4 D. Keilin and E. F. Hartree, *Proc. Roy. Soc., B, 127,* 167 (1939).
5 J. D. Alben, P. P. Moh, F. G. Fiamingo and R. A. Altschuld, *Proc. Nat. Acad. Sci., 78,* 234 (1979).
6 T. H. Stevens, G. W. Brudvig, D. F. Bocian and S. I. Chan, *Proc. Nat. Acad. Sci., 76,* 3320 (1979).

B24

Europium(III) Laser Luminescence Excitation Spectroscopy of Calcium-Modulated Proteins: Parvalbumin and Calmodulin

WILLIAM DEW. HORROCKS, Jr.*, PAUL MULQUEEN, MOO-JHONG RHEE, PATRICK J. BREEN and ERICH K. HILD

Department of Chemistry, The Pennsylvania State University, University Park, Pa. 16802, U.S.A.

Tripositive lanthanide ions, symbolically Ln(III), have been used for more than a decade to probe calcium-binding sites in proteins [1]. Efforts in this laboratory [2] have been devoted to the development of methods to exploit the luminescence properties of certain of the lanthanide ions [3, 4], notably Eu(III) and Tb(III), in the study of this class of protein. Central to our research is the use of a tunable, pulsed dye laser to excite directly the metal ion levels. The Eu(III) ion, with its nondegenerate $^7F_0$ ground state, is particularly useful as a probe species. Excitation spectroscopy of the $^7F_0 \rightarrow {}^5D_0$ transition in the 578–581 nm region (monitored via the $^5D_0 \rightarrow {}^7F_2$ emission at ~612 nm) has been useful in detecting and characterizing individual, distinct Eu(III) ion binding sites in proteins [2, 5]. The observation of more than one peak in such an excitation spectrum is unambiguous evidence for the existence of more than one type of binding site. In addition, sites can be characterized via their Eu(III) (or Tb(III)) excited state lifetimes in H_2O and D_2O solution in order to determine how many water molecules are coordinated to the ion at each site [6]. Laser spectroscopic methods can also be used to measure distances between Ca(II) ion binding sites by monitoring Förster-type nonradiative energy transfer between emissive (Eu(III), Tb(III)) and absorbing (Pr(III), Nd(III), Ho(III), Er(III)) ions occupying nearby sites [7–9].

Calcium-modulated proteins are an important class of macromolecule found in the cytosol [10]. These proteins, which include calmodulin, troponin C, myocin light chains, parvalbumin and intestinal calcium-binding protein, all bind Ca(II) ions with pK_d values ($-\log K_d$) between 5.0 and 6.5 in the presence of millimolar concentrations of Mg^{2+}. Ln(III) ions bind even more tightly and spontaneously replace bound Ca(II) ions. Two members of this class of protein, namely parvalbumin and calmodulin, are the objects of the present study.

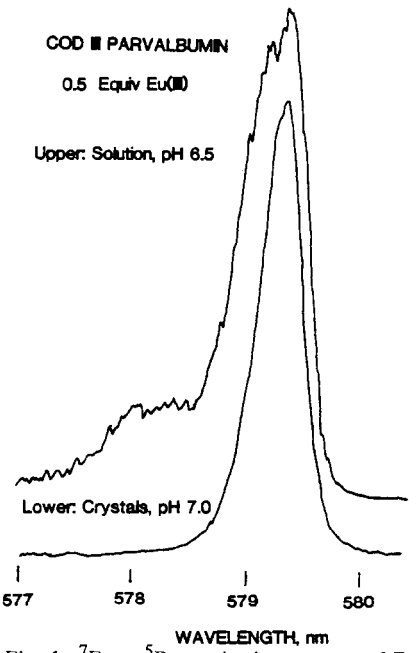

Fig. 1. $^7F_0 \rightarrow {}^5D_0$ excitation spectra of Eu(III) in parvalbumin in solution and crystalline states.

Results and Discussion

Parvalbumin. This protein binds two Ca(II) ions strongly. Earlier work in this laboratory [7, 11] has shown that at pH 4.0 the principal CD and EF sites are filled simultaneously during a titration with Eu(III), while at pH 6.5 a third site is occupied as well. Excited state lifetime results suggest that one water molecule is coordinated to the CD and EF Eu(III) ions while three are involved at the third site. The occupancy of this third site was shown to be responsible for the 'quenching' of Tb(III) luminescence after more than 1.5–1.8 equivalents of this ion have been added. The solution-state results are in apparent disagreement with X-ray diffraction studies [12], which show that if parvalbumin is crystallized in the presence of less than one equivalent of Tb(III) only the EF site is occupied while with more Tb(III) present both CD and EF sites are filled. No evidence for a third metal ion binding site was found in the X-ray study [12]. Figure 1 shows the $^7F_0 \rightarrow {}^5D_0$ excitation spectrum of a parvalbumin solution (upper trace) containing 0.5 equivalents of Eu(III) which provides evidence for the occupation of all three sites. This solution, when dialyzed against 2.8 *M* $(NH_4)_2SO_4$ buffer at pH 7.0, yielded crystals whose excitation spectrum is shown in the lower trace. Only a single sharp peak indicative of the sole occupancy of the EF site is

