CIRCULARLY POLARIZED LUMINESCENCE AS A PROBE OF METAL IONS BINDING SITES IN CALMODULIN*

Nursen CORUH and James P. RIEHL**

Department of Chemistry, University of Missouri-St. Louis, St. Louis, MO 63121, U.S.A.

Received July 19, 1991
Accepted July 23, 1991

Circularly polarized luminescence (CPL) from dilute solutions of Tb(III) bound to the Ca-binding protein calmodulin is reported. The dissymmetry ratio, g_{em}, at 543·5 nm can be monitored as a function of equivalents of metal ion concentration. Competitive and consecutive addition of Ca(II) versus Tb(III) yield results which are consistent with previous results that suggest that Tb(III) and Ca(II) have a preferred affinity for different metal-ion binding sites in this protein.

Due to the inherent sensitivity and selectivity of luminescence techniques, and the explicit dependence of optical activity on local and macromolecular conformation changes, it is our belief that circularly polarized luminescence (CPL) studies involving luminescent biomolecular systems will be among the most important future applications of this spectroscopic technique. In this work we describe specific applications of CPL to only one such system, viz., the ubiquitous calcium binding regulatory protein calmodulin. This system is in many ways prototypical of the type of system for which, we believe, CPL is uniquely suited, and justifies the specific emphasis on calmodulin described in this pilot study. Calmodulin has the additional advantage that it is well characterized[1-3], so that the CPL results can be compared to those obtained by other chemical and spectroscopic methods to ascertain the importance and usefulness of this type of spectroscopy used in this type of investigation.

A large amount of research has been devoted to understanding and characterizing the conformational changes that calmodulin undergoes when it binds to Ca(II) and other metal ions, as well as a range of enzymes and drugs. Although it has not been established that differences between the four Ca(II) binding sites are physiologically relevant, a number of researchers have been concerned with the sequence of binding. Special attention has been given to comparing the binding of Ca(II) ion with that of luminescent lanthanide(III), particularly Tb(III) (refs[4-7]). The most recent results on this topic, which indicate that Tb(III) [and Eu(III)] have a higher affinity for sites I and II (than III and IV), and that this is opposite to the

* Presented at the 3rd International Conference on CD Spectroscopy, August 1989, Prague, Czechoslovakia.

** Address correspondence to this author.

affinity of Ca(II), is somewhat surprising, and apparently controversial[8]. Obviously, this result has serious implications in other studies using Tb(III) as a substitutional probe of Ca(II) binding sites.

EXPERIMENTAL AND RESULTS

Calcium-free bovine brain calmodulin (CAM) (SIGMA) was prepared using double deionized water and suitable care was taken to avoid contamination with metal ions. Solutions were buffered at a pH of 6·9 using PIPES, and KCl added to a concentration of 0·5 mol l^{-1}. Concentrations of CAM were determined by absorption at 277 nm (ε 3 300). Total emission and CPL were recorded on a differential photon counting instrument constructed in our laboratory. Quartz sample cells (volume 300 ml, pathlength 0·4 mm) were used in the work reported here. CPL spectra (I and ΔI) for a 7 μM solution of CAM containing two equivalents of Tb(III) is presented in Fig. 1. Excitation of Tb(III) was via the 488 nm line of a Coherent INNOVA-4 Argon-ion laser, corresponding to the $^7F_6 \rightarrow {}^5D_4$ absorption, and emission was monitored via the $^7F_5 \leftarrow {}^5D_4$ transition (λ_{max} 543·5 nm).

The binding of metal ions has a clear effect on the conformation of CAM as evidenced by a number of CD studies in the near and far UV (refs[9,10]). Mammalian CAM has only two tyrosine residues and no tryptophans, thus CD in the spectral range from 250–310 nm is interpretable in terms of changes in the chiral environment only in the vicinity of tyrosine-99 and tyrosine-138. Titration studies have

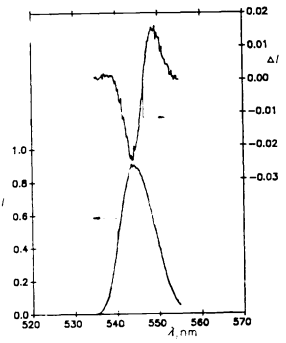

FIG. 1

Circularly polarized luminescence (ΔI) and total emission (I) for a 7 μM solution of calmodulin containing 2 equivalents of Ca(II) and 2 equivalents of Tb(III). λ_{exc} 488 nm

