Circularly Polarized Luminescence from Terbium(III) as a Probe of Metal Ion Binding in Calcium-Binding Proteins†

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ABSTRACT: A number of different experimental techniques have been used to probe the details of structural changes on the binding of Ca(II) to the large number of known calcium–binding proteins. The use of luminescent lanthanide(III) ions, especially terbium(III) and europium(III), as substitutional replacement for calcium(II), has led to a number of useful experiments from which important details concerning the metal ion coordination sites have been obtained. This work is concerned with the measurement of the circularly polarized luminescence (CPL) from the $^5D_4 \rightarrow {}^7F_5$ transition of Tb(III) bound to the calcium binding sites of bovine trypsin, bovine brain calmodulin, and frog muscle parvalbumin. It is demonstrated that it is possible to make these polarization measurements from very dilute solutions (<20 μM) and monitor structural changes as equivalents of Tb(III) are added. It is shown that the two proteins that belong to the class of "EF-hand" structures (calmodulin and parvalbumin) possess quite similar CPL line shapes, whereas Tb(III) bound to trypsin has a much different band structure. CPL results following competitive and consecutive binding of Ca(II) and Tb(III) bound to calmodulin are also reported and yield information concerning known differences between the sequence of binding of these two species.

The trivalent lanthanide ions have found widespread use as substitutional replacements for Ca(II) ions in a fairly wide variety of protein systems, due to their chemical and physical similarities to Ca(II). Of special importance is the use of lanthanide(III) ions as spectroscopic probes of the metal ion coordination environment (Horrocks & Sudnick, 1981; Richardson, 1982; O'Hara, 1987). Whereas Ca(II) has no accessible transitions in the ultraviolet–visible part of the spectrum, due to its inert-gas electron configuration, a number of lanthanide(III) ions possess accessible, albeit weak, absorptions and emissions derived from intraconfigurational f $\leftrightarrow$ f transitions. By far, the most often used lanthanide ions in these kinds of studies are Tb(III) and Eu(III), because they are the most luminescent of the series. Two other useful features of these ions are that the hydrated ions are not very luminescent, resulting in the intensity of luminescence being a direct measure of binding, and that, even though the absorptions are weak, these ions can be efficiently excited either directly, using nondamaging visible laser light, or, in the case of Tb(III), indirectly through energy transfer from neighboring aromatic residues. In recent years, spectroscopic applications of lanthanide(III) luminescence to biochemical systems have, for the most part, been centered on using intermetal energy transfer to probe metal–metal site distances, and on luminescence lifetime measurements and site-selective Eu(III) excitation spectroscopy to probe metal ion coordination differences. The latter experiments are based on the ability to measure the small wavelength dependence of the position of the very sharp nondegenerate $^5D_0 \rightarrow {}^7F_0$ transition.

The first report of the measurement of the usually small net circular polarization in the luminescence (CPL) of lanthanides bound to proteins appeared in 1974 (Gafni & Steinberg, 1974). In this study CPL was measured in the emission of Tb(III) bound to transferrin and conalbumin. An extensive survey was reported by Brittain, Richardson, and Martin in 1976, and a detailed study of Tb(III) bound to trypsin was presented by Epstein et al. in 1977. More recently, we have published some preliminary results from our laboratory (Çoruh et al., 1988; Çoruh & Riehl, 1991). CPL is the emission analog of circular dichroism (CD) and, as such, reflects the chirality of the luminescent state (Riehl & Richardson, 1986; Richardson & Riehl, 1977). It is conventional to report CPL results in terms of the emission dissymmetry ratio, g_{em}. This quantity is related to the observed intensity of left (I_L) and right (I_R) circularly polarized light through the following simple relationship:

$$g_{em} = \frac{2\Delta I}{I} = \frac{I_L - I_R}{(1/2)(I_L + I_R)}$$

where ΔI is referred to as the differential emission intensity and I is the total intensity of emitted light. The factor of 2 present in this equation allows the emission dissymmetry ratio to be directly compared to the analogous quantity in absorption (circular dichroism) measurements, g_{abs}. In the case of CPL measured from lanthanide ions, no excited state/ground state structural differences are expected to be observed in simple excitation of f electrons, and therefore, the luminescence results also probe the ground-state conformation. The advantages of CPL over CD are in the selectivity afforded by localized luminophores in isolated spectral regions, and in the inherent sensitivity of luminescence techniques.

In the early experiments by Brittain et al. (1976), more than 30 different proteins were examined. The experiments were all performed with fairly concentrated protein solutions (>1 mM), with a large excess of Tb(III), and with excitation of aromatic residues in the ultraviolet. In this work the total emission intensity was correlated to whether or not the binding site was an external or internal site, and to the proximity of the coordination site to the donor aromatic residue. In approximately one-fourth of these proteins, CPL from Tb(III) was detected. The net circularly polarized emission intensities were also correlated with whether or not the metal ion site

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was on the exterior and, therefore, not in a very chiral environment, or in the interior, in which, presumably, the helical nature of the protein provided a more chiral coordination sphere for the lanthanide ion. Due to the inherent sensitivity and selectivity of luminescence techniques, and explicit dependence of optical activity on local and macromolecular conformation changes, CPL studies involving luminescent biomolecular systems have enormous potential at providing unique important information concerning secondary and tertiary structure of these systems.

