Terbium(III) Emission as a Probe of Calcium(II) Binding Sites in Proteins

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Abstract: Upon addition of Tb^{3+} to 40 proteins, many but not all known to interact with Ca^{2+}, 36 show a characteristic green emission from Tb^{3+} upon excitation in the aromatic region. Energy transfer to Tb^{3+} is observed from tryptophan in a majority of cases, but transfer from tyrosine and phenylalanine also occurs. Circular polarization of Tb^{3+} emission is observable in nine cases including the muscle proteins, carp parvalbumin and troponin-C. All the serine proteases and their zymogens examined display Tb^{3+} emission, a result which suggests that they also bind Ca^{2+} relatively specifically. The Tb^{3+} emission from elastase is partially circularly polarized as well. Weak partially circularly polarized Tb^{3+} emission also appears with pronase, α-amylase, and thermolysin. Due to the unexpected high frequency of nearby aromatic chromophores and Tb^{3+} added in proteins, Tb^{3+} emission promises to be a useful probe for delineating features of protein structure.

Many proteins either contain Ca^{2+} as a basic structural and functional entity or utilize this ion in carrying out their biological function. In contrast to proteins bearing transition metal ions, those involving Ca^{2+} are considerably more difficult to investigate by spectroscopic techniques. The electronic transitions of Ca^{2+} cannot be studied by conventional optical absorption and emission spectroscopy, and the absence of unpaired electrons precludes the use of magnetic resonance techniques in probing the chemical and structural nature of calcium ion binding sites.

Effective ionic radii of Ca^{2+} ions vary with coordination number, being 1.00, 1.07, and 1.12 Å for six-, seven-, and eightfold coordination, respectively.[1] Trivalent lanthanide ions (Ln^{3+}) possess effective ionic radii that show a gradual contraction from La^{3+} to Lu^{3+}—1.06–0.85 Å for sixfold coordination and 1.18–0.97 Å for eightfold coordination. As shown in Table I, these values span those for Ca^{2+} with the values for Nd^{3+} equaling those of Ca^{2+}. These data suggest that in those cases where ion size is of significant importance in determining binding characteristics, Ln^{3+} ions may be used to replace Ca^{2+} without causing serious structural modifications. Perhaps of greater importance to the potential use of Ln^{3+} ions as replacement species for Ca^{2+} in protein systems is the strong propensity of both Ln^{3+} and Ca^{2+} ions for oxygen donor groups (charged or uncharged). In forming complexes both Ca^{2+} and Ln^{3+} ions prefer charged or uncharged oxygen donor groups over nitrogen donor atoms. In aqueous solution, except for multidentate ligands, intervention of hydroxide coordination almost always occurs before amine nitrogen coordination can take place.[2]

Another similarity between Ca^{2+} and Ln^{3+} ions in their coordination chemistry is the lack of strong directionality (geometrical preference) in binding donor groups in the first coordination sphere and the apparent variability in coordination number. These characteristics, along with those cited above (size and preference for oxygen donors), clearly mark Ln^{3+} ions as suitable replacement species for Ca^{2+} in calcium binding proteins. The one obvious difference between Ln^{3+} and Ca^{2+} is the larger charge to ionic radius ratio for Ln^{3+} over Ca^{2+}. It is unlikely that this charge:ionic radius incompatibility will lead to undesirable structural consequences in the replacement of Ca^{2+} with Ln^{3+} in protein systems (where charge compensation should not be a serious problem). In fact, the greater charge to ionic radius ratio for Ln^{3+} over Ca^{2+} suggests that Ln^{3+} will frequently bind more strongly than Ca^{2+}, facilitating the replacement process. Charge to ionic ratios are comparable for Mg^{2+} and La^{3+}, a result which suggests that in some situations the smaller tripositive lanthanide ions may substitute for Mg^{2+}.

Replacement of Ca^{2+} with Ln^{3+} ions has been demonstrated by x-ray diffraction for a number of calcium binding protein systems.[3,4] Spectroscopic properties of the substituted Ln^{3+} ions have been used in several studies to probe the nature of the metal binding sites. Except for La^{3+} and Lu^{3+}, all the other trivalent lanthanides contain unpaired f electrons, and magnetic resonance methods may be employed to probe their environments. Pr^{3+}, Eu^{3+}, Ho^{3+}, and Yb^{3+} are convenient chemical shift probes in nuclear magnetic resonance (NMR) while Gd^{3+} with seven unpaired f electrons is a broadening probe in NMR and may also be investigated by electron spin resonance.