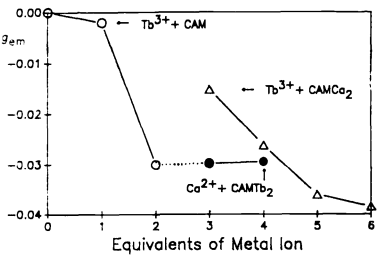

FIG. 2

Emission dissymmetry ratios g_{em} at 543·5 nm ($^7F_5 \leftarrow {}^5D_4$) from Tb(III) bound to calmodulin (concentration 7 μmol l^{-1}) as a function of equivalents of metal ion [Ca(II) and Tb(III)]. λ_{exc} 488 nm

demonstrated that there is a cooperativity in binding of the first two metal 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 two equivalents of metal ions. This result is in agreement with recent experiments from our laboratory in which the CPL from Tb(III) was monitored as a function of equivalents of metal ion added to an initially metal-free calmodulin sample[11].

Tyrosine-sensitized luminescence of Tb(III), in which excitation at 280 nm leads to Tb(III) fluorescence through radiationless energy transfer, as well as direct excitation of Tb(III) have been widely used[4,5]. The two tyrosine residues are in close proximity to sites III and IV, thus sensitized luminescence of Tb(III) can be used to distinguish between the pairs of sites, and establish the order of binding for this ion[12]. The generally accepted result is I and II, then III and IV.

Additional information about this system can be obtained through competitive or consecutive binding of Ca(II) and Tb(III). In Fig. 2, the dissymmetry ratio, $g_{em} = \Delta I/(I/2)$, at 543·5 nm is plotted as a function of equivalents of metal ion. As can be seen from this data, the addition of 2 equivalents of Ca(II) has little effect on the CPL from previously bound Tb(III). In this figure, it is also seen, that after two equivalents of Tb(III) have been added to CAM–Ca_2, g_{em} is essentially the same as that measured in the previous procedure, suggesting that reversing the order of addition does not affect the location of Tb(III) and Ca(II) binding. This result is consistent with several previous studies in which it has been concluded that Ca(II) and Tb(III) prefer to bind to different pairs of sites. Continued addition of Tb(III) results in replacement of Ca(II) by Tb(III) as evidenced by the decrease in the dissymmetry ratio.

DISCUSSION

It is important to emphasize that the spectra displayed in Fig. 1, and the g_{em} data plotted in Fig. 2, are for very dilute solutions, approaching physiological concentrations. The individual points plotted in Fig. 2 are the mean of 20 g_{em} readings each consisting of $2 \cdot 10^5$ photons, and required in some cases a 1 h collection time. In order to ensure that the single wavelength results displayed in Fig. 2 were not simply due to small frequency or lineshape changes, complete spectra were obtained for selected samples. Complete spectra, as in Fig. 1, require two full days of data collection. Nevertheless, these experiments are important, and illustrate the usefulness of the CPL technique as a very sensitive and specific probe of conformational structure in biochemical systems. Additional selectivity can be obtained by combining the measurement of CPL with wavelength- and polarization-selective excitation spectroscopy. These experiments are in progress.

REFERENCES

1. Potter J. D., Strang-Brown P., Walker P. L., Iida S.: Methods Enzymol. *107*, 135 (1983).
2. Cox J. A., Comte M., Malone A., Burger D., Stein E. A.: Met. Ions Biol. Syst. *17*, 215 (1984).
3. Forsen S., Vogel H. J., Drakenberg T. in: *Calcium and Cell Function* (W. Y. Cheung, Ed.), Vol. 6, p. 113. Academic Press, New York.
4. Kilhoffer M. C., Demaille J. G., Gerand D.: FEBS Lett. *116*, 269 (1980).
5. Kilhoffer M. C., Gerard D., Demaille J. G.: FEBS Lett. *120*, 99 (1980).
6. Lamboog P. K., Steiner R. F., Steinberg H.: Arch. Biochem. Biophys. *217*, 517 (1982).
7. Kretzinger R. H., Rudnick S. E., Weismann L. J.: J. Inorg. Biochem. *28*, 289 (1986).
8. O'Hara P. B.: Photochem. Photobiol. *46*, 1067 (1987).
9. Dedman J. R., Potter J. D., Jackson R. L., Johnson J. D., Means A. R.: J. Biol. Chem. *252*, 8415 (1977).
10. Crouch T. H., Klee C. B.: Biochemistry *19*, 3692 (1980).
11. Coruh N., Hilmes G. L., Riehl J. P.: J. Lumin. *39*, 3203 (1988).
12. Buccigross J. M., O'Donnell C. L., Nelson D. J.: Biochem. J. *235*, 677 (1986).