A wide variety of proteins are known to bind Ca(II) and lanthanide(III) ions (Martin & Richardson, 1979). A number of these Ca-binding proteins are classified as having an "EF-hand" structure (Kretsinger & Nockolds, 1973). These all have a helix–loop–helix structure with a loop consisting of 6–12 residues, including several carboxylates in the coordination sphere of the metal, and almost all the loop ends are stabilized with a β-turn. The crystal structures of several of these have been determined [for example, Babu et al. (1985, 1988), Hertzberg and James (1985) and Moews and Kretsinger (1975)]. Only a very few of these structures, however, yield sufficient detail such that specific information concerning the coordination environment of the calcium binding sites is known, and of course, no specific information concerning the structure of the proteins in solution can be inferred from the crystal results. Much of the spectroscopic interest in proteins is concerned with developing reliable probes of conformation and conformational changes. The binding of metal ions has a clear effect on the conformation of Ca-binding proteins, as evidenced by the number of CD studies in the near- and far-UV (Closset & Gerday, 1975). Of special interest has been studies aimed at understanding the structural characteristics of the small acidic high-affinity multifunctional calcium-binding protein calmodulin (CaM). For example, CD studies have been reported for CaM, and from these results it has been concluded that there is a cooperativity in binding of the first two Ca(II) ions and that most of the conformational changes, as reflected in the CD of the phenylalanine and tyrosine residues, are complete after the addition of 2 equiv of metal ions (Crouch & Klee, 1980). Interest in the details of conformational changes as a function of metal ion concentration in this system has been increasing, since many recent studies have demonstrated that the regulation of CaM requires the intracellular control of Ca(II) concentration. In addition, during the last 5 years there have been several studies emphasizing the microconformational changes that occur following the binding of various species to Ca(II)–CaM and calcium-free CaM [for example, Bucigross and Nelson (1988)]. Other techniques aimed at elucidation of conformation of these and related systems include magnetic resonance (Levine et al., 1983) and, more recently, FTIR (Trewhalla et al., 1980; Jackson et al., 1991). The CD and FTIR techniques suffer from the disadvantage that one by necessity measures the average conformation, since so many absorbing species contribute to the observed signal. EPR measurements provide more localized information, but rely on the chemical attachment of spin labels.

In this work we report the measurement of CPL from Tb(III) bound to three calcium-binding proteins, namely, calmodulin, parvalbumin (Parv), and trypsin (Tryp). Two of these proteins (CaM and Parv) are very similar and possess an "EF-hand" structure; the third is from a quite different class of intestinal calcium-binding proteins. All three proteins have been extensively studied by other techniques, and their sequences and crystal structures are known. The important and unique aspect of this work is the ability to study these systems at very low concentrations (5–20 μM) of protein and Tb(III). The use of dilute protein solutions avoids problems associated with aggregate formation, and the fact that equally dilute Tb(III) concentrations can be studied allows one the ability to probe conformational effects as a function of individual metal ion binding. Special attention is given to CaM, in which it is generally accepted that Tb(III) and Ca(II) have a preference for different binding sites.

MATERIALS AND METHODS

CPL and total luminescence spectra were recorded on an instrument constructed in our laboratory operating in a photon-counting mode (Richardson & Riehl, 1977). Laser excitation of Tb(III) was accomplished at 488 nm with a Coherent INNOVA-70 argon ion laser. The emitted light was directed through a 50-kHz photoelastic quarter-wave modulator (Morvue), which together with a linear polarizer acts as a circular analyzer. The 50-kHz modulation in the transmitted beam corresponds to alternately left then right circularly polarized emitted light. The polarization-modulated beam was then focused onto the entrance slits of a 0.22-m double monochromator (SPEX) and detected by a cooled EMI-9558QB photomultiplier tube. Output pulses were passed through an amplifier/discriminator and counted by a specially built differential gated photon counter, which was phase referenced to the 50-kHz modulator frequency. The ratio of the differential count (corresponding to an up/down counter) to the total count (a separate up counter) was input to a personal computer after a fixed number of total counts were collected. $TbCl_3$ was obtained from Aldrich and used without additional purification. Tb(III) stock solutions were standardized using EDTA titrations with arsenazo indicator. Frog muscle parvalbumin, bovine pancreatic trypsin, and bovine brain calmodulin were purchased from Sigma. No interference due to residual chelating agent or other impurities was observed. All solutions were prepared in double deionized water using PIPES buffer (pH = 7) and 0.1 M KCl, and suitable care was taken to avoid metal ion contamination. Protein concentrations were determined spectrophotometrically.

