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Terbium Luminescence in Synthetic Peptide Loops from Calcium-binding Proteins with Different Energy Donors*

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John P. MacManus, Christopher W. Hogue, Brian J. Marsden, Marianna Sikorska, and Arthur G. Szabo

From the Division of Biological Sciences, National Research Council, Ottawa K1A 0R6, Canada

Fourteen 14-mer peptides corresponding to a consensus sequence of metal-binding loops from proteins of the calmodulin family were synthesized. The effect of varying both the position in the binding loop, and the type of aromatic side chains as energy donors for enhancement of terbium luminescence, was studied. It was concluded that tryptophan in loop position 7 gave optimal luminescence enhancement, and that the additional inclusion of a tyrosine in the loop at positions 2 or 4 could further boost emission from the bound terbium. In all further cases energy transfer from aromatic residues at positions other than 7 was markedly less efficient. These results suggest that the peptides assume a configuration which allows a hexadentate ligand structure around the bound terbium ion. This is consistent with a Dexter-type electron exchange model of energy transfer.

A family of proteins, such as calmodulin, parvalbumin, and troponin C, with metal binding loops composed of linear sequences of 12 residues, has been described (Strynadka and James, 1989). These binding loops contain six amino acids in positions 1, 3, 5, 7, 9, and 12 which donate oxygen for metal coordination. Glutamic acid is invariant in position 12 and donates both of its side chain oxygens. Thus, seven oxygen ligands are at the seven vertices of a pentagonal bypyramid, or distorted octahedron, at approximately 2.4 angstroms from the central bound metal ion.

In addition to binding calcium and magnesium, these metal binding proteins can bind members of the lanthanide series (Brittain *et al.*, 1976; Evans 1983). Terbium has been used extensively as a luminescent isomorphous substitute of calcium (Dockter, 1983). Enhancement of the terbium luminescence can occur by energy transfer from an aromatic residue located close to the binding site. Many studies using terbium binding to the proteins of the calmodulin family have been undertaken. Studies with parvalbumin were some of the first and showed that energy transfer from Phe in position 7 of a metal binding loop can enhance terbium luminescence (Sowadski *et al.*, 1978; Nelson *et al.*, 1979; De Jersey *et al.*, 1981; Rhee *et al.*, 1981; Henzl *et al.*, 1985). Later studies suggested that other possible energy donors in parvalbumin could be Tyr-48 or Trp-102, both of which lie outside the loops in the adjacent helical stretches (Horrocks and Collier, 1981; Eberspach *et al.*, 1988). Terbium luminescence was also enhanced by energy transfer from Tyr in loop position 7 in calmodulin (Kilhoffer *et al.*, 1980a, 1980b; De Jersey *et al.*, 1981; Wallace *et al.*, 1982) and oncomodulin (Henzl *et al.*, 1986). Tyr in loop position 10 can also enhance terbium luminescence in calmodulin (Kilhoffer *et al.*, 1980b; Wallace *et al.*, 1982). Tryptophan has been introduced into calmodulin by site-specific mutagenesis in loop position 7 and leads to dramatic enhancement of terbium luminescence (Kilhoffer *et al.*, 1988).

In an effort to understand the factors governing metal binding free of the complication of the entire protein structure, studies have been performed on isolated loops obtained by peptide synthesis. The first studies of synthetic loop peptides with flanking helices yielded useful information on calcium binding (Reid *et al.*, 1980). Such studies have continued with smaller peptides restricted to loops (Marsden *et al.*, 1988). Only a few reports exist on terbium addition to short synthetic peptides. Peptides of 12 amino acids based on a loop from troponin C had either a Phe or a Tyr in position 10 of the loop and could enhance terbium luminescence (Kanellis *et al.*, 1983; Malik *et al.*, 1987). Synthetic peptide loops have also been based on sequences from calmodulin. Peptides of 16 amino acids with Tyr in loop position 7 or 10 (Buchta *et al.*, 1986) or 12-mer peptides with Tyr in position 7 (Borin *et al.*, 1989) were also capable of enhancing terbium luminescence.

In all these studies with proteins or cognate synthetic peptides no comparison of the effectiveness of a particular aromatic donor or its loop position has been possible. Qualitative information on the potential of a certain donor residue in a limited number of loop positions is all that can be concluded. We undertook therefore a quantitative comparative study to ascertain which donor configuration in a metal-binding loop is optimal for maximal enhancement of terbium luminescence.

MATERIALS AND METHODS

Peptide Synthesis and Purification—The protected peptide resins were synthesized using a *p*-methylbenzhydrylamine resin (100–200 mesh, 4–8 meq/g) and N^{α}-*tert*-butoxycarbonyl amino acids by the method of simultaneous multiple peptide synthesis (Houghten, 1985). The peptides were cleaved by the conventional HF/anisole procedure (Houghten *et al.*, 1986).

The freeze-dried peptides were purified by ion exchange high performance liquid chromatography on a SynChropak Q300 (250 × 4.6 mm) column equilibrated in 5 mM KH_2PO_4, pH 6.5, and eluted by a gradient of 1%/min at a flow rate of 1 ml/min of 1 M KH_2PO_4, pH 6.5, for 30 min. This step was necessary because of the presence in all peptides of a Asp-Gly sequence which can cyclize during HF cleavage, but the cyclic derivative can be readily removed by ion exchange chromatography (Marsden *et al.*, 1988). Care was taken prior to purification to maintain the pH below 7 during KOH addition to the peptide stock to avoid cleavage of the cyclic derivative which could lead to isomerization of aspartyl residues. The major peak with absorbance at 280 nm was collected and subsequently applied to a reverse phase SynChropak RPP (250 × 4.1 mm) column equilibrated in 0.1% trifluoroacetic acid and eluted with a gradient of acetonitrile (1%/min for 40 min at a flow rate of 1.5 ml/min). Stocks from

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lyophilized peptide were prepared for spectroscopy in distilled water and a sample taken for quantitation by measurement of leucine content by standard amino acid analysis on an acid hydrolysate. Another sample was hydrolyzed by methanesulfonic acid for tryptophan estimation.

Fluorescence Spectroscopy—The remaining purified peptide stocks in water were diluted to give a final concentration of 10 mM PIPES[1] buffer, pH 6.5, with an absorbance at 285 nm of 0.1. Absorption spectra were recorded on a Varian DMS 200 UV spectrophotometer. Fluorescence measurements were made on a SLM 8000C spectrofluorimeter equipped with a Neslab Endocal refrigerated bath at 20 °C. Emission spectra were taken with 4 nm band pass for both the excitation and emission monochromaters. Excitation spectra were taken with excitation band pass at 4 nm and emission band pass at 8 nm, with a cutoff filter (Corning 3603-75C) to eliminate second-order wavelength interference. Spectra were collected at 1-nm intervals with a 0.5-s integration at each interval.