With the exception of La^{3+} and Lu^{3+}, each of the trivalent lanthanide ions exhibit absorption spectra due to intraconfigurational f-f transitions which are easily accessible to study by conventional optical absorption techniques. However, the extinction coefficients associated with these transitions are generally so low that for the study of Ln^{3+}-protein systems in which Ln^{3+} concentrations are $<10^{-2}$ M, optical absorption spectroscopy can be done only near the limits of instrumental sensitivity. On the other hand, emission due to intraconfigurational f-f transitions in Tb^{3+} ions bound to protein systems remains very strong (significantly above detection limits) even when Tb^{3+} is present at concentrations as low as $\sim 10^{-5}$ M. Although other Ln^{3+} ions and Ln^{3+} complexes are known to emit rather strongly in solution media, only Tb^{3+} has been found to retain a relatively high emission intensity when bound to protein systems in aqueous solution.

It is the purpose of the present study to provide a general assessment of the utility of Tb^{3+} as a luminescent probe of Ca^{2+} sites in a wide variety of calcium binding proteins. As shown in Table I, effective ionic radii for Tb^{3+} are 0.08 Å less than those for Ca^{2+} for the same coordination number. In the experiments reported here excitation of the Tb^{3+}-protein complex is in the near-ultraviolet region—into an absorption band of one of the three aromatic amino acids: phenylalanine, tyrosine, or typtophan—and emission is monitored over the 535–555-nm region. The overall process is then described as follows: (1) absorption (by an aromatic side chain of phenylalanine, tyrosine, or tryptophan); (2) energy transfer between closely spaced donor (absorber subunit) and acceptor (Tb^{3+}) species; and, (3) emission (from Tb^{3+}). Tb^{3+} emission in the 535–555-nm region may be assigned to the $^5D_4 \rightarrow {}^7F_5$ radiative transition (phosphorescence). Both total (unpolarized) emission intensities and differential circularly polarized emission intensities were measured over the 535–555-nm spectral region. In these experiments the emission spectra provide information on the state of binding of the Tb^{3+} ions and the structural characteristics of the binding sites, while the exci-

Table I. Effective Ionic Radii, Å[a]

Atomic no.	Ion	Coordination no. 6	8
47	La^{3+}	1.06	1.18
60	Nd^{3+}	1.00	1.12
20	Ca^{2+}	1.00	1.12
65	Tb^{3+}	0.92	1.04
71	Lu^{3+}	0.85	0.97
12	Mg^{2+}	0.72	0.89

[a] From ref 1.

tation spectra provide information on the donor-acceptor couples responsible for the nonradiative transfer of energy to the Tb^{3+} ions.

In this study the intensity of Tb^{3+} emission was observed for successive additions of Tb^{3+} to a variety of proteins with which Ca^{2+} is known to interact. We are observing Tb^{3+} binding to proteins using only one probe, that of electronic emission spectroscopy. Therefore, only Tb^{3+} that receive energy from a nearby aromatic side chain and then emit are evident. If two Tb^{3+} are bound with equal strengths and only one emits, the titration curve will suggest that 2 equiv of Tb^{3+} are bound. If the emitting Tb^{3+} is bound much more weakly, an induction section will appear in the Tb^{3+} titration curve. However, care must be taken to ensure that induction sections are not due to weak interactions and the presence of competitive Tb^{3+} reagents such as buffer components, EGTA, and EDTA.

Experimental Section

The total emission (TE) and circularly polarized emission (CPE) measurements were performed on an apparatus constructed in these laboratories.[5] Samples were irradiated with the frequency-selected output of a 1000-W xenon–mercury arc lamp and a Spex "minimate" monochromator. In all cases emission was detected in the forward direction (head-on) and transmitted exciting radiation was eliminated by use of appropriate solution filters prior to its entering the emission monochromator. An excitation band-pass of 2 nm was used. Emission measurements (TE and CPE) were restricted to the 535–555-nm region ($^5D_4 \rightarrow {}^7F_5$ Tb^{3+} emission) and Tb^{3+} titration studies were conducted by monitoring the emission intensity at 544.5 nm and subtracting any background emission due to a long wavelength "tail" of intrinsic protein fluorescence.

Both TE and CPE are reported in arbitrary units. We denote TE intensity by $I = I_L + I_R$ and CPE intensity by $\Delta I = I_L - I_R$ (where I_L and I_R are, respectively, the observed intensities of left and right circularly polarized emission). Although I and ΔI were measured in relative intensity units, absolute values of the ratio $\Delta I/(I/2)$ ($= g_{em}$ or emission anisotropy factor) were measured. This definition of g_{em} is twice that given in our previous protein papers.[8,9]

Unless stated otherwise the emission experiments were conducted at pH 6.5 in a piperazine buffer at 0.1 M ionic strength controlled with KCl. At pH 6.5 hydroxy complexes of Tb^{3+} do not appear[2] and Tb^{3+} does not complex to the buffer which contains only nitrogen donor atoms. The subtilisins and phospholipase of *Apis mellifica* were obtained from Sigma. Pronase is a Calbiochem product. The total light chains of rabbit myosin were furnished by Dr. S. Lowey; prothrombin and derivatives by Dr. G. L. Nelsesteun; S-100 by Dr. P. Calissano; the porcine phospholipases by Dr. G. H. deHaas; the *Crotalus adamanteus* phospholipase by Dr. M. A. Wells; the vitamin D dependent Ca binding protein by Dr. K. J. Dorrington; the pyrophosphatase by Dr. B. S. Cooperman; the Ca ATPase by Dr. C. M. Grisham; and the concanavalin A by Dr. G. N. Reeke. Other protein samples were high-grade commercial products.