RESULTS

The lowest-energy multiplets associated with the $4f^8$ electronic configuration of Tb(III) are shown in Figure 1 (each state is labeled according to the dominant $^{2S+1}L_J$ component). Transitions between these terms are intraconfigurational in nature and are, therefore, formally forbidden in the free ion. In solution, the emissive state for Tb(III) is the $^5D_4$ state as shown in this figure. Excitation of Tb(III) may be accomplished either through direct Ar ion laser excitation at 488 nm, by excitation of aromatic residues in close proximity to the binding site followed by radiationless transfer to an excited state of Tb(III), or, in a few cases, by metal–metal energy transfer between two different metal binding sites. The emission spectra of Tb(III) consist of a series of sharp isolated transitions arising from emissions to the manifold of J-levels within the ^7F ground-state term. The relative intensities and line splitting patterns (band structure) are known to be sensitive to the detailed nature of the ligand environment about the metal ion.

The most intense transition from Tb(III) is the $^5D_4 \rightarrow {}^7F_5$ transition centered at approximately 544 nm. This transition has two important characteristics that make it an ideal probe of metal ion coordination. First, since the states involved correspond to $J = 4$ and $J = 5$, there is the possibility of having

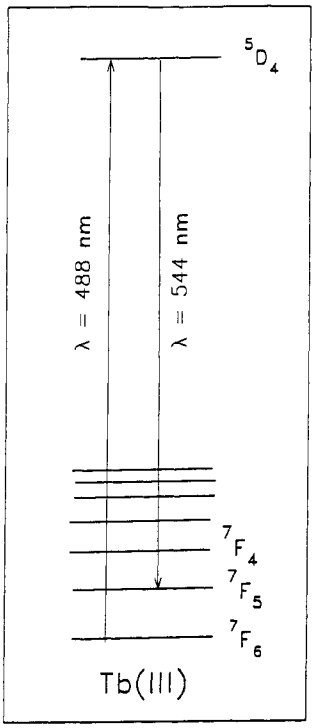

FIGURE 1: Schematic approximate energy level diagrams for Tb(III).

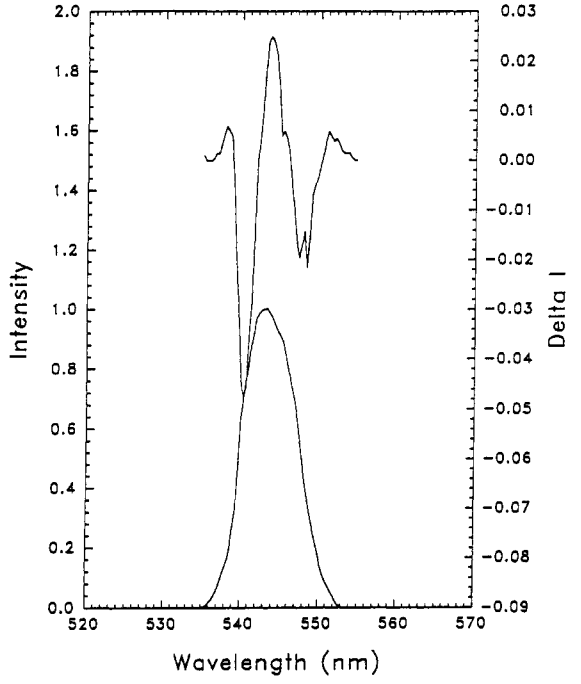

FIGURE 2: Circularly polarized luminescence (upper curve) and total luminescence (lower curve) for 10 μM trypsin plus 10 μM Tb(III) at pH = 7. Excitation wavelength = 488 nm.

9 crystal field components in the initial state ($2J + 1$) and 11 components in the final state in low-symmetry environments. Second, f ↔ f transitions that obey magnetic dipole selection rules, i.e., $\Delta J = \pm 1$, are expected to possess unusually large dissymmetry ratios (Richardson, 1980). These two attributes taken together can make this transition particularly sensitive to subtle structural changes in the chiral metal ion environment.

CPL from Tb(III) Bound to Trypsin. In Figure 2, we plot the measured total emission and CPL for 1 equiv of Tb(III) ion bound to bovine pancreatic trypsin. Tryp is known to have one Ca(II) binding site, and the binding is known to inhibit enzymatic autodigestion of the protein. The concentrations of Tryp and Tb(III) are 10 μM, and excitation was accomplished via the 488-nm line of an Ar ion laser. This wavelength corresponds to the transition $^7F_6 \rightarrow {}^5D_4$. The spectral region displayed corresponds to the $^5D_4 \rightarrow {}^7F_5$ transition. The CPL spectrum shown in this figure is very similar to the CPL measurement of Tryp–Tb(III) reported by Epstein et al. (1977), although, in their study, protein concentrations were between 0.05 and 0.1 mM, and Tb(III) ions were excited indirectly at 295 nm through tryptophan → Tb(III) energy transfer. These authors postulated the existence of one or more low-affinity secondary Tb(III) binding sites from observed changes in CPL line shape and intensity as a function of Tb(III) concentration. Small differences in the magnitude of the CPL between bovine trypsin and porcine trypsin were also noted by these authors; the line shapes for these two proteins were, however, very similar. As shown in this figure, the total emission spectrum, I (lower curve), shows no structure from the local crystal field; however, the CPL spectrum, ΔI (upper curve), consists of a number of peaks of varying signs and magnitudes.

