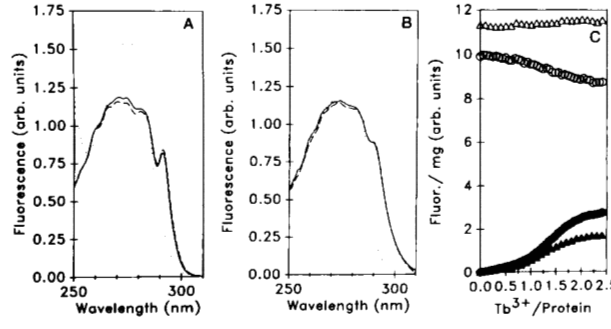

FIG. 4. **Excitation spectra and Tb^{3+} titrations of oncomodulin mutants with Trp outside metal binding loops.** *Panels A* and *B* are excitation specra of F102W and Y65F oncomodulin, respectively. Excitation spectra are normalized at 285 nm. ——, Ca^{2+}, 350 nm emission; – – –, $+Tb^{3+}$, 350 nm emission; ····, $+Tb^{3+}$, 545 nm emission. *Panel C, filled symbols* are data at 545 nm emission, *open symbols* at 350 nm emission. Tb^{3+} titration of holo·Y65W OM (○, ●) and holo·F102W OM (▲, △).

the addition of Tb^{3+}, suggesting a conformational change in the core of the protein.

Cod III Parvalbumin—The Cod III parvalbumin has Trp in position 102, homologous to the F102W OM mutant (Hutnik *et al.*, 1990). It has a Phe in position 57, rather than Tyr. The excitation spectra of PV at 350 nm emission (Fig. 5*A*) has a prominent shoulder at 291 nm, with little change after the addition of Tb^{3+}. The excitation spectra at 545 nm emission indicate a prominent Phe contribution to Tb^{3+} luminescence enhancement, along with a contribution by Trp. This implies that the Trp-102 in PV contributes more to Tb^{3+} luminescence than the Trp-102 in the OM mutant F102W. The fact that the PV tryptophan transition at 291 nm is more prominent may suggest there is a difference in orientation of these 2 residues to the metal binding loops. However, the tyrosine component of the F102W spectrum may simply mask a weak Trp contribution at 291 nm. The 2 Tyr residues of OM would need to be removed by selective mutations to make a proper comparison.

The Tb^{3+} titration of PV at 285 nm excitation (Fig. 5*B*) shows the contribution of PV Trp-102 to Tb^{3+} luminescence. This is shown compared to the titration of F102W and brOM. The PV titration is clearly linear, with a sharp plateau. F102W OM provides a greater Tb^{3+} luminescence enhancement at 285 nm excitation than PV, due to the presence of Tyr-57. The titration of PV at 260 nm excitation results in a more intense Tb^{3+} signal (Fig. 5*C*), because Phe is the most efficient donor. This titration can be compared to that of the OM mutant Y57F, which has no efficient Tyr→Tb^{3+} ET. Both have Phe in position 57. The titration of PV shows a linear response, but the titration of Y57F shows a biphasic one. The corrected Trp fluorescence of PV *increases* slightly as Tb^{3+} is added (data not shown), similar to the OM mutant F102W, in contrast to the data of Horrocks and Collier (1981) in which it decreased slightly.

The comparison of the Tb^{3+} titrations of F102W at 285 nm and Y57F at 260 nm to that of PV illustrate the differences in metal binding of these proteins. The sequential filling of sites by Tb^{3+} in the presence of Ca^{2+} is clearly distinguished by either Trp or Phe reporters in the OM mutants. Sequential filling of Cod III PV by these same ET donors was not evident under the same conditions, suggesting similar affinities of the EF and CD sites for Tb^{3+}.

Tryptophan in the Loops of Calmodulin—In this study, mutants of calmodulin, VU-9 (F99W SYNCAM CaM) (Kilhoffer *et al.*, 1988), and VU-32 (T26W SYNCAM CaM) were compared. These proteins have four metal binding loops and should chelate four Tb^{3+}. VU-9 has Trp in loop position 7 of the domain III, and VU-32 has Trp in loop position 7 of domain I. Each Tb^{3+} may contribute to the overall luminescence intensity, depending on its proximity to a suitable donor. Since both VU-9 and VU-32 have a single Trp in a loop position 7 at one end of the protein, and no energy donor in the other end, two of the sites will make only a very small contribution to luminescence intensity. The Tb^{3+} effectively probes 2 of the 4 metal binding sites, hence they are comparable in terms of luminescence intensity to the OM mutants described here.