Results and Discussion

For the first entry in Table II, carp parvalbumin, the results of an x-ray structure determination indicate that the aromatic ring of Phe-57 is closely juxtaposed to a Ca^{2+} in the EF site.[6] Upon substitution of Tb^{3+} for this Ca^{2+}, irradiation in the phenylalanyl absorption region at 259 nm yields energy transfer to Tb^{3+} and emission from Tb^{3+} in the 545-nm region which is partially circularly polarized.[7,8] Tb^{3+}-substituted troponin-C from two different species yields CPE spectra similar to that observed for carp parvalbumin, but in this case energy transfer to Tb^{3+} occurs from excitation of a tyrosyl residue.[8,9] Of the two tyrosyls in rabbit and three tyrosyls in bovine cardiac troponin-C, only one, Tyr-109 (111), is homologous in the two species. Furthermore, this tyrosyl residue is homologous with Phe-57 in carp parvalbumin. The closely similar CPE spectra and g_{em} values for all three species suggest that the face of the tyrosyl ring interacts with Tb^{3+} in troponin-C, as does the phenylalanyl ring in carp parvalbumin. Ionization of the tyrosyl phenolic group is not necessary to produce Tb^{3+} emission.

Table II. Tb^{3+}–Protein Emission Summary

Protein	Equiv of Tb^{3+}	Excitation	TE[a]	CPE[a]
Parvalbumin, carp	1	Phe	s	m
Troponin-C, rabbit skeletal	1	Tyr	s	m
Troponin-C, bovine cardiac	1	Tyr	s	m
Trypsinogen	1	Trp	m	n
Trypsin	1	Trp	m	n
Chymotrypsinogen	1	Trp	m	n
α-Chymotrypsin	1	Trp	m	n
δ-Chymotrypsin		Trp	m	n
Subtilisin Carlsberg	1	Tyr	m	n
Subtilisin BPN′	2	Tyr	m	n
Pronase		Trp	s	w
Elastase, porcine	1	Trp	vs	s
Collagenase	~20	Tyr + Trp	s	s
Prothrombin, bovine	11	Trp + Tyr	s	n
Prothrombin fragment I	7	Trp	m	n
S-100, calf brain	2	Trp	w	n
Prophospholipase A, porcine	1	Tyr	m	n
Phospholipase A, porcine and equine	1	Tyr	m	n
Phospholipase A, rattlesnake	1	Tyr	m	n
Phospholipase A, honey bee	1	Trp	m	n
Vitamin D Ca binding protein, porcine	1	Tyr	m	n
α-Amylase, bacterial	2	Trp	s	m
α-Amylase, porcine	2	Trp	s	w
Lysozyme, hen egg	1	Trp	w	n
Lactalbumin, bovine	1	Trp	w	n
Deoxyribonuclease, bovine	2	Trp + Tyr	s	n
Pyruvate kinase, rabbit	4	Trp	w	n
Creatine phosphokinase, rabbit		Trp	w	n
Pyrophosphatase, yeast	2	Trp	w	n
Ca ATPase, rabbit	2	Trp	m	n
Thermolysin	1	Trp	s	w
Concanavalin A		Trp	m	n

[a] vs, very strong; s, strong, m, medium; w, weak; n, none.

The emission properties characteristic of a Tb^{3+} ion interacting with an ionized phenolate group were investigated with the model ligand, *N,N*-ethylenebis[2-(*o*-hydroxyphenyl)]-glycine (EHPG).[10,11] This ligand contains two carboxylate, two amino, and two phenolic groups. Ionization of the phenolic groups in the free ligand shifts the absorption maximum from 276 nm at pH 6 to 296 nm at pH 12. In the presence of 1 equiv of Tb^{3+}, all six groups are in their basic forms by pH 8, where the absorption maximum appears at 291 nm. At pH 6 the absorption maximum occurs at 278 nm, indicating that Tb^{3+} has not displaced the phenolic protons. The corrected, integrated excitation spectra for Tb^{3+} emission at 545 nm display maxima at 294 nm at pH 8 and near 280 nm at pH 6. Thus an ionized phenolate group bound to Tb^{3+} yields an excitation maximum near 295 nm. Since energy transfer occurs from EHPG to Tb^{3+} at pH 6, where the absorption maximum at 278 nm indicates

that phenolic ionization has not yet taken place, the excitation maximum near 280 nm is evidently due to interaction of Tb^{3+} with the face of the aromatic chromophore.