All emission spectra were corrected and normalized to an absorbance of 0.1 at the excitation wavelength (285 nm) in a quartz cell of 1 cm pathlength. All excitation spectra were normalized to 1.0 at 285 nm. Emission and excitation spectra were collected before and after titration of each peptide with terbium. Spectra were also taken after the titrations following N_2 purging to reduce quenching by dissolved oxygen. Terbium working solutions were diluted from a 1 M stock of $TbCl_3$ (Aldrich). This stock was calibrated with an EDTA microtitration using Xylenol Orange as indicator (Pribil, 1967).

Fluorescence quantum yields of Tyr or Trp in the peptides were measured using L-tyrosine or NATA (*N*-acetyl-L-tryptophanimide, both from Aldrich) (quantum yield = 0.14; Szabo and Rayner, 1980) as secondary standards. Each standard was dissolved in the PIPES buffer, pH 6.5, at an $A_{285\,nm}$ of approximately 0.1 and bubbled with nitrogen for 10 min before measurement. Quinine sulfate in 0.05 M sulfuric acid (quantum yield = 0.70 (Secrist *et al.*, 1972)) was used to obtain the quantum yield of each peptide-Tb complex at 490 nm. Since no standard had suitable emission overlapping the major terbium emission at 545 nm, the measurement of quantum yield was done relative to that of a complex of EHPG (*N,N*-ethylenebis[2-(2-hydroxyphenyl)]glycine, also known as EDTA-b-HPA, Aldrich) and terbium. A stock 1:1 molal complex of EHPG·Tb in PIPES buffer, pH 6.5, was diluted to an absorbance of approximately 0.1 at 285 nm. To rank the quantum yield of luminescence at 545 nm, the ratio of quantum yields of peptide and EHPG was used, to give a relative quantum yield. Spectra of all three of these standards, quinine, tyrosine or NATA, and EHPG·Tb were obtained before each peptide experiment.

The titration data were obtained with the aid of programs for the control of the spectrofluorimeter written in the SLM8000 macro command language. This permitted luminescence measurements at several separate excitation/emission wavelength pairs. The band pass was 4 nm for both excitation and emission monochromators. The macro was programmed with an integration interval of 5 s for each separate wavelength. The following excitation/emission pairs were monitored for peptides with Phe or Tyr: 260/280, 260/310, 260/490, 260/545, 285/310, 285/350, 285/490, 285/545, and 285/570. For peptides containing Trp the excitation/emission pairs were 260/490, 260/545, 285/350, 285/490, 285/545, 285/570, 295/350, 295/490, and 295/545. Aliquots of 2 μl of stock (1.0 or 2.5 mM $TbCl_3$ (Aldrich) were added with continuous stirring to 1.5 ml of peptide solution (30–150 μM), and fluorescence measurements taken after each addition. Each reading at each wavelength was normalized to an $A_{285\,nm}$ of 0.1, corrected for instrument response sensitivity, and finally adjusted for the incremental dilution and expressed per peptide unit by the use of Microsoft Excel spreadsheets.

A sensitivity index was obtained for each peptide at both 490 and 545 nm by multiplying the quantum yield of fluorescence at these wavelengths by the respective extinction coefficient at 285 nm.

RESULTS

Design from Consensus Sequences—Rather than synthetically modeling a particular binding loop sequence from a particular protein such as calmodulin or troponin C, we undertook a generic approach. The amino acid sequences of more than 200 species-variant loops are known and can be used as a design data base (Marsden *et al.*, 1990). We examined the sequences of all known loops and derived a most common consensus sequence of DKDGDGYIDFEE.

This consensus sequence was altered as follows in such a way as to maximize the metal ion affinity and thereby maximize terbium luminescence. The Asp residue at position 3 in the loop sequence was changed to Asn on the basis of the studies of Marsden *et al.* (1988), where removal of potential charge repulsion gave rise to the highest affinity for lanthanide ion binding. Thus, the consensus sequence was modified to DK*N*GDGYIDFEE. Finally, with the wish to both maximize the affinity of binding and eliminate the possibility of fluorescence quenching by the water molecule bridge at loop position 9 (Strydnadka and James, 1989), this consensus sequence was further modified to DKNGDGYI*E*FEE.

Because loop sequences in the data base with D-N-D as the first three oxygen donors have an Ala in position 4, we also choose to study some peptides of structure DKN*A*DGYIEFEE. To minimize the influence of the charged end groups on the liganding efficiency of residues 1 and 12, an additional residue was placed at each end of the loop sequence. A Gly was placed at the N terminus and a Leu at the C terminus, making the peptides of study 14 residues in length.

The aromatic residues Phe, Tyr, or Trp were placed at position 7 of the loop to act as energy donors to bound terbium. The same residues were also incorporated at position 10, and Tyr or Trp was placed at position 2 or 4 of the loop sequence to determine whether the presence of more than 1 aromatic residue in the loop sequence would further enhance terbium luminescence. Fig. 1 shows the sequences of the 14 peptide variants used in this investigation. The 14 peptides can be divided into three groups depending on which positions contain the potential energy donors Tyr or Trp. The first group consisted of peptides with Tyr substitutions, namely peptides 3, 4, 36, and 37. The second group consisted of peptides with Trp substitutions, that is peptides 2, 33, 9, 34, and 35. The third group consisted of peptides which contained both Tyr and Trp, peptides 5, 16, 17, 18, and 19.

Emission of Peptide 16 and Fluorescent Standards—Data from peptide 16 is shown as a first example of the experiments performed with each of the 14 synthetic peptides. Fig. 2 shows the emission of peptide 16, along with the standards NATA, quinine sulfate and EHPG·Tb complex. Without terbium

	X	2	Y	4	Z	6	−Y	8	−X	10	11	−Z
Peptide 3	G	D	K	N	G	D	G	Y	I	E	F	E	E	L
Peptide 4	G	D	K	N	G	D	G	F	I	E	Y	E	E	L
Peptide 36	G	D	K	N	Y	D	G	F	I	E	F	E	E	L
Peptide 37	G	D	Y	N	A	D	G	F	I	E	F	E	E	L
Peptide 2	G	D	K	N	G	D	G	W	I	E	F	E	E	L
Peptide 33	G	D	K	N	A	D	G	W	I	E	F	E	E	L
Peptide 9	G	D	K	N	G	D	G	F	I	E	W	E	E	L
Peptide 35	G	D	K	N	W	D	G	F	I	E	F	E	E	L
Peptide 34	G	D	W	N	A	D	G	F	I	E	F	E	E	L
Peptide 5	G	D	K	N	G	D	G	Y	I	E	W	E	E	L
Peptide 19	G	D	W	N	A	D	G	Y	I	E	F	E	E	L
Peptide 18	G	D	K	N	A	D	G	W	I	E	Y	E	E	L
Peptide 16	G	D	Y	N	A	D	G	W	I	E	F	E	E	L
Peptide 17	G	D	K	N	Y	D	G	W	I	E	F	E	E	L

FIG. 1. **Synthetic peptides based on the consensus of most common sequences of calcium-binding loops.** The *X*, *Y*, and *Z* refer to the Cartesian positions of the coordinating ligands. The peptides are listed in three groups. First, those peptides containing Tyr; second, those peptides containing Trp; and third, those containing both Tyr and Trp.