The Tb^{3+} titration of VU-9 (Fig. 6*A*) were similar to those published earlier (Kilhoffer *et al.*, 1988). However, the largest increase in Tb^{3+} luminescence enhancement of apo·VU-9 occurred over the Tb^{3+}/protein ratio of 4:1 rather than the published 6:1. Titrations of CaM with Tb^{3+} or Eu^{3+} have shown ratios ranging from 4:1 at pH 6.5 (Wallace *et al.*, 1982), 5:1 at pH 7 (Mulqueen *et al.*, 1985), and 6:1 at pH 7.5 (Craig *et al.*, 1987; Kilhoffer *et al.*, 1988). This range of values may suggest that the number or affinity of lanthanide binding sites of CaM is pH-dependent. Alternatively, it may be the result of inconsistencies in protein determination. Nonetheless, this variation indicates some caution needs to be exercised. Tb^{3+} titrations are now being cited as a means of quantifying calcium binding proteins due to the high affinities and stoichiometric filling (Treviño *et al.*, 1990, 1991). The lanthanide titration end point can be a more precise indication of concentration of these proteins (in the apo state) than a commercial protein assay.

The largest change in Tb^{3+} luminescence occurred between a ratio of 3:1 to 5:1 for Ca^{2+}·VU-9 and 2:1 to 4:1 for apo·VU-9. Ca^{2+}·VU-9 was filled at a 5:1 ratio, with most Tb^{3+} emission occurring when the last site was filled. Apo·VU-9 was filled at 4:1, with the majority of Tb^{3+} luminescence arising from filling the third binding site and less from filling of the fourth site. This indicates that in the absence of Ca^{2+}, Tb^{3+} fills domain III as the third site as described previously (Buccigross and Nelson, 1986). In the presence of Ca^{2+}, however, Tb^{3+} fills domain III as the fourth site. Hence, Ca^{2+} affinity for site III is significantly greater than for site IV.

The titration of VU-32 with Tb^{3+} (Fig. 6*B*) shows the result of placing the ET donor in domain I of CaM. The Ca^{2+}·VU-32 titration shows the largest change in Tb^{3+} emission coming during the fill of the second site. The apo·VU-32 Tb^{3+} titration shows a linear increase in Tb^{3+} emission while filling both the first and second sites. This result shows that apo·VU-32 binds Tb^{3+} to domains I and II with comparable

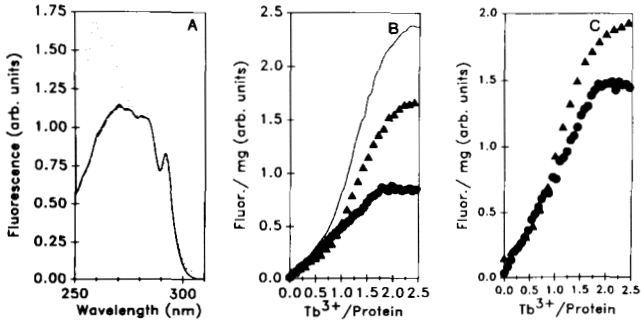

FIG. 5. **Excitation spectra and Tb^{3+} titrations of Cod III parvalbumin compared to oncomodulin mutants.** *Panel A*, excitation spectra of Cod III PV normalized at 285 nm. ——, Ca^{2+}, 350 nm emission; – – –, +Tb^{3+}, 350 nm emission; ····, +Tb^{3+}, 545 nm emission. *Panel B*, comparison of Tb^{3+} titrations of holo-PV (●), holo-F102W OM (▲), and holo-brOM (——) at 285 nm excitation, 545 nm emission. *Panel C*, comparison of Tb^{3+} titrations of holo-PV (●) and holo-Y57F (▲) OM at 260 nm excitaion, 545 nm emission.

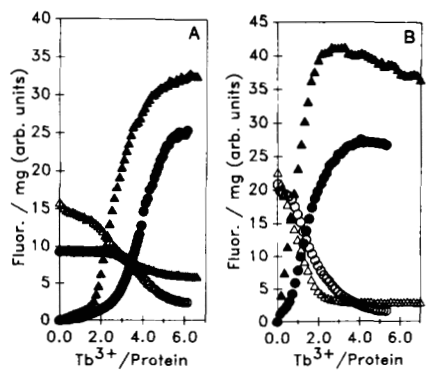

FIG. 6. **Tb^{3+} titrations of calmodulin mutants with Trp in site 7 of a metal binding loop.** In *panels A* and *B*, *filled symbols* are data at 545 nm emission, *open symbols* at 350 nm emission. *A*, Tb^{3+} titration of holo·VU-9 (○, ●) and apo·VU-9 (▲, △) SYNCAM mutants. *B*, Tb^{3+} titration of holo·VU-32 (○, ●) and apo·VU-32 (▲, △) SYNCAM mutants.

affinities. In the presence of Ca^{2+}, however, VU-32 binds Tb^{3+} in domain II first, domain I second; hence, the Ca^{2+} binding affinities are not equivalent.