Energy transfer from the face of an aromatic chromophore to Tb^{3+} yields excitation maxima at 259 nm for phenylalanine, 280 nm for tyrosine, and 295 nm for tryptophan. Three examples support a value near 280 nm for a facial tyrosine interaction: EHPG at pH 6, troponin-C discussed above, and porcine phospholipase A mentioned below. Interaction of Tb^{3+} with an ionized phenolate group yields an excitation maximum at 295 nm, in the same region as does the facial tryptophan interaction. We do not distinguish between this pair of possibilities in this paper. Since most of our experiments were performed near pH 6.5, tyrosyl phenolic ionization is unlikely to have occurred, and we identify an excitation maximum near 295 nm with a facial interaction between tryptophan and Tb^{3+}. Because of its weak absorption, phenylalanine would not be evident in energy transfer in the presence of significant contributions from tyrosine or tryptophan. The distinction between the last two aromatic side chains, the excitation spectra of which overlap, is made by comparing the intensity of Tb^{3+} emission upon excitation at 295 nm to that at 280 nm after correction for the relative spectral outputs of the lamp/excitation monochromator combination at 295 and at 280 nm. Emission intensity ratios, $I(295)/I(280)$, which are observed to be <0.9 implicate tyrosine, whereas ratios >1.2 indicate that tryptophan is involved in the absorption and energy-transfer process. The only examples with intermediate values (i.e., between 0.9 and 1.2) are those in which more than one Tb^{3+} is bound, so that several Tb^{3+} ions associated with both tryptophan and tyrosine are probably emitting.

Perusal of Table II indicates that a large number of proteins known to interact with Ca^{2+}, upon substitution of Tb^{3+}, yield the characteristic emission from Tb^{3+}. Of all the proteins tested only four—β-amylase, phosvitin, thrombin, and the total light chains from rabbit myosin—failed to yield Tb^{3+} enhanced emission. With α-casein, emission occurs from a large number of Tb^{3+}, but the protein displays an unusual photosensitivity. All proteins showed signs of intrinsic emission from tyrosine and tryptophan residues.

Emission (phosphorescence) in the 535–555-nm region originates with Tb(III) transitions derived from the $^5D_4 \rightarrow {}^7F_5$ free-ion radiative process. The $^5D_4 \rightarrow {}^7F_5$ transition is magnetic dipole allowed and one may anticipate, therefore, that it will exhibit substantial optical activity when Tb(III) resides in a chiral ligand environment. The degeneracy of this transition will be lifted in the presence of low symmetry ligand fields, and one may further expect to observe splittings in the associated spectral observables. The circularly polarized emission (CPE) spectra of carp parvalbumin and troponin-C reveal three components split out of the $^5D_4 \rightarrow {}^7F_5$ Tb(III) transition in the Tb(III)-protein complexes.[7-9] Although emission from Tb^{3+}-substituted Ca^{2+} proteins occurs, the data of Table II indicate that only occasionally are the circularly polarized components sufficiently strong to be detected in our experiments (i.e., $g_{em} > 10^{-3}$). The criteria for generation of observable CPE are uncertain. In order for the magnetic dipole allowed $^5D_4 \rightarrow {}^7F_5$ Tb^{3+} transition to become optically active, it is clear that this transition must be mixed with, or coupled to, an electric dipole allowed transition. Furthermore, the electric dipole transition vector must possess a component which is parallel to the magnetic dipole transition vector (associated with the $^5D_4 \rightarrow {}^7F_5$ Tb^{3+} transition). It is likely that these conditions will be satisfied if the Tb^{3+} ion is bound at a site in which the "crystalline field" environment provided by the protein (through donor group arrangement) is highly chiral or in which chromophoric groups with strong electric dipole allowed transitions (such as aromatic moieties) are dissymmetrically disposed about the Tb^{3+} ion. Interior protein sites

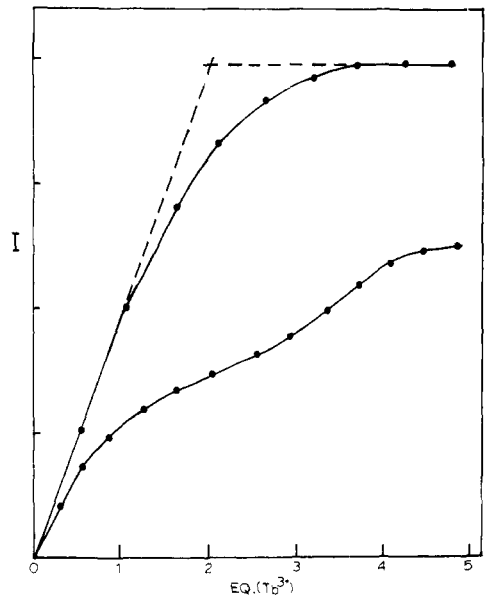

Figure 1. Tb^{3+} emission titrations of subtilisin BPN′ (upper curve) and subtilisin Carlsberg (lower curve). Excitation spectra for the Carlsberg enzyme indicate tyrosine as the absorber at 1 equiv while tryptophan also contributes at 4 equiv.