[1] The abbreviations used are: PIPES, disodium piperazine-*N*, *N*′-bis(2-ethanesulfonic acid); NATA, *N*-acetyl-L-tryptophanimide; EHPG, *N,N*-ethylenebis[2-(2-hydroxyphenyl)]glycine.

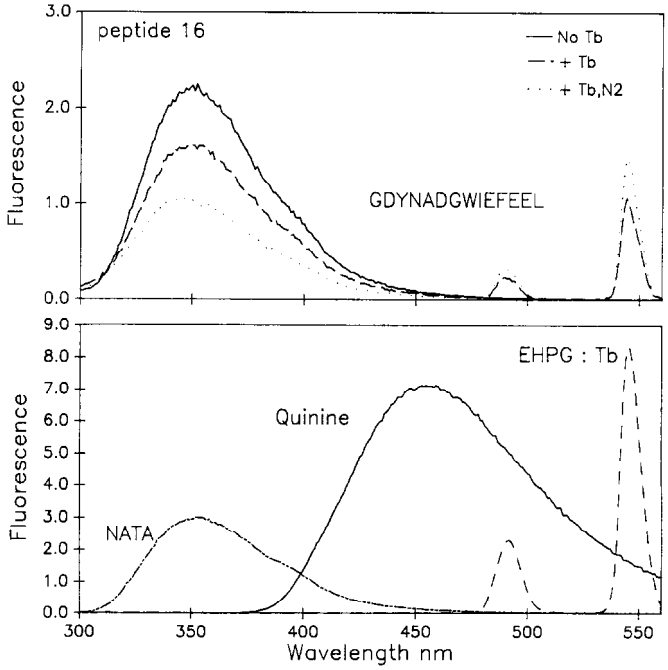

FIG. 2. **Emission spectra of peptide 16 and the various standards used for estimation of quantum yield of fluorescence of both donor aromatic residue (*e.g.* Trp), and the acceptor terbium at 490 and 545 nm.**

there was no emission at 490 or 545 nm, and only the fluorescence of tryptophan at 350 nm was observed. Upon the addition of excess terbium (greater than 1:1 Tb:peptide) a signal of enhanced luminescence of terbium appears at 490 and 545 nm. A concomitant decrease in the fluorescence of the tryptophan was observed. The standard NATA was used to calculate the quantum yield of fluorescence of tryptophan. In the case of other peptides where only Tyr was present, the standard L-tyrosine was used to determine quantum yield. Quinine sulfate was used to determine the quantum yield of the peptide-terbium complex at 490 nm. Removal of dissolved oxygen in the sample buffer by nitrogen purging increased the terbium luminescence at both 490 and 545 nm. For peptide 16 this was accompanied by a further decrease of tryptophan fluorescence at 350 nm.

Peptides with Tyr as Energy Donor—The emission spectra of peptides containing only Tyr substitutions can be seen in Fig. 3. The peak height of the luminescence due to terbium at both 490 and 545 nm was dependent on the position of the Tyr donor in the peptide. Tyr at loop position 7 gave the most terbium luminescence (Fig. 3*A*), but Tyr at loop position 2 was also effective (Fig. 3*D*). A decrease in the Tyr fluorescence was observed in proportion to the terbium intensity at 545 nm. The peptides with Tyr at positions 4 or 10 were much less effective at enhancing terbium luminescence (Fig. 3, *B* and *C*). Bubbling of the peptide solutions with nitrogen had no effect on terbium emission in peptide 3 but significantly increased emission in peptide 37. The addition of calcium in ratios up to 14:1 to peptides 3 and 37 was without effect on the Tyr fluorescence.

The shape of the excitation spectra was very informative as to the source of the energy transfer to terbium, and confirmed the suggestion that loop position 7 was best for terbium enhancement (Fig. 4). The excitation spectra of peptide 3 (Fig. 4*A*) showed that it was the single Tyr at position 7 which enhanced terbium emission at 545 nm. However, in the case of the other three peptides it was not principally Tyr which acted as donor, but Phe at position 7 as seen by the pronounced vibronic structured excitation spectra which could be assigned to phenylalanine (Fig. 4, *B, C,* and *D*). Each peptide was titrated with terbium and the emission of both the donor aromatic residue, and the acceptor terbium, was recorded. For example, peptide 3 gave a maximal terbium fluorescence at a 1:1 molal ratio of Tb:peptide and a maximal quenching of the Tyr donor (Fig. 5*A*).

Peptides with Trp as Energy Donor—The placement of Trp in loop positions 2, 4, 7, or 10 as possible energy donor gave essentially the same conclusion as the Tyr peptides, that is that position 7 was the optimal donor position. The emission spectra (Fig. 6) of these peptides showed that terbium emission was greatest when the Trp was in position 7 (Fig. 6, *A* and *B*). There was a small effect of the Gly to Ala change in position 4 of peptides 2 and 33. Surprisingly, purging with nitrogen caused quenching of the Trp fluorescence and an increase in terbium emission, except for peptide 34. On the other hand addition of calcium in ratios up to 14:1 had no effect on Trp fluorescence of peptide 2.

Again the shape of the excitation spectra were very informative in pinpointing the source of energy donation to terbium (Fig. 7). Trp was the major source of energy transfer when in position 7. However, a significant terbium output could be obtained by exciting Phe in position 7 between 250 and 270 nm in peptide 34 (Fig. 7*D*) or peptide 35 (not shown). The quantum yield data (excitation = 285 nm) showed that terbium bound to the latter peptides had a low quantum yield (see below). Titration of these peptides gradually quenched the Trp emission (for example, see Fig. 5*B*). Saturation did not occur at 1:1 for donor or terbium emission.

Peptides with Tyr in Addition to Trp as Energy Donor—The last group of peptides containing both Tyr and Trp were used to investigate the possibility of increasing the emission from terbium at 490 and 545 nm above that of groups one or two. The emission spectra are shown in Fig. 8. All had a Tyr or a Trp at the optimal position 7. Peptides 5 and 19 had a Tyr at loop position 7, and the placement of a Trp at position 2 was more effective than at position 10 (Fig. 8, *A* and *B*). Peptides 16–18 had a Trp at position 7. The additional placement of a Tyr at position 2 (peptide 16) (Fig. 2), position 10 (peptide 18) (Fig. 8*C*), or position 4 (peptide 17) (Fig. 8*D*) was effective at increasing the terbium emission over peptides 2 or 33 which contained no Tyr. Bubbling with nitrogen caused a decrease in Trp fluorescence and an increase in terbium emission, particularly in peptide 16 (Fig. 2) and peptides 17 and 18 (Fig. 8, *C* and *D*).

The excitation spectra supported the idea of major energy donation to terbium from position 7. Even with Trp at position 2 or 10, it was the Tyr that dominated the excitation spectra of peptides 5 and 19 (Fig. 9, *A* and *B*). Likewise Trp dominated such spectra in peptides 17 and 18 (Fig. 9, *C* and *D*) and 16 (not shown). The donor fluorescence was gradually quenched in all these peptides during titration with terbium (Fig. 5, *C* and *D*).