A decrease in apo·VU-32 Tb^{3+} emission occurs after the first two sites are filled. From the 350 nm emission we see that the maximum Trp emission quenching occurs after 2 eq of Tb^{3+} have been added to apo·VU-32 and does not change further. Thus, further quenching of Trp was not causing the decrease in Tb^{3+} emission past 2 eq of Tb^{3+}. This decrease must be due to a conformational change of apo·VU-32 that decreases Tb^{3+} emission either by reducing the coordination efficiency of Tb^{3+} in domain I or by increasing the distance between Trp-26 and Tb^{3+} in domain I. This conformational change is not seen in Ca^{2+}·VU-32, probably because it is already in such a conformation due to bound Ca^{2+}. Simultaneous monitoring of scattering intensity did not reveal any precipitation that might otherwise cause such a decrease.

The picture of Tb^{3+} binding to VU-9 and VU-32 CaM suggests that the order of fill of apo·CaM by Tb^{3+} is domains I and II (simultaneously), then domain III, then IV. The order of fill of Ca^{2+}·CaM by Tb^{3+} is domain I, II, IV, then finally III. The differences in these orders must be due to conformational differences between apo· and Ca^{2+}·CaM or affinity differences between Ca^{2+} and Tb^{3+}.

Ranking of Calcium Binding Proteins by Tb^{3+} Sensitivity— Central to this study is the ranking of the proteins in their Ca^{2+}-bound form for Tb^{3+} luminescence efficiency (Fig. 7). This is the product of the extinction coefficient at 285 nm and the quantum yield at 490 nm relative to quinine sulfate, and at 545 nm relative to an EHPG·Tb^{3+} complex as detailed under "Materials and Methods." The proteins with Trp in loop position 7 were the most efficient.

Cod III PV and F102W OM both have Trp in a homologous position within the hydrophobic core. F102W was expected to have a Tb^{3+} luminescence sensitivity equal to the sum of the sensitivities of PV (1 Trp, position 102) and brOM (2 Tyr, positions 65 and 57). In fact, it was the least efficient of all the proteins with a Tyr in position 57. This result, together with spectral evidence, shows that Trp-102 acts as a "sink" for ET, robbing Tb^{3+} of energy transfer from Tyr or Phe. This result is of consequence, indicating that energy transfer processes can easily work *against* Tb^{3+} luminescence enhancement.

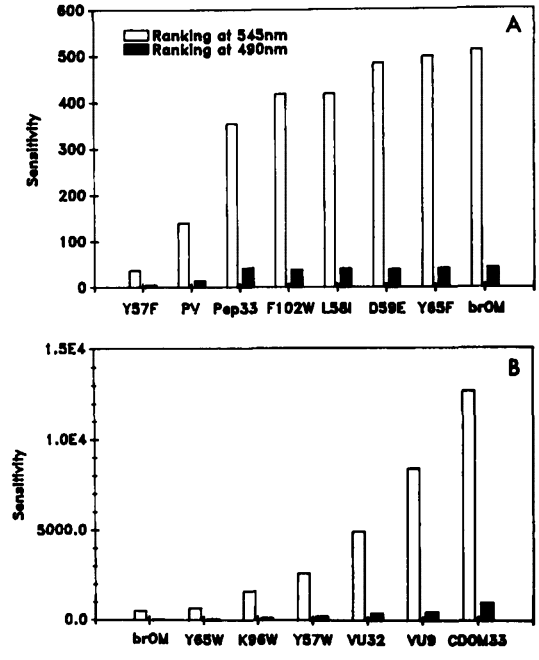

FIG. 7. **Ranking of sensitivity of Tb^{3+} luminescence of metal binding proteins.** *Open bars* are sensitivity (product of extinction coefficient at 285 nm and relative luminescence yield) at the Tb^{3+} luminescent emission at 545 nm emission, *closed bars* at 490 nm emission. Peptide 33 from MacManus *et al.* (1990) was included as a comparison with previous work.

The sensitivity of the most efficient synthetic peptide (peptide 33) of the previous work is *very interesting* when compared against this field of intact proteins. While it has the same energy donor and ligands as CDOM33, it is less sensitive than most of the proteins shown here. CDOM33 provides 37.5 times more Tb^{3+} luminescence than peptide 33. It is possible to extract from Fig. 3*B* that the CD loop in the protein contributes 86% of the total Tb^{3+} luminescence. This reduces the sensitivity difference such that the CD loop in CDOM33 is 32 times more luminescent with Tb^{3+} than the same loop as a 14-mer synthetic peptide. The inherent stability and conformation of metal binding loops in these proteins must account for the difference. Since the only luminescence variable that differs between these two is quenching as a result of inefficient coordination, the constrained protein binding loop must provide more efficient coordination to Tb^{3+}. Therefore, water molecules would have fewer opportunities to enter the Tb^{3+} coordination sphere in the protein loop.