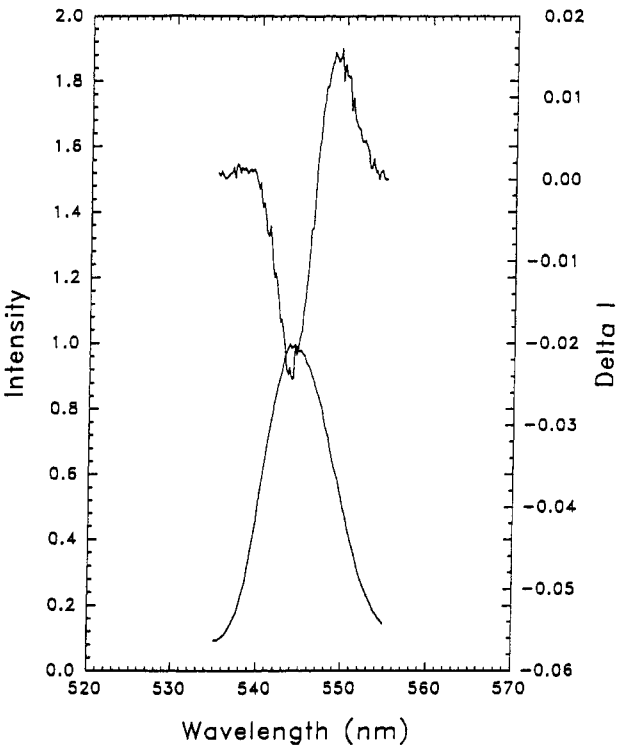

FIGURE 3: Circularly polarized luminescence (upper curve) and total luminescence (lower curve) for 4 μM calmodulin and 16 μM Tb(III) at pH = 7. Excitation wavelength = 488 nm.

CPL from Tb(III) Ion Bound to Calmodulin. The three-dimensional crystal structure of CaM has been known for many years (Babu et al., 1985, 1988), and numerous publications on the function and nature of metal binding have appeared in the literature. Calmodulin has four Ca(II) binding domains, two in the N-terminal region and two in the C-terminal region, each with a helix–loop–helix structure. Figure 3 shows the CPL and total luminescence spectra in the spectral region corresponding to the $^5D_4 \rightarrow {}^7F_5$ transition after addition of 4 equiv of Tb(III) ions. The results displayed are also for direct excitation of Tb(III) at 488 nm. The CaM concentration was 5 μM, and the pH = 7.0. It should be noted that all bound Tb(III) ions contribute to the spectra shown and that these results must be interpreted in terms of the average coordination environment of all four sites.

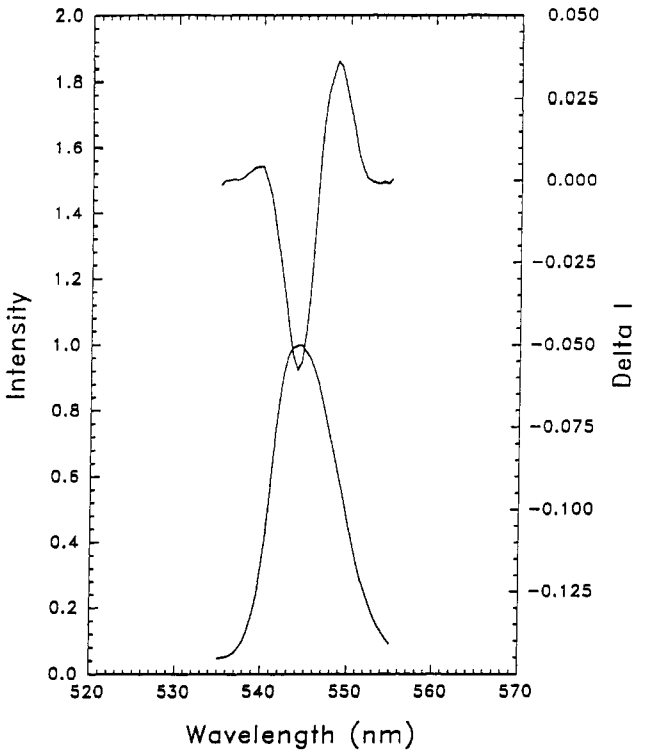

FIGURE 4: Circularly polarized luminescence (upper curve) and total luminescence (lower curve) for 5 μM parvalbumin and 10 μM Tb(III) at pH = 7. Excitation wavelength = 488 nm.