Quantum Yields—The quantum yield of fluorescence for the donor Tyr or Trp, and both the 490 and 545 nm peaks of terbium are shown in Fig. 10. The donor's quantum yield was decreased where significant energy transfer to terbium occurred. The overall pattern of quantum yield changes of terbium at 490 or 545 nm were similar, with the notable exception of peptide 2. Even though in general, bubbling with nitrogen caused a further increase in terbium luminescence, the calculation of the quantum yield after such treatment was not considered meaningful. This was because the nitrogen

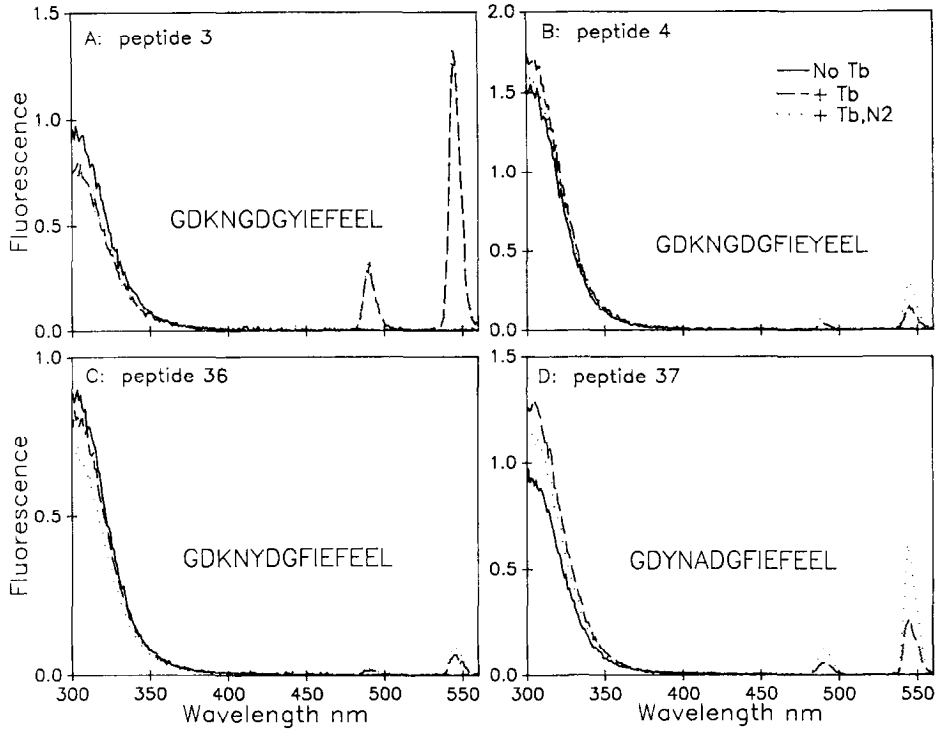

FIG. 3. **Emission spectra of peptides 3, 4, 36, and 37 which contain Tyr as possible energy donor (excitation = 285 nm).**

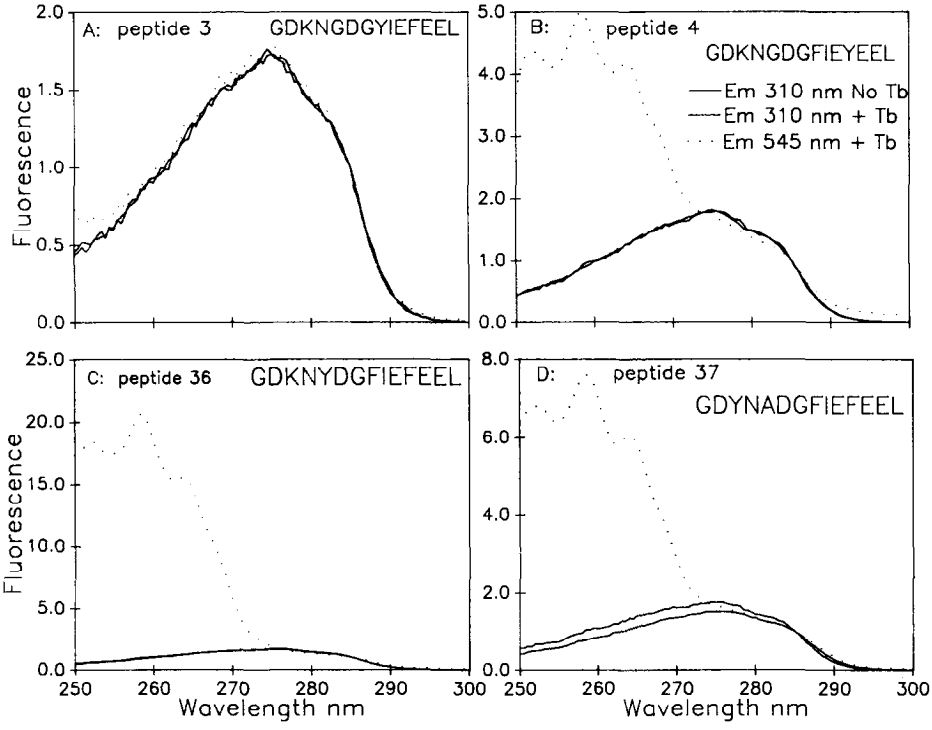

FIG. 4. **Excitation spectra of peptides 3, 4, 36, and 37 which contain Tyr as possible energy donor.**

also caused an increase in the scattered light signal at 570 nm (not shown), probably caused by peptide precipitating out of the solution.

The quantum yield data in Fig. 10 did not give a ranking of the efficacy or sensitivity of each peptide, since by definition it is the efficiency of light emission per photon absorbed. A ranking was obtained, by calculating a sensitivity index based on the product of the quantum yield and the extinction at 285 nm, which is shown in Fig. 11. Peptide 36 was the lowest on such a scale and was set equal to unity. Clearly, the peptides containing Trp at loop position 7 were the most sensitive as a group, and the inclusion of an additional Tyr, particularly at positions 2 and 4 was able to maximize the terbium luminescence.