CONCLUSIONS

Data from Tb^{3+} luminescence studies of calcium binding proteins can reveal their conformational differences and similarities. A Tb^{3+} binding protein possessing good donor-acceptor overlap integral and short donor-acceptor distance would have a high Tb^{3+} luminescence output providing that the coordination efficiency was suitable for excluding quenching moieties. The mutant and wild-type calcium binding proteins examined here show a wide range of Tb^{3+} luminescence levels.

The mutants Y57F and Y65F help explain the changes in Tb^{3+} luminescence that occur in OM. Tyr-57 is responsible for 95% of the ET to the two bound Tb^{3+} in OM. The luminescence of the Tb^{3+} occupying the CD site was greater than that of Tb^{3+} occupying the EF site due to the position of the donor Tyr-57 in the CD site. OM mutants with a Trp outside a binding loop and with Tyr in CD loop position 7 have a similar Tb^{3+} luminescence sensitivity, regardless of the position of Trp. In these cases, Tyr-57 provided the predominant portion of Tb^{3+} enhancement as demonstrated by excitation spectra. Further, the excitation spectra of Cod III PV, F102W, and Y65W demonstrate that a donor residue in position 7 of a metal binding loop will dominate the ET process to Tb^{3+}. Even a Phe can dominate over a Trp, as seen by Cod III PV. This information would suggest to other workers that it is prudent to run excitation spectra before attempts at titration, in order to take advantage of the most sensitive Tb^{3+} donor.

Previous work with peptides suggests that Trp in the Ca^{2+} binding loop position 7 should give the best Tb^{3+} luminescence enhancement, owing to the combination of spectral overlap and donor-acceptor distance. The Y57W mutant showed a 5.1-fold increase in Tb^{3+} luminescence sensitivity over brOM. At 285 nm, Trp has an extinction coefficient 6.8-fold larger than Tyr. This suggests that there was no gain owing to better donor fluorescence overlap with Tb^{3+} absorbance, contradicting our expectations. It is possible that the Trp in loop position 7 causes a reduction in chelation efficiency, leading to other deactivation processes which might offset any gain from a better spectral overlap. Alternatively, this might imply that the fluorescence spectral overlap with Tb^{3+} is not an

important determinant, which would favor a triplet state mechanism of ET.

The K96W mutant, with 1 Trp and 2 Tyr, was expected to have higher sensitivity than Y57W, due to the presence of an aromatic donor in each of the binding loops. However, its Tb^{3+} sensitivity was less than Y57W. The close proximity of Trp-96 and Tyr-57 in K96W might lead to destabilizing interactions in the two sites that increase accessibility to quenchers of Tb^{3+}. In addition, Tyr-57→Trp-96 ET and the low fluorescence efficiency of Trp-96 processes may decrease the Tb^{3+} luminescence enhancement. Although Tyr and Phe appear in many natural EF hand loops, there are no paired EF hand loops with aromatics in ligand position 7 of *both* loops (Marsden *et al.*, 1990).

The difference in sensitivity between Y57W and CDOM33 is striking, since they both have the same ET donors in position 57. The difference between these two mutants demonstrates the importance of coordination efficiency for Tb^{3+} luminescence emission. The replacement of the OM CD site residues DNDQSGYLDGDE with DKNADGWIEFEE *completely reverses* the order of fill of Tb^{3+} as detected by the Trp donor in the CD site. The relative affinity of the two sites in oncomodulin has been reversed so that the new CD site has a higher affinity than the existing EF site.

Results of this work caution strongly against the use of the Förster equation in approximating donor-lanthanide distances in biological systems. The Förster equation should only be used when distances of 10 Å or greater separate donor and acceptor (Birks, 1970). For CDOM33, the center-to-center donor-acceptor distance computed using the Förster equation for Trp and Tb^{3+} was 2.24 Å ± 12%, which would have the two superimposed. Förster theory *underestimates* distances when it is applied to cases of Dexter electron-exchange and triplet-triplet ET (Turro, 1978; Lamdla and Turro, 1969). The Dexter process is distance-dependent as r^{-n} where $n > 6$, accounting for a shorter range of interaction. The F102W mutant described here demonstrates that a long range Förster Tyr→Trp ET (11–15 Å) can compete with a short range Tyr→Tb^{3+} ET (5–6 Å) process.

In the case of CDOM33, the replacement of the OM CD loop with one based on Tb^{3+} luminescence enhancement of model peptides has yielded a more highly luminescent Tb^{3+} binding protein. A large database of naturally occurring sequence variations used in picking a consensus sequence can give a protein engineering effort the experience of countless trials of evolution. This study and the previous work support the usefulness of making a series of smaller peptide models of a site and testing their properties before committing to cassette mutagenesis.

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