CPL from Tb(III) Ion Bound to Parvalbumin. Parvalbumins possesses 6 α-helical regions (A–F); binding loops CD and EF are known to bind Ca(II). The helix–loop–helix structure of these regions also has the so-called "EF-hand" conformation. The CPL and total luminescence spectra of a solution containing 2 equiv of Tb(III) ion in 5 μM parvalbumin are shown in Figure 4. The Tb(III) emission displayed again is in the $^5D_4 \rightarrow {}^7F_5$ region, and the sample was excited directly to the $^5D_4$ state with 488-nm radiation. The CPL line shape of Tb(III)–Parv looks very similar to the CPL of Tb(III)–CaM as shown in Figure 3.

Titration of Tb(III) Ions into Parvalbumin and Calmodulin. At the low concentrations used in the studies reported here, an extremely long time (>20 h in some cases) is required to collect a sufficient number of photons such that a complete CPL spectrum can be determined with sufficient precision. Although use of visible laser excitation virtually eliminates photochemical damage to the proteins under investigation, it is still not possible to perform a long series of experiments on one sample that might require the measurement of several CPL spectra lasting for several days, due to slow gradual photodegradation of the sample. However, information concerning changes in coordination environment can be probed by monitoring the CPL at specific wavelengths. For example, in systems in which more than one metal binding site exists, it is possible to monitor the dissymmetry factor at the peak wavelength as a function of equivalents of added metal ion. Results from just such an experiment are shown in Figure 5, in which the emission dissymmetry ratio, g_{em}, measured at the total intensity maximum (543.5 nm), is plotted (filled circles) as a function of equivalents of Tb(III) added to a solution of parvalbumin. The excitation wavelength was again 488 nm. g_{em} is a direct measure of the chirality around the coordination sphere of the metal ion, and thus, variations in sign and magnitude are directly related to the occurrence of local conformational changes. It is important to note again that, in general, all bound Tb(III) ions contribute to the measured g_{em}. Thus, any observed differences in the value of g_{em} could be due to differences in chirality of the two sites, or due to protein conformational changes taking place as a function of metal ion addition. From the results displayed in this figure, it is apparent that the chirality around the metal binding sites does not change appreciably during the addition of Tb(III). This is consistent with a cooperative model of metal ion binding in which each protein has essentially either 0 or 2 equiv of bound metal ion, or an alternative explanation is that the local chirality of the two different sites are the same within the accuracy of this measurement.

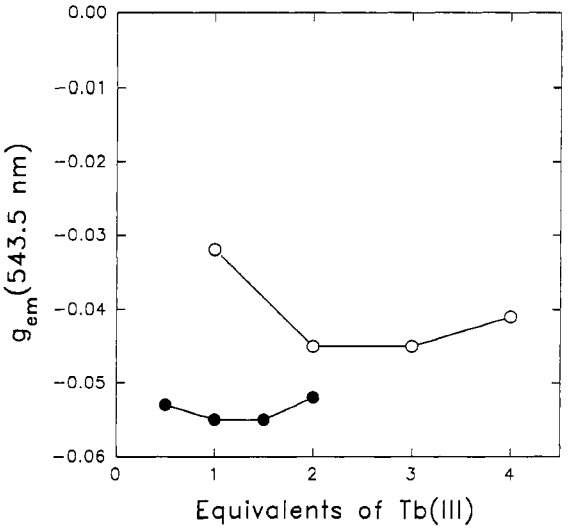

FIGURE 5: Emission dissymmetry ratio, g_{em} (543.5 nm) versus equivalents of Tb(III) for 5 μM parvalbumin (filled circles) and 4 μM calmodulin (open circles).

In Figure 5 we also show the changes in the Tb(III) emission dissymmetry ratio at 543.5 nm upon metal binding to 10 μM CaM (open circles). As can be seen in this figure, g_{em} decreases from 0 [no Tb(III) present, and therefore no emission] and reaches a minimum after 2 equiv have been added. Additional equivalents of Tb(III) lead to more total emission (not displayed in this figure), but g_{em} slightly decreases in magnitude. This result is especially noticeable in the results plotted for the fourth equivalent. As mentioned above, CD results for the addition of Ca(II) to CaM have also indicated that this protein has essentially attained its final conformation after the addition of 2 equiv of metal ion (Crouch & Klee, 1980).

Differences between the Binding of Ca(II) and Tb(III) to Calmodulin. Figure 6 presents the total emission spectra for Tb(III) ion binding to calmodulin. In this experiment Tb(III) excitation was accomplished through indirect excitation at 280 nm. This wavelength corresponds to the maximum in the absorption of two tyrosine residues which are in close proximity to the binding sites designated III and IV in calmodulin. Therefore, an increase in the Tb(III) luminescence can be attributed to Tb(III) binding at sites III and IV. Note that, as can be seen in this figure, very little emission is observed until more than 2 equiv of Tb(III) are added. This result has been described previously (Wang et al., 1982; Wallace et al., 1982). A proportional increase in luminescence intensity can be observed as additional Tb(III) is added. During this experiment, 30-min periods were allowed for complete binding of each equivalent of Tb(III) ions before recording the emission spectrum. Clearly, the binding of Tb(III) in calmodulin is first to the sites (designated I and II) which are not close to the tyrosine residues, and not until more than 2 equiv have