DISCUSSION

Despite many demonstrations of terbium luminescence enhancement in both calcium-binding proteins and cognate synthetic peptide-binding loops, little is known about the optimal requirements for energy transfer in such a loop. Obviously, (*a*) the coordination efficiency, (*b*) the donor receptor spectral overlap integral, and (*c*) the distance from donor

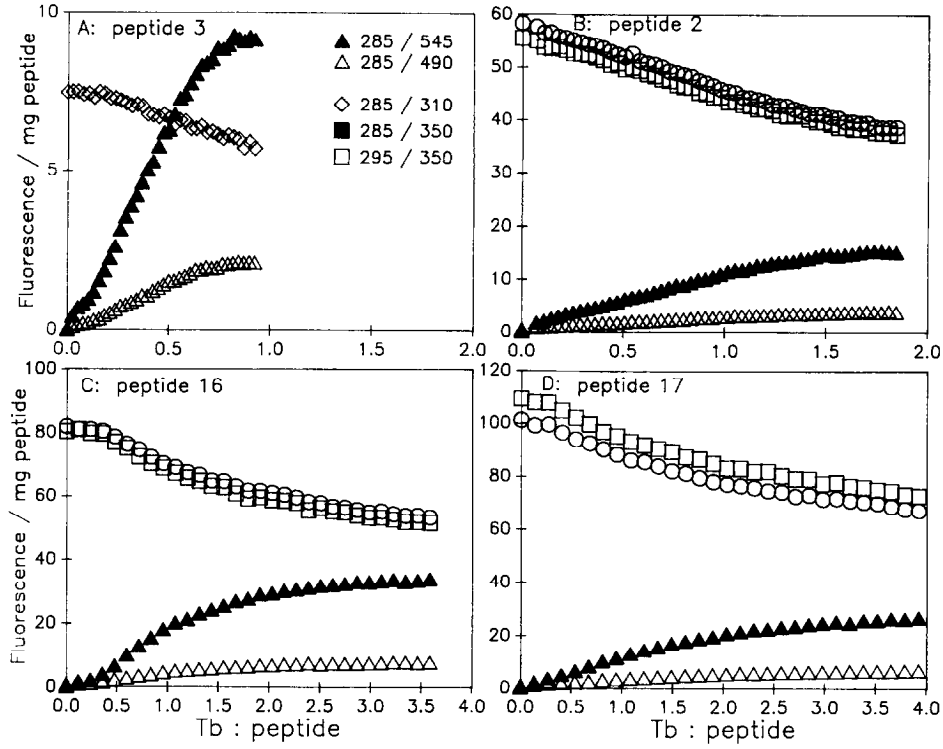

FIG. 5. **Examples of terbium titration of peptides 2, 3, 16, and 17 which serve to illustrate the quenching of the aromatic donor as the emission of terbium increased.**

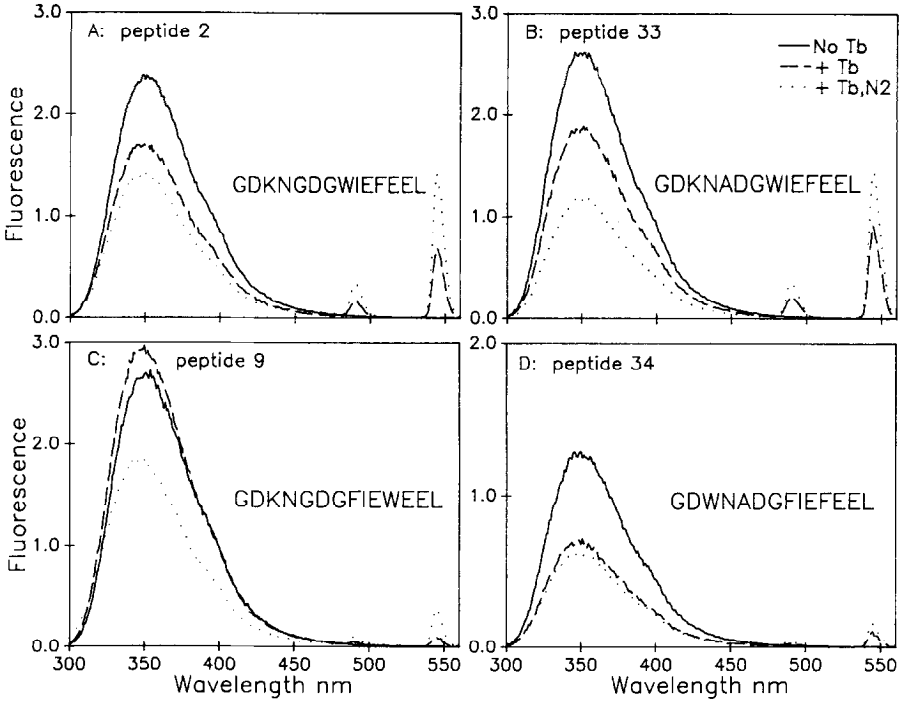

FIG. 6. **Emission spectra of peptides 2, 9, 33, and 34 which contain Trp as possible energy donor (excitation = 285 nm).**

to acceptor, will all influence the subsequent terbium luminescence. The present study was undertaken to try to discern experimentally what position in a metal-binding loop was the most efficient for energy transfer to terbium bound to the loop center. No study has been undertaken on the proximity of more than one aromatic residue in such a metal-binding loop.

The oxygen donors are all the same in the 14 peptides studied and have been chosen to maximize metal binding affinity. When the loops are present in proteins such as calmodulin or troponin C, the distance from these coordinating oxygen atoms to the bound calcium averages 2.4 angstroms (Babu *et al.*, 1988; Strydnadka and James, 1989). Studies on the conformation of synthetic peptide loops in solution show that a lanthanide, especially the ones of ionic radius close to that of calcium, fold the peptide into the same three-dimensional structure as exists in the intact protein (Gariepy *et al.*, 1985; Marsden *et al.*, 1989). It is assumed therefore that all of

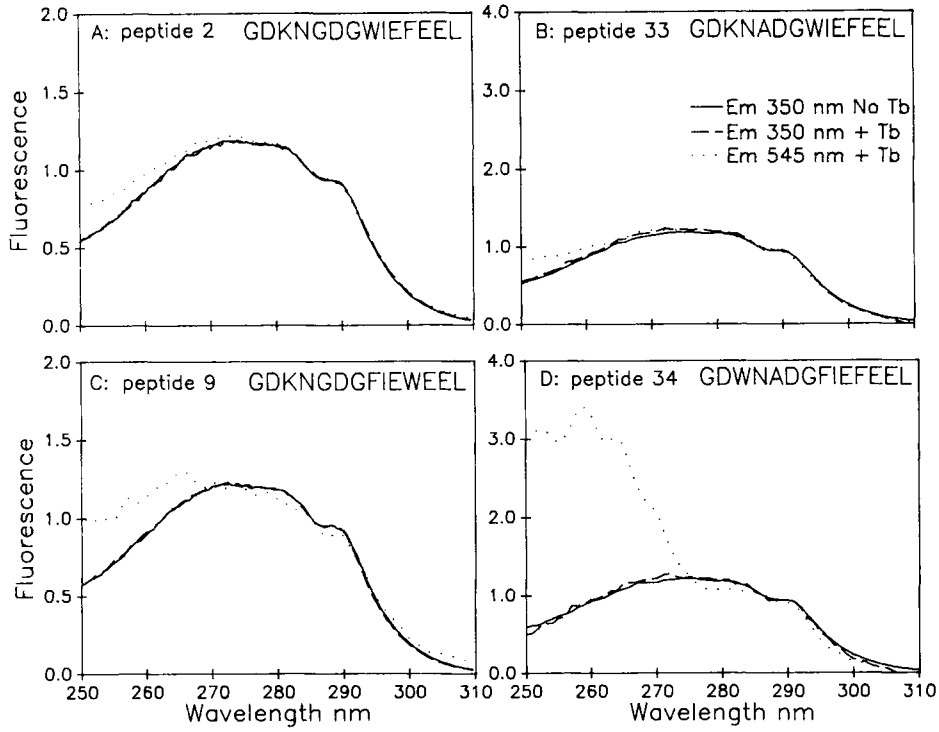

FIG. 7. **Excitation spectra of peptides 2, 9, 33, and 34 which contain Trp as possible energy donor.**