are most likely to provide one or both of these environmental features. Exterior binding sites will, in general, provide a coordination sphere which is in part occupied by nonchiral solvent molecules. Coordination by side chains of threonine, serine, cysteine, and aspartate residues and by amide carbonyl oxygens would place Tb^{3+} only a few bond lengths from an asymmetric carbon. However, the oppositely signed CPE curves for the muscle proteins and α-amylase on one hand, and elastase and thermolysin on the other (see below), all composed of L-amino acid residues, suggest that absolute configuration at asymmetric α-carbon atoms plays a lesser role in determining chirality at the Tb^{3+} binding sites than do other structural factors such as disposition of donor groups and spatial arrangement of aromatic groups in the immediate vicinity of the binding site.

Except for roles for Ca^{2+} in the activation of trypsinogen to trypsin and in the resistance of the latter to autolysis, metal ions are not usually considered with the serine proteases. Yet all the serine proteases listed in Table II clearly bind at least 1 equiv of Tb^{3+} according to the total emission titration curves. In a detailed emission study, trypsin has been shown to bind 1 equiv of Tb^{3+} and other Ln^{3+}.[12] Trypsinogen and chymotrypsinogen each show binding of 1 equiv of Tb^{3+}, but instead of leveling off, the titration curves for Tb^{3+} emission continue to rise after the addition of 1 equiv. The continuing rise is probably due to Tb^{3+}-aided activation of the zymogens to the enzymes, in which the Tb^{3+} ions are stronger emitters. Ln^{3+} ions are more effective than Ca^{2+} in accelerating conversion of trypsinogen to trypsin.[13]

Soybean and chicken ovomucoid trypsin inhibitors do not themselves give emission with Tb^{3+}, and equivalent amounts added to the Tb^{3+}-trypsin complexes are without effect on the Tb^{3+} emission. Excess of the inhibitor benzamidine reduces the Tb^{3+} emission of Tb^{3+}-trypsin to just less than half and shows little effect upon the emission from trypsinogen. The inhibitor formyltryptophan completely quenches the emission from chymotrypsin, but this could be due to radiationless energy transfer directly from the donor site to the inhibitor (thus blocking Tb^{3+} excitation) or possibly to mechanisms involving significant alterations of the metal ion binding site by inhibitor binding to the enzyme.

The emission titration curve for subtilisin Carlsberg is irregular and is shown in Figure 1. In addition to the tyrosine

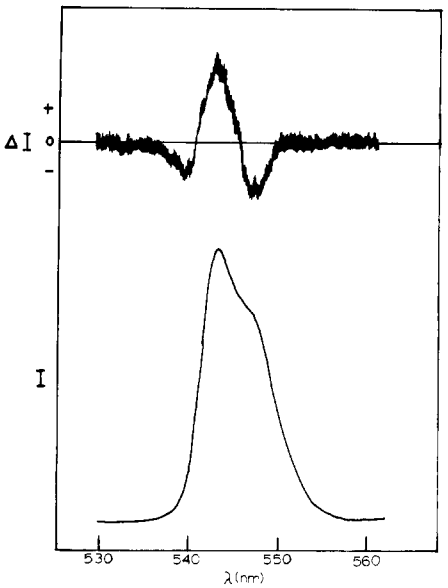

Figure 2. Elastase circularly polarized emission and total emission for Tb^{3+}. The emission anisotropy factors (g_{em}) for Tb^{3+}-elastase are +0.062 at 544 nm and −0.022 at 550 nm.

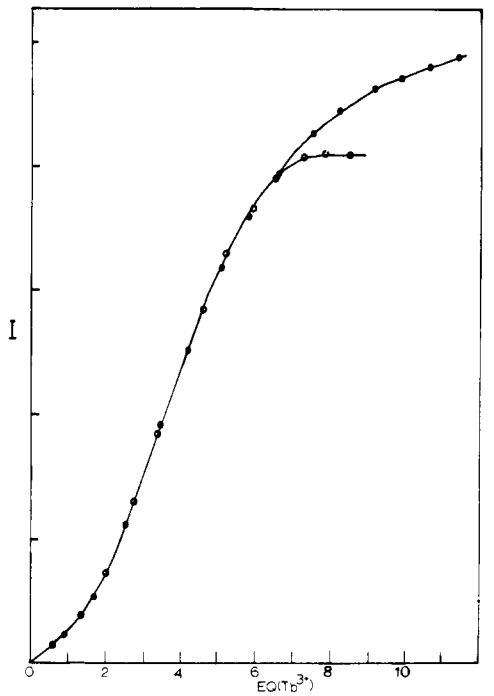

Figure 3. Tb^{3+} emission titrations of bovine prothrombin (solid circles) and prothrombin fragment 1 (open circles).