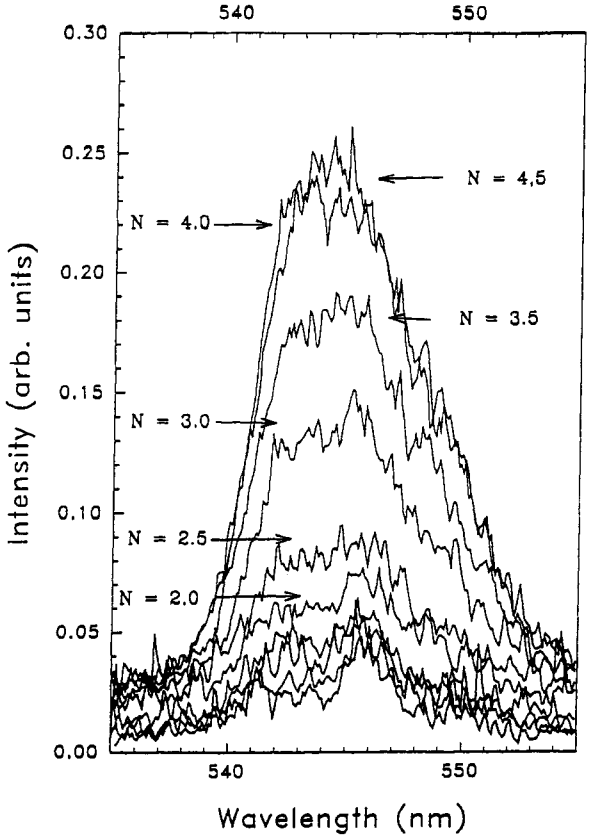

FIGURE 6: Total emission from Tb(III)–CaM. *N* equals the number of equivalents of Tb(III). Excitation wavelength = 280 nm.

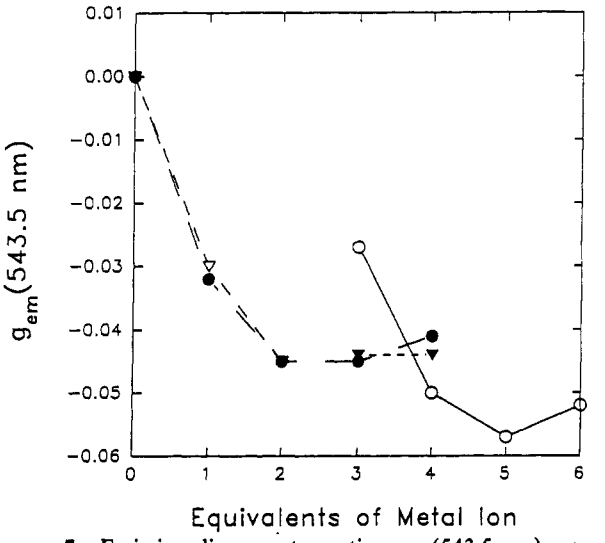

FIGURE 7: Emission dissymmetry ratio, g_{em} (543.5 nm), versus equivalents of Tb(III) for 10 μM calmodulin. Filled circles denote Tb(III) additions; open circles denote addition of Tb(III) to Ca_2–CaM; open triangles denote Tb(III) addition to apocalmodulin followed by (filled triangles) Ca(II) additions to Tb_2–CaM.

been added does a significant amount of this ion bind to sites III and IV.

In order to probe the specific differences between the binding sites in CaM, and between the differences in binding between Ca(II) and Tb(III), we have performed a number of competitive and sequential binding experiments in which g_{em} at the total emission maximum (543.5 nm) was monitored. Results are presented in Figure 7, in which g_{em} at 543.5 nm is plotted as a function of equivalents of added metal ion for a series of consecutive metal ion additions. In this figure, filled circles represent the addition of equivalents of Tb(III) to CaM (this is the same data plotted in the Figure 5); open circles represent Tb(III) additions to a solution of CaM containing 2 equiv of Ca(II); and open and filled triangles show the results for Tb(III) additions to CaM followed by Ca(II) additions to $Tb(III)_2$–CaM, respectively. A number of conclusions can be gleaned from these data. Addition of 1 equiv of Tb(III) to CaM which already has 2 equiv of bound calcium ions yields the same value for g_{em} obtained when Tb(III) was added without Ca(II) present. This suggests that Ca(II) and Tb(III) are binding to different domains in this protein. As mentioned above, this has been the subject of some controversy in the literature, and it is now believed, from a number of other experiments, that this is indeed the case. These same data also illustrate that there is little effect on the chirality of the terbium binding site (presumably site I) by the presence of Ca(II) bound to sites III and IV. Note that a second equivalent of Tb(III) to Ca_2–CaM also yields a g_{em} value close to that obtained without Ca(II) being present.