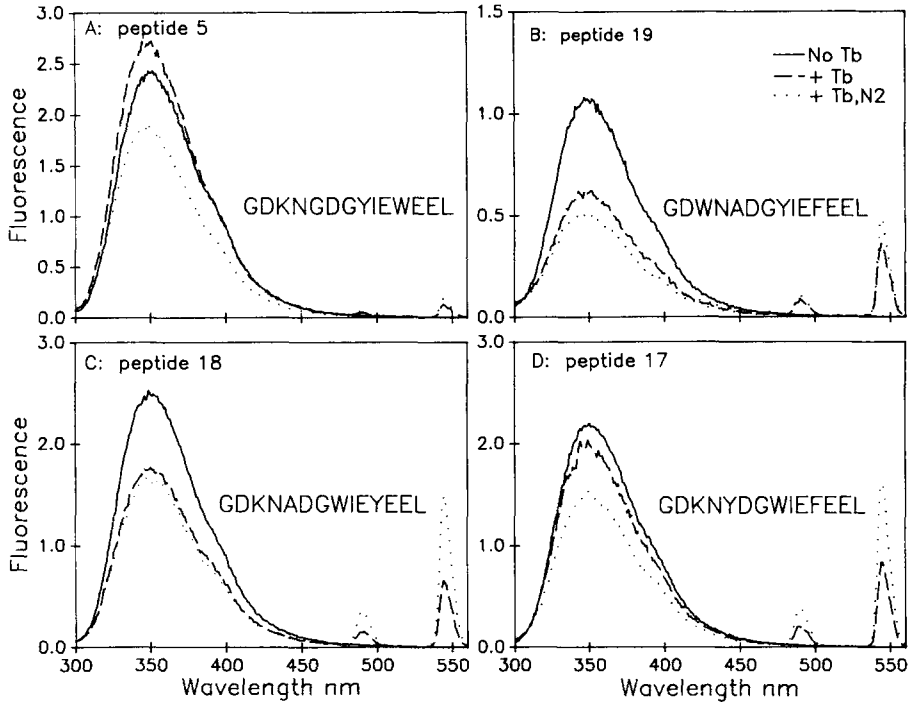

FIG. 8. **Emission spectra of peptides 5, 17, 18, and 19 which contain both Tyr and Trp as possible energy donors (excitation = 285 nm).**

the peptides in this study folded around the bound terbium in a similar manner. Analysis of the solution conformation by NMR is in progress.

Terbium has 12 absorptions peaks which lie between 250 and 400 nm (Dockter, 1983). The fluorescence emission spectrum of Phe or Tyr overlaps with the higher energy bands of these terbium absorption peaks, but the emission spectrum of Trp overlaps to a much greater extent between 300 and 350 nm. Coupled to the increasing extinction coefficient at 280 nm, it has long been recognized that the order of efficiency of terbium enhancement would be from Phe < Tyr < Trp (Brittain *et al.*, 1979; De Jersey *et al.*, 1981).

The side chains which are not involved in coordination of the metal tend to point outward from the bound metal into solution in loops of calcium-binding proteins whose crystallographic structure has been determined (Strydnadka and James 1989). The distance from the terbium to its energy donor in parvalbumin has been estimated to be approximately 5 angstroms in the case of Phe-57 in loop position 7 (Nelson *et al.*, 1979), and approximately 12 angstroms in the case of Trp-102 which lies just outside the loop (Rhee *et al.*, 1981). Thus, if a donor in a loop is within 5–12 angstroms of the bound metal, then terbium luminescence enhancement can result albeit with different efficiencies. From examination of

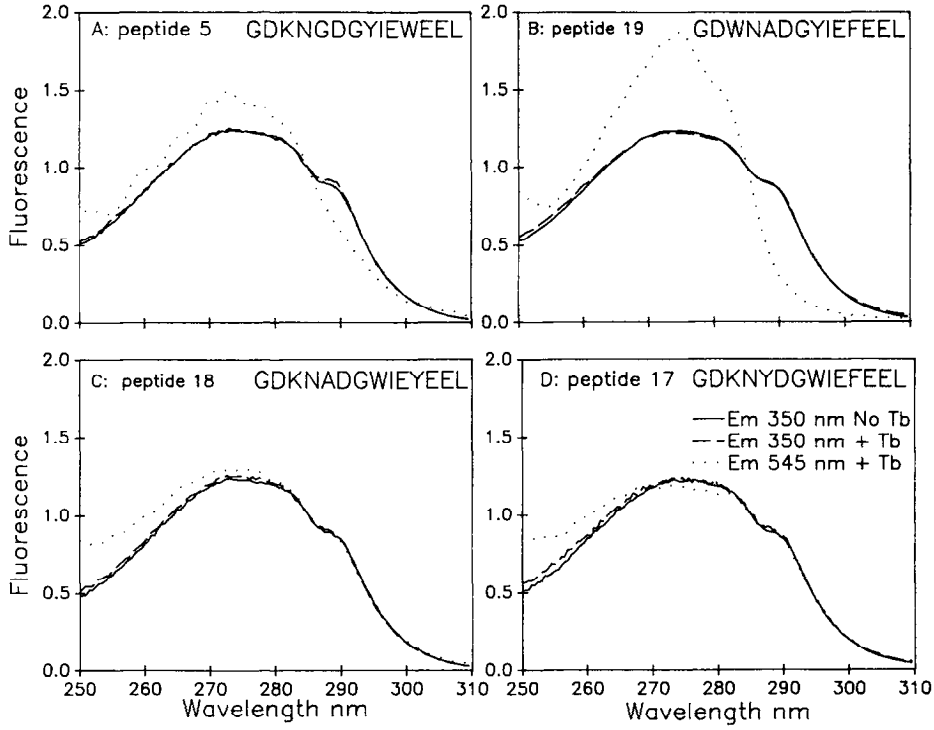

FIG. 9. **Excitation spectra of peptides 5, 17, 18, and 19 which contain both Tyr and Trp as possible energy donors.**

the known three-dimensional structures of proteins containing such binding loops (Babu *et al.*, 1988; Strydnadka and James, 1989), it can be estimated that the ring center of an aromatic residue in loop position 2, 4, or 10 would be approximately 8, 9, or 12 angstroms, respectively, from the bound metal. The corresponding distance from an aromatic amino acid at position 7 to the bound metal averaged 5 angstroms. Thus, enhancement of terbium luminescence is expected to occur from all positions of aromatic residues in these peptides.