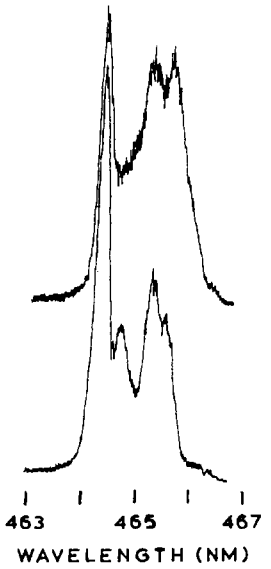

Fig. 2. $^7F_o \rightarrow {}^5D_2$ excitation spectra of Eu(III) bound to calmodulin in pH 5.7 in the presence of 4.0 (upper trace) or 2.0 (lower trace) equivalents of added Eu(III).

evident, consistent with the X-ray findings. Furthermore, lifetimes measured on crystals grown from both H_2O and D_2O are virtually identical indicating the absence of coordinated water in the crystalline state. This corroborates the X-ray diffraction results [12] which reveal a peak in the Fourier map which suggests that a sulfate ion has replaced the coordinated water molecule at the EF site. The excitation spectra (not shown) of crystals grown in the presence of larger amounts of Eu(III) show evidence for the occupancy of both CD and EF sites, but not for a third site. Dissolving the Eu(III)-substituted crystals results in the reformation of the solution state spectra.

Calmodulin. This important regulatory protein contains four Ca(II) ion binding sites, one each in domain numbers I–IV. Studies of tyrosine-sensitized Tb(III) luminescence [13–15] suggest that Ln(III) ions bind initially at sites I and II and subsequently at sites III and IV. The $^7F_o \rightarrow {}^5D_o$ excitation spectra of Eu(III) during a titration are consistent with the binding of four Eu(III) ions, but do not resolve differences between the two classes of sites [15]. Figure 2 shows, however, that there are significant differences between the excitation spectra of the $^7F_o \rightarrow {}^5D_2$ transition depending upon whether two or four equivalents of Eu(III) ions are bound. This illustrates the potential of excitation spectroscopy of higher transitions in the study of multiple classes of binding sites.

Using the same protocol as employed earlier with parvalbumin [8], Förster-type non-radiative energy transfer distance measurements were carried out between sites I and II of calmodulin. In the absence of an energy acceptor ion the reciprocal excited state lifetime of Tb(III) in these sites, τ_o^{-1}, is 0.94 ms^{-1}. In the presence of Ho(III) or Nd(III) in the adjacent site respective τ^{-1} values of 1.18 and 1.25 ms^{-1} were obtained. Using the Förster theory parameters established for parvalbumin [8], these results lead to respective distances of 11.6 and 10.8 Å between sites I and II. These values are close to the CD–EF site separation in parvalbumin (11.8 Å) and support the idea that the calmodulin structure is closely related to that of parvalbumin.

Additional results regarding Ln(III) ion binding to these proteins, including relative binding constants across the Ln(III) series will be presented.

Acknowledgment. This research was supported by the National Institutes of Health through grant GM 23599.

1 W. DeW. Horrocks, Jr., *Advan. Inorg. Biochem., 4,* 201 (1982).
2 W. DeW. Horrocks, Jr. and D. R. Sudnick, *Acct. Chem. Res., 14,* 384 (1981).
3 R. B. Martin and F. S. Richardson, *Quart. Rev. Biophys., 12,* 181 (1979).
4 W. DeW. Horrocks, Jr. and M. Albin, *Progr. Inorg. Chem., 31,* in press.
5 W. DeW. Horrocks, Jr. and D. R. Sudnick, *Science, 206,* 1194 (1979).
6 W. DeW. Horrocks, Jr. and D. R. Sudnick, *J. Am. Chem. Soc., 101,* 334 (1979).
7 W. deW. Horrocks, Jr., M.-J. Rhee, A. P. Snyder and D. R. Sudnick, *J. Am. Chem. Soc., 102,* 3650 (1980).
8 M.-J. Rhee, D. R. Sudnick, V. K. Arkle and W. DeW. Horrocks, Jr., *Biochemistry, 20,* 3328 (1981).
9 A. P. Snyder, D. R. Sudnick, V. K. Arkle and W. DeW. Horrocks, Jr., *Biochemistry, 20,* 3334 (1981).
10 R. H. Kretsinger, *CRC Crit. Rev. Biochem., 8,* 119 (1980).
11 W. DeW. Horrocks, Jr. and W. E. Collier, *J. Am. Chem. Soc., 103,* 2856 (1981).
12 J. Sowadski, G. Cornick and R. H. Kretsinger, *J. Mol. Biol., 124,* 123 (1978).
13 M.-C. Kilhoffer, J. D. DeMaille and D. Gerard, *FEBS Lett., 116,* 269 (1980).
14 M.-C. Kilhoffer, D. Gerard and J. G. DeMaille, *FEBS Lett., 120,* 99 (1980).
15 C.-L. A. Wang, R. R. Aquaron, P. C. Leavis and J. Gergely, *Eur. J. Biochem., 124,* 7 (1982).

B25

Model and Enzymatic Studies with Cytochrome P-450

DAVID DOLPHIN*, BRIAN R. JAMES and TAK LEUNG

Department of Chemistry, University of British Columbia, Vancouver, B.C., Canada V6T 1Y6

The cytochromes P-450 which have only been recognized for the past two decades are now the most