Additional information can be obtained by comparison of the g_{em} values when 2 Tb(III) equiv are added to Ca_2–CaM (first two open circles) to the results for the addition of 2 equiv of Ca(II) to Tb_2–CaM (filled triangles). The addition of 2 equiv of Ca(II) to Tb_2–CaM has virtually no effect on the chirality of the Tb(III) binding sites. It should be noted that, in experiments in which equivalents of metal ions are added to cuvettes containing protein, the precision of the results is quite high. For the measurements described here, the standard deviation (or error) in g_{em} values may be obtained from simple photon statistics (Richardson & Riehl, 1977). The error in these data is approximately 2×10^{-3} for g_{em} values of around 5×10^{-2}. However, for different sample preparations, as combined in this figure, we estimate an actual uncertainty for g_{em} at these extremely low concentration samples of approximately 15%. Caution must be observed when monitoring g_{em} at only one wavelength, since changes in band structure brought about by significant coordination geometry changes might go undetected. For this reason, complete total emission and CPL scans have been performed at regular intervals during the course of the metal additions. In Figure 8 we display the CPL and total emission spectra for the equivalent additions of 2 Tb(III) and 4 Tb(III) ions to Ca_2-CaM. These spectra required more than 24 h of data collection. The g_{em} ratios as plotted in Figure 5 and as calculated from these figures are within the error estimate (15%) given above. As can be seen in this figure, the CPL line shapes for the two spectra are virtually identical and are indistinguishable from the spectra displayed in Figures 3 and 4. This similarity in line shapes is also seen in CPL measurements following the addition of only 1 equiv of Tb(III), and therefore, even though this site is associated with a smaller $|g_{em}|$ value, the overall chiral environment as reflected in crystal field splitting and CPL line shape is very similar to that of the other three sites.

As discussed above, and as shown in Figure 7, a significant contribution to the Tb_2–CaM conformation was not observable upon Ca(II) ion addition. It should also be kept in mind that, even if there is a further change in the conformation, it may not affect the binding domains which are occupied by Tb(III) ions, simply because the observation is limited to the binding sites which were occupied by Tb(III) ions. Although not large, small differences in the spectral region associated with changes in α-helicity can be recognized upon Ca(II) ion additions to Tb_2–CaM from CD measurements.

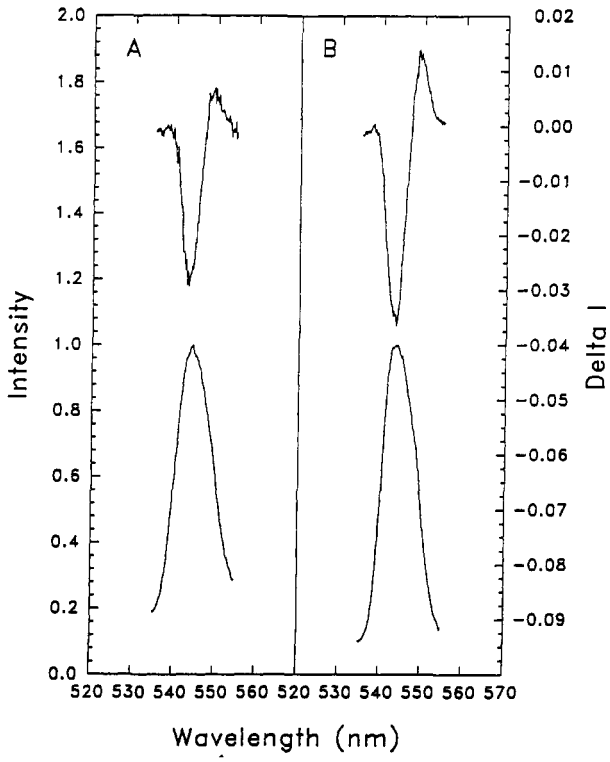

FIGURE 8: Circularly polarized luminescence (upper curves) and total luminescence (lower curves) for 10 μM calmodulin (A) corresponds to the addition of 2 equiv of Tb(III) and 2 equiv of Ca(II); (B) corresponds to the addition of 4 equiv of Tb(III) and 2 equiv of Ca(II). pH = 7, excitation wavelength = 488 nm.

DISCUSSION

CPL spectroscopy involving Tb(III) ions has been shown to be a useful probe for the solution structure and dynamics of optically active complexes in solution. Variations in crystal field splittings, polarizations, and intensities have provided useful information concerning the coordination environment of the central Tb(III) ion and on the existence of multiple emitting species. Comparison of the CPL measured for Tb(III) bound to Tryp to that of CaM and Parv illustrates the usefulness of this technique to probe differences in coordination environment. The binding site in Tryp is very much different than that in the "EF-hand" structures. Although the total emission spectra for the three species are indistinguishable, the magnitude and polarization of the crystal field transitions result in very different CPL spectra. Considering the very complex nature of the transition involved ($^5D_4 \rightarrow {}^7F_5$), it is remarkable that the CPL line shapes for this transition are essentially identical for Tb(III)–CaM and Tb(III)–Parv. Furthermore, it is even more striking that the CPL results shown for various Tb(III)–Parv and CaM ratios show little variability in line shape as the number of bound Tb(III) ions increases. Variations in magnitude are observed as a function of equivalents of Tb(III), and these must be interpreted in terms of the average CPL from each emissive Tb(III).