All 14 synthetic peptides in this study demonstrated enhancement of terbium luminescence. Some were better than others. The overriding conclusion from these peptides was that irrespective of whether Phe, Tyr, or Trp occupied loop positions 2, 4, or 10, it was the aromatic ring in loop position 7 which dominated the enhancement of terbium luminescence. Despite Phe having the lowest extinction coefficient, when it was placed at position 7 Phe made the major contribution in peptides 4, 36, and 37 (Fig. 4) or peptide 34 (Fig. 7). Tyr made a major contribution in the presence of Trp in peptide 19 (Fig. 9). Since this position 7 has an aromatic ring closest to the metal at 5 angstroms, this should not be a surprise because energy transfer is very sensitive to distance between donor and acceptor (Rhee *et al.*, 1981; Dockter, 1983). In the case when the distance is less than 10 angstroms, Forster energy transfer theory no longer applies. Rather, a Dexter-type electron exchange transfer may be operative (Birks, 1970).

However, aromatic residues in other positions can also transfer energy to the bound terbium. Thus, Tyr in position 2 or 10 (Fig. 4, *B* and *C*), or Trp in position 2 or 10 (Fig. 7, *C* and *D*), also enhanced terbium emission but with significantly lower efficiency than in position 7. It was not unexpected that the most sensitive five peptides had Trp at position 7 (Fig. 11). The inclusion of a Tyr at position 10 in addition to the Trp at position 7 did not increase terbium luminescence (compare peptides 33 and 18 in Fig. 11). However, the presence of Tyr in position 2 or 4, with Trp at position 7, did lead to a 20–40% increase in terbium luminescence output (compare peptide 33 with 16 and 17 in Fig. 11). Although a Tyr in position 2, 4, or 10 can clearly directly donate some energy to terbium (peptides 36 and 37, Fig. 4, *C* and *D*; peptide 4, Fig. 4*B*), in the case of peptide 16 or 17 another mechanism must apply. If Tyr (position 2 or 4) in these peptides was transferring electronic energy directly to bound terbium then the quantum yield of terbium luminescence should be a weighted mean value of the quantum yields of peptides 33 and 36 or 37. The weighting would be according to the fractional extinction coefficients of Tyr and Trp at the excitation wavelength (285 nm) in those peptides containing both residues. For example it can be calculated that the quantum yield of peptide 16 would be 0.07 rather than the observed value of 0.12, if both Tyr (position 2) and Trp (position 7) were transferring energy directly to the bound terbium. The results clearly suggest that an energy transfer mechanism must be operative where Tyr (position 2) transfers energy to Trp (position 7) which in turn transfers its electronic energy to bound terbium. In the case of peptide 18 the quantum yield of terbium luminescence is lower than that of peptide 33 because Tyr (position 10) absorbs light energy which is not transferred to terbium.

The results for peptide 19 are noteworthy. Its excitation spectrum indicates that terbium luminescence largely results from direct energy transfer from Tyr (position 7). Furthermore, based on a quantum yield of 0.11 for peptide 3 (Tyr, position 7) and a fractional extinction coefficient for Tyr of ~0.14 one would estimate that direct tyrosine energy transfer to terbium should lead to a quantum yield of 0.015, significantly lower than the measured value of 0.04. This peptide must then adopt a configuration where there is inefficient Tyr to Trp energy transfer and the Tyr residue is even closer to the bound terbium.

The excitation spectrum of peptide 5 suggests that both Tyr (position 7) and Trp (position 10) both transfer energy to bound terbium. The quantum yield value of 0.02 is consistent with this conclusion. Direct energy transfer from both the aromatic residues in peptides 4, 9, are suggested by the excitation spectra.

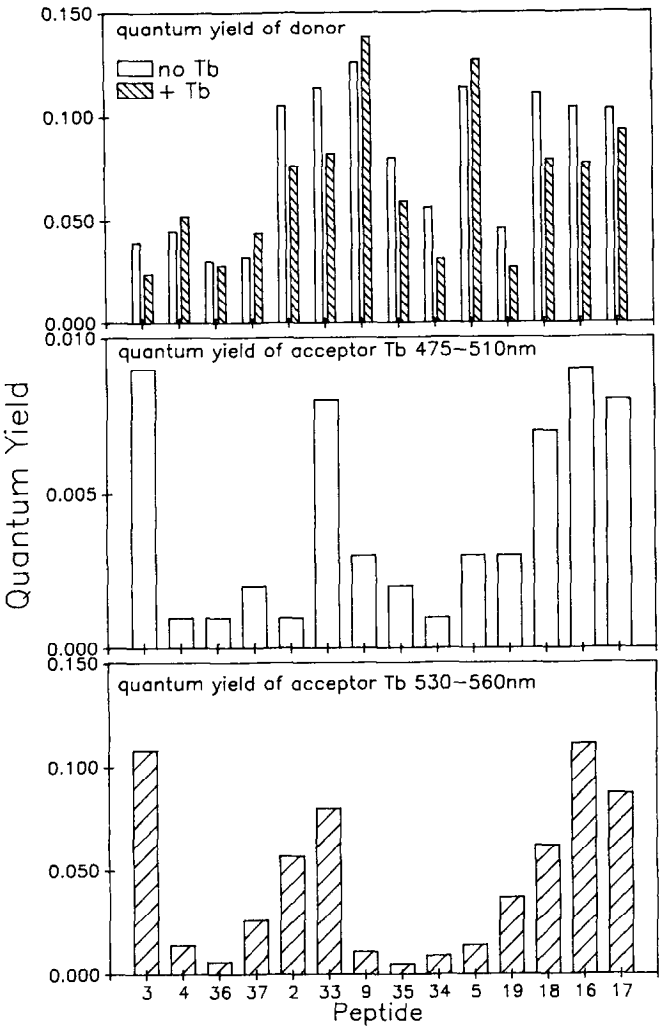

FIG. 10. **Quantum yield of fluorescence of donor aromatic residues and acceptor terbium at both 490 and 545 nm with excitation at 285 nm.**

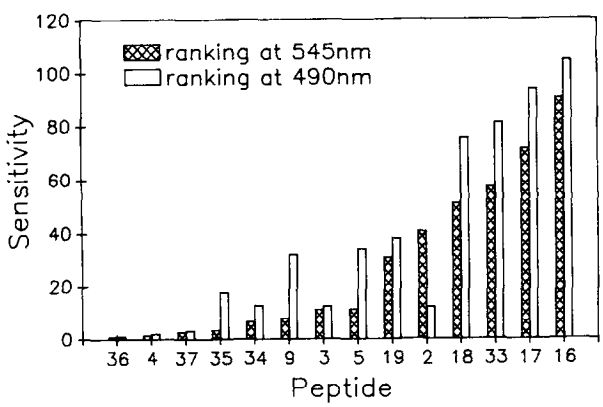

FIG. 11. **Sensitivity index (product of quantum yield and extinction coefficient) of terbium emission for all peptides.** Peptide 36 set equal to 1.