excitation at 1 equiv, the enzyme also shows both tyrosine and tryptophan excitation when a plateau in the emission intensity is reached at 4 equiv of Tb^{3+}. This result may be accounted for by weak binding of a second Tb^{3+} near the sole tryptophan residue. Though the emission titration curve for subtilisin BPN′ (Figure 1) suggests binding of 2 equiv of Tb^{3+}, it may also be accounted for by binding of 1 equiv (see introduction). Both subtilisins have recently been shown by another method to bind Ca^{2+}.[14]

The 545-nm emission intensity of Tb^{3+}-elastase is very strong. Furthermore, elastase is the only member of the serine protease class examined in the present study which yields CPE in the Tb^{3+} emission. The CPE spectrum of Tb^{3+}-elastase shown in Figure 2 is nearly the inverse (with respect to sign) of those observed for parvalbumin and for troponin-C.[8,9] The emission anisotropy factors (g_{em}) measured for Tb^{3+}-elastase are $g_{em} = +0.062$ (at 544 nm) and $g_{em} = -0.022$ (at 550 nm). Addition of excess inhibitor L-Ala-L-Ala-L-Ala yields a 50% enhancement of total emission (TE) at pH 6.5 and an increase in emission anisotropy factors to $g_{em} = +0.11$ at 544 nm and to −0.046 at 550 nm. Thus the protonated inhibitor enhances CPE more than TE at pH 6.5. At pH 8.1, the inhibitor and its *N*-acetyl and *N*-acetyl methyl ester derivatives cause nearly complete quenching of Tb^{3+} emission. Thus removal of the positive charge at the amino terminus leads to quenching of emission rather than its enhancement.

Though binding of Ca^{2+} to trypsin, chymotrypsin, and their zymogens has been suggested,[15] only one of two x-ray structure determinations of trypsin has revealed a specific Ca^{2+} binding site.[16] Gd^{3+} binds competitively to Ca^{2+},[17] suggesting that Ln^{3+} and Ca^{2+} occupy the same site. The strong similarity of the Tb^{3+} emission curves for trypsin and chymotrypsin and their zymogens suggests that there should also be a specific Ca^{2+} binding site in chymotrypsinogen and chymotrypsin. The binding of Tb^{3+} to the two subtilisins and elastase as indicated in Table II also suggests that specific Ca^{2+} binding may be a general property of serine proteases.

Calcium ions activate collagenase (*Clostridium histolyticum*) and several Ca^{2+} ions may be bound to the enzyme. Upon addition of Tb^{3+}, a weak emission occurs corresponding to energy transfer from both tyrosine and tryptophan, the intensity of which grows with each added equivalent, finally producing a weak circularly polarized emission after addition of a large excess of Tb^{3+}.

Prothrombin is known to bind about 11 Ca^{2+}, the first three cooperatively.[18,19] This behavior is also reflected in the Tb^{3+} emission titration, shown in Figure 3, which exhibits an induction region for the first 3 equiv and begins to level off at about 11 equiv, where both tryptophan and tyrosine contribute to energy transfer. At 5:1 and 18:1 Tb^{3+} to prothrombin molar ratios, the half-life for development of the full Tb^{3+} emission is an identical 9 min at 22 °C. Thus the rate-limiting process for Tb^{3+} binding is protein refolding. The amino terminal prothrombin fragment I yields a Tb^{3+} emission titration curve which is superimposable on that of prothrombin up to 7 equiv, where the curve for the fragment levels off (Figure 3). Thus, fragment I contains the strong Tb^{3+} prothrombin binding sites. The excitation spectrum indicates tryptophan as the predominant absorber in the plateau region of the fragment. The results for Tb^{3+} correspond closely to the 6 equiv of Ca^{2+} found to bind cooperatively to fragment I.[19] Intermediate I exhibits emission from four Tb^{3+} upon tryptophan excitation. No emission is produced upon addition of Tb^{3+} to thrombin.

Calf brain S-100 protein binds several Ca, two to high affinity sites at pH 7.6 which enhance fluorescence of the single tryptophan. K^+ competitively interacts with the Ca^{2+} sites as indicated by a cooperative increase of tryptophan fluorescence upon addition of Ca^{2+}.[20] Upon titration of the protein with Tb^{3+} at pH 6.5 or 7.5, irradiation of the tryptophan produces a weak Tb^{3+} emission which increases until 2 equiv is added when it abruptly levels off. The increase of this Tb emission exhibits an induction region in the presence of KCl as shown in Figure 4. Thus in the cases of prothrombin, its amino terminal fragment, and S-100 protein, subtle cooperative effects produced in tryptophan fluorescence by Ca^{2+} binding may also be evident in the induction regions of Tb^{3+} emission upon tryptophan irradiation. These results strongly suggest that Tb^{3+} is substituting in a Ca^{2+} site, which is close to a tryptophan, with a minimum of other alterations.