The observed similarities in CPL line shape for Tb(III) bound to CaM and Parv indicate that the coordination environments in these two proteins are very closely related. Previous structural studies have determined that sites III and IV of CaM are very similar to the two binding sites in the CD and EF domains of parvalbumin (Cheung, 1982). Sites I and II of CaM also possess the "EF-hand" structure, with the major difference being that site I possesses an extra carboxylate group and, therefore, an extra negative charge. These observations are very much consistent with evolutionary speculations concerning these two proteins. Additional studies are underway on other Ca binding proteins that possess this type of structure in order to determine if measurement of CPL from Tb(III) can be used as an easy diagnostic probe of the existence of an "EF-hand" type binding site.

Although it has not been established that differences between the four Ca(II) binding sites in CaM are physiologically relevant, a number of researchers have been concerned with the sequence of binding. Special attention has been given to comparing the binding sequence of Ca(II) ion with that of Ln(III) ions, particularly Tb(III). One obvious focus of these studies is to investigate the reliability of Tb(III) as a fluorescent probe in this and other related systems. For example, kinetic results on this topic (Martin et al., 1986) indicate that Tb(III) [and Eu(III)] have a higher affinity for sites I and II (than III and IV) and that this is, indeed, opposite to the affinity of Ca(II). As shown in Figure 7, tyrosine-sensitized luminescence of Tb(III), in which excitation at 280 nm leads to Tb(III) fluorescence through radiationless energy transfer, also leads one to the conclusion that the Tb(III) ions are not binding to the sites in close proximity to the tyrosine residues until more than 2 equivalents have been added. The two tyrosine residues are in close proximity to sites III and IV. The generally accepted order of binding for Tb(III) is, therefore, I and II, and then III and IV. The data displayed in Figure 7 are also consistent with a situation in which Tb(III) and Ca(II) ions are not competing for the same sites. That is, the addition of Ca(II) has no effect on the chirality of Tb(III), and the addition of Tb(III) to a solution already containing 2 equiv of Ca(II) yields the same result as that of a solution which contained no Ca(II).

As discussed previously, CD measurements have indicated that some cooperativity exists in the binding of the first two Ca(II) ions to CaM (Crouch & Klee, 1980). From the data presented in Figures 5 and 8, it is apparent that there is no strong cooperativity in the binding of the first 2 equiv of Tb(III), since if metal binding to the first site caused binding to the second site to be much more favorable than binding to another molecule of CaM, then g_{em} (which is an intensive quantity) should be constant during the first equivalent additions, and this is not observed. From the observed decrease in g_{em} from 0 to 2 equiv of added Tb(III), one can conclude that the intrinsic value for g_{em} is different for the two sites. Recent site-selective experiments on the binding of Eu(III) to calmodulin conducted in our laboratory (Çoruh, 1991) have indicated that the first binding site for Eu(III), and presumably Tb(III), is site I. From previous studies, and as shown in this work, the binding of the first Tb(III) ion in site I does have has a clear effect on the binding affinity of Tb(III) in site II, since no binding to sites III or IV is observed until more than 2 equiv have been added. As described above, site I is a significantly different chemical environment than the other three sites, which are very similar. Nevertheless, binding in site I clearly makes binding in site II much more favorable than binding in sites III or IV. No further details can be learned concerning this cooperativity from these experiments, since even though sites I and II are clearly different, the contribution to g_{em} from each site is unknown.

One of the major questions in CaM binding studies, such as this one, is why do Ca(II) and Tb(III) prefer different sites? Falke et al. (1991) have very recently presented results on the binding affinities of a large number of metal ions on the EF-hand-like binding site in *Escherichia coli* D-galactose and D-glucose receptor in which very large differences in the

binding constants between +2 and +3 ions were observed following synthetic modification of the binding loop. For purposes of our work, the most interesting result presented by these authors is the fact that although the binding of Tb(III) changed very little as a result of changing the size and charge of one of the loop residues, the binding constant of Ca(II) varied more than 5 orders of magnitude. The interpretation of these results by Falke et al. is based on a very simple model in which the Coulombic attractive forces of the positively charged central ion with the negatively charged donor oxygens are balanced against the Coulombic repulsive interactions between the donor oxygens. The range of charge separation distances is, of course, controlled to some extent by the size of the binding site cavity. This type of analysis may be used to explain the differences in binding affinity observed in CaM, where site I has an extra negative charge as compared to the other three sites. Model calculations on the relative contributions of the Coulombic interactions in the various sites of CaM are currently underway.

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