The excitation spectrum of peptides 4, 34, 36, and 37 shows that Phe (position 7) must be transferring energy directly to bound terbium. The absorption spectrum (data not shown) of these peptides resembled that expected for a peptide containing a Trp or Tyr residue with only some additional vibronic pattern on the high energy side resulting from the absorption of the single Phe residue consistent its significantly lower extinction coefficient. The clear Phe-like spectral pattern of the excitation spectra and the very low contribution of the Trp or Tyr spectral pattern to the total excitation spectra strongly suggest that both Phe and Trp or Tyr are directly transferring their excitation energy to the bound terbium. If there were energy transfer from Phe to Trp to terbium in peptide 34 or Phe to Tyr to terbium, comparable to that proposed in peptides 16 and 17, then excitation spectra closely resembling the absorption spectra would be expected. This also explains the very low quantum yield of terbium luminescence in this peptide since Phe has negligible absorbance at 285 nm which was the excitation wavelength where the quantum yield was determined.

Peptides 34 and 19 have a common feature in that their excitation spectra reveal that there is significant and direct energy transfer from the aromatic residue at position 7 to the bound terbium and little energy transfer to the Trp residue at position 2. Yet in peptide 16 there is energy transfer from a Tyr residue in position 2 to the Trp in position 7. For some structural reason in the alternate arrangement as in peptide 19 the same energy transfer between Tyr and Trp is not very important.

These results taken together demonstrate that only when an aromatic residue is located in position 7 does significant energy transfer to bound terbium occur. Energy transfer efficiency from positions 2, 4, and 10 is significantly less but a ranking may be determined from the data. If one compares peptides 4, 36, and 37 where the Tyr residue occupies positions 10, 4, and 2, respectively, the order of energy transfer efficiency by Tyr to terbium is $2 > 10 > 4$. For the comparable series of Trp-containing peptides, peptides 9, 35, 34, the order was position $10 > 2 > 4$.

The quenching of the fluorescence of the Phe, Tyr, or Trp donor has been reported many times following terbium addition to either proteins or peptides (Borin *et al.*, 1989; Henzl *et al.*, 1985, 1986; Kilhoffer *et al.*, 1988; Wallace *et al.*, 1982). The usual suggestion is that energy transfer is the main cause of donor quenching, although there is some controversy on this point (Borin *et al.*, 1989; Kilhoffer *et al.*, 1980a; Wallace *et al.*, 1982). In this study, such quenching of the donor was seen in all cases where significant terbium luminescence was observed (*e.g.* Fig. 5). It cannot be entirely ruled out that quenching of Tyr, or particularly Trp, may have been due to an environmental change of the donor induced by metal binding, or to direct lanthanide quenching of indole side chains (Anantharaman and Chrysochoos, 1983; Tine *et al.*, 1986). Peptides 3, 37, and 2 showed no Tyr or Trp quenching even when a 14:1 molal excess of calcium was added. This suggests that metal binding *per se*, in the absence of energy transfer, did not quench the aromatic fluorescence. This is in contrast to the findings of Borin and co-workers (1989) using a single calmodulin-like peptide where calcium did cause quenching of Tyr fluorescence. It was not possible to eliminate the possibility of direct quenching. But, since the quenching of Tyr or Trp (Fig. 5), was correlated to terbium luminescence, and since this quenching did not occur in peptides 4, 36, 37, or 9 even though comparable concentrations of terbium were present, it seems most likely that energy transfer is the major cause of quenching of the Tyr or Trp fluorescence.

These findings confirm NMR results which suggest that the small peptides fold into a hexadentate configuration in the presence of terbium which is similar to that of the binding loops in the intact calcium-binding proteins (Gariepy *et al.*, 1985; Marsden *et al.*, 1989). If such a configuration were not achieved then one would not expect the specificity of significant energy transfer from an aromatic residue located at

position 7 of the peptide-binding loop. For if simple electrostatic binding of terbium to any of the acidic residues of the peptide occured then one might expect comparable enhancement of terbium luminescence when an aromatic residue was located at any position in the loop.

When synthetic peptide loops have been titrated with lanthanides and monitored by proton NMR, very sharp saturation at ratios of 1:1 has been seen (Gariepy *et al.*, 1983; Marsden *et al.*, 1988). In this study, a sharp inflection point in the fluorescence titration signal at 545 nm was not observed, with the notable exception of peptide 3 (Fig. 5*A*). In general, the reports on titration of terbium luminescence do not show sharp 1:1 saturation but have a signal plateau between 1.5 and 2:1 (Buchta *et al.*, 1986; Borin *et al.*, 1989). Several of these peptides exhibit an unusual lag phase, for example, peptide 16 (Fig. 5*C*). Up to 0.25 equivalent of terbium could be added without apparent effect. Titrations were rarely carried out beyond ratios of 2.5–3.0 terbium-peptide because of precipitation, which was continually monitored by light scatter at 570 nm. Such precipitation of peptides by terbium has been previously reported (Buchta *et al.*, 1986).

There are some significant differences in the relative quantum yield of terbium at 490 nm compared to the quantum yield at 545 nm of these peptides (Fig. 10). This is reflected in Fig. 11 which compares the efficiency of all of the peptides to provide enhancement of terbium luminescence. These differences may have as their origin some subtle photophysical properties, which we are not able to rationalize at this time. Similarly, we have made no attempt to explain the differences in the aromatic amino acid quantum yields in the absence of bound terbium (Fig. 10, *top panel*). Without further work such as time resolved fluorescence, circular dichroic or NMR spectroscopy, any explanation would be excessively speculative.

The effect of N_2 bubbling on the emission spectrum requires rationalization. In the majority of cases, with the notable exception of peptide 3, bubbling with N_2 caused an increase in the terbium luminescence at both 490 and 545 nm. This can be easily seen in peptide 37 (Fig. 3*D*), peptides 2, 33, and 9 (Fig. 6), and peptides 16 (Fig. 2), 17, and 18 (Fig. 8, *C* and *D*). This was presumably due to the removal of oxygen gas, a quencher of long lifetime luminescence from the vicinity of the excited terbium. However, the puzzle was that N_2 bubbling also caused a further decrease in donor fluorescence in all cases. This decrease was not proportional to the increase in terbium luminescence. It may have been due to precipitation of the peptide since an increase in scattered light emission at 570 nm was usually observed after purging with N_2. This suggests that if an alternative degassing technique were used which did not result in precipitation of the peptide-terbium complex, that even higher terbium luminescence output would have been observed.

In conclusion, these studies show that the efficiency of energy transfer, from aromatic amino acid residues to terbium bound to binding loops comparable to the calcium-binding loops in proteins, can be determined from studies with synthetic peptides. This obviates the necessity of preparing a series of mutant proteins with aromatic amino acid residues in the different positions of the binding loops. In this way the results obtained from the peptides can be used to assist in the efficient preparation of mutant proteins with desired properties. In this study it has been shown that such small peptides fold to form a metal ligand complex similar to that occurring in the intact protein. The aromatic residue in position 7 is most effective in transferring its electronic energy to terbium bound to these loops. This must reflect the very short range interaction required for such energy transfer. A necessary conclusion is that the energy transfer no longer is consistent with the theory of Forster since it takes place over a distance of less than 10 angstroms.

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