Porcine phospholipase A_2 and its zymogen bind one Ca^{2+} which influences the tyrosine but not the tryptophan difference spectra of the proteins.[21] Irradiation in the tyrosine region (280 nm) produces emission from added Tb^{3+}, the intensity of which levels off after the addition of 1 equiv. The emission intensities from Tb^{3+} are nearly identical in the porcine enzyme and its

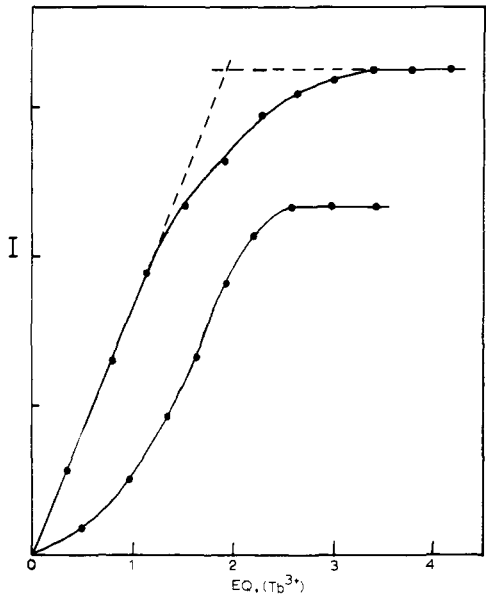

Figure 4. Tb^{3+} emission titrations for bacterial α-amylase (upper curve) and calf brain S-100 protein (lower curve, $I \times 5$). The titration for S-100 protein was performed at pH 7.5 in the presence of 0.1 M KCl. Equivalents of Tb^{3+} are per mole of protein.

Table III. Relative Intensities of Tb^{3+} Emission

		Phospholipase A	Zymogen
No analogue		1.0	1.0
Decylphosphorylcholine	<cmc[a]	1.4	1.1
	>cmc	2.8	1.9
Hexadecylphosphoryl-choline	>cmc	1.8	

[a] cmc = critical micelle concentration.

zymogen. The intensity from the equine enzyme is comparable, but that from the bovine enzyme is less than 10% as great. According to preliminary x-ray diffraction results the Ca(II) in porcine prophospholipase cannot be bound to the phenolic oxygen and is normal to the aromatic ring of Tyr-35.[22] Results for the addition of two substrate analogues, which do not undergo hydrolysis, below and above their critical micelle concentrations (cmc) are shown in the Table III. Though both the porcine enzyme and its zymogen substrates are of comparable effectiveness in catalyzing the hydrolysis of substrates at less than the cmc, the enzyme is much more effective at greater than the cmc.[23]

Phospholipase A_2 from rattlesnake and honey bee venoms also show emission upon binding of 1 equiv of Tb^{3+}. Energy transfer is from tyrosine in the snake and tryptophan in the bee venoms. A consistent sequence homology may be constructed which places Tyr-27 in the snake, Tyr-28 in the porcine, and Trp-8 in the bee enzymes in similar positions.[24] It will be interesting to learn if x-ray crystal structures show these residues to be near Ca^{2+} ions. The sequence homology with the bee venom may be overdrawn as the alignment proposed does not match the active centers.

The vitamin D dependent Ca^{2+} binding protein from porcine intestinal mucosa contains five Phe, one Tyr, and no Trp.[25] The bovine protein is similar.[26] Ultraviolet difference spectra obtained upon Ca^{2+} removal show peaks characteristic of a tyrosine perturbation.[25] Addition of Tb^{3+} to the Ca^{2+} free porcine protein produces Tb^{3+} emission which spans several equivalents but which may be interpreted as due to weak coordination of a single Tb^{3+}. A measurable CPE was not found.

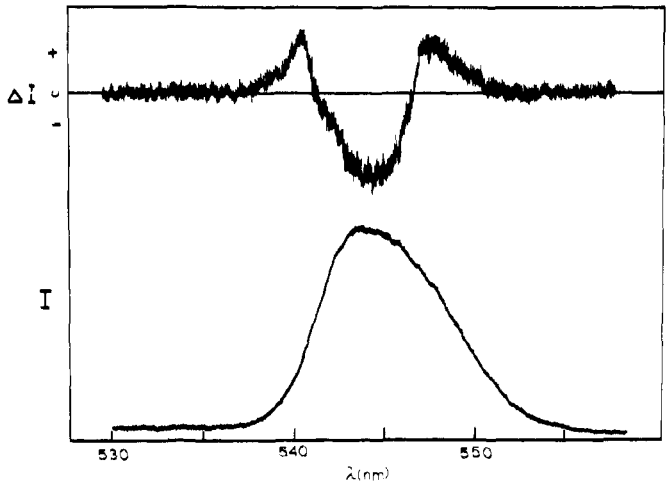

Figure 5. Bacterial α-amylase circularly polarized emission and total emission for Tb^{3+}. The emission anisotropy factors (g_{em}) are −0.062 at 544 nm and +0.030 at 550 nm.

Bacterial α-amylase consists of two identical subunits each of which is thought to bind two Ca^{2+}. Substitution of Ca^{2+} by Ln^{3+} yields active enzymes.[27] The emission titration curve levels off after the addition of 2 mol of Tb^{3+} per mole of protein (Figure 4) for both the bacterial and porcine enzymes. Thus at most only one of two Ca^{2+} appears to be displaced by Tb^{3+}. The Tb^{3+} emission in both enzymes is partially circularly polarized. The CPE and TE spectra for the bacterial enzyme are shown in Figure 5.

Lysozyme crystals are known to bind several metal ions including Gd^{3+} at a specific site near a tryptophan (108).[28] Lanthanides bound at this site have been shown to serve as chemical shift and broadening probes for the enzyme in solution.[29] When Tb^{3+} is added to a lysozyme solution a weak emission from Tb^{3+} appears upon tryptophan excitation. Addition of excess inhibitor *N*-acetylglucosamine, which binds near the metal ion site, doubles the intensity of the Tb^{3+} emission. This doubling may be accounted for by greater Tb^{3+} binding, a movement of Trp (108) closer to the bound Tb^{3+}, or increased protection of the bound Tb^{3+} from solvent quenching action. Lactalbumin exhibits photosensitivity.

Depending upon pH, deoxyribonuclease binds one to two Ca^{2+}, leading to a red shift in the near-ultraviolet absorption features attributable to tryptophan and tyrosine residues.[30] Though strong Tb^{3+} emission is produced by predominantly tryptophan (but also tyrosine) excitation, the enzyme is unusually photosensitive and was not investigated further.

Gd(III) and Tb(III) bind to pyruvate kinase to form ternary complexes with phosphoenolpyruvate.[31] The Tb^{3+} emission titration shows binding of 4 equiv of Tb^{3+} per mole of enzyme corresponding to one Tb^{3+} per each of four subunits. Addition of phosphoenolpyruvate, pyruvate, or ADP results in emission quenching. Creatine phosphopinase was too photosensitive for extended study but excess creatine enhanced the total emission and excess ADP reduced it.

Pyrophosphatase binds Gd^{3+} more strongly than Ca^{2+}.[32] The emission titration curve may be interpreted as due to binding of two Tb^{3+} per mole of enzyme or one Tb^{3+} per subunit. Addition of 1 or 2 equiv per mole of Tb–enzyme of the inhibitor methylene diphosphonate reduces the Tb^{3+} emission by one-half.

Thermolysin binds four Ca^{2+}, a pair at a double site and two singly at separate sites. X-Ray diffraction results show that three lanthanide ions replace all four Ca^{2+}, one Ln^{3+} replacing both Ca^{2+} in the double site.[33] Solution studies reveal that the first Tb^{3+} replaces the two Ca^{2+} at the double site and is strongly bound.[34] This Tb^{3+} is not displaced by EDTA which

removes the other two Ca^{2+} in the Tb^{3+} protein. Upon titration with Tb^{3+} the TE curve exhibits an irregular increase in intensity until 5 equiv has been added, in agreement with a published curve.[34] CPE also appears, but reaches full value after the addition of 1 equiv of Tb^{3+}. The CPE spectrum exhibits a shape similar to that of elastase in Figure 2 with emission anisotropy factors of +0.14 at 544 nm and −0.092 at 550 nm. The CPE is to be identified with the Tb^{3+} in the double site which has a tryptophan (186) nearby. Addition of excess EDTA and/or the inhibitor L-phenylalanyl-L-phenylalaninamide does not alter the CPE band shape or intensity. Thus removal of the other two Ca^{2+} and/or addition of an inhibitor do not alter sensitive details of environment about Tb^{3+} in the double site.

For concanavalin A the emission titration curve shows only weak Tb^{3+} binding upon tryptophan excitation. The emission is less quenched in D_2O indicating access of water to bound Tb^{3+}. A tryptophan side chain does not lie near the Ca^{2+} site of con A. Instead of displacement of Ca^{2+} by Tb^{3+}, we suggest binding of Tb^{3+} at Glu-87, where Sm^{3+} has been found in an x-ray diffraction investigation.[35] Energy transfer from a nearby tryptophan side chain to Tb^{3+} at Glu-87 may occur from Trp-182 and possibly Trp-88.

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