Spectroscopic Studies of Metal Ion Binding to a Tryptophan-Containing Parvalbumin†

Patrick J. Breen, Erich K. Hild, and William DeW. Horrocks, Jr.*
Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802
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ABSTRACT: The binding of Ca(II) and members of the trivalent lanthanide ion, Ln(III), series to apoparvalbumin (isotype pI = 4.75) from codfish (*Gadus callarius L*) results in the development of a distinctive sharp feature in the UV absorption spectrum at about 290 nm. Titration curves obtained by monitoring the spectral change in this region reveal a change in slope after the addition of 1 equiv of metal ion and no further rise after 2 equiv has been added, consistent with sequential binding to the principal EF and CD sites. Laser-induced luminescence excitation spectra of the $^7F_0 \rightarrow {}^5D_0$ transition of bound Eu(III) demonstrate the quantitative binding of this ion to the principal sites and disclose the presence of a subsidiary site at pH values greater than 6. Metal ion competition experiments monitored by means of this excitation transition show that the early members of the Ln(III) ion series bind more tightly than those at the end. Tryptophan-sensitized Tb(III) luminescence reveals that this ion binds sequentially to the EF and CD sites, in that order. The intrinsic tryptophan fluorescence of apoparvalbumin is increased in a stepwise fashion as Ca(II) or Ln(III) ions bind sequentially, with the exceptions of Eu(III) and Yb(III). The binding of the latter two ions causes quenching of the protein fluorescence via an energy-transfer process which involves low-lying charge-transfer bands. The distance dependences of the tryptophan to Tb(III) and tryptophan to Eu(III) energy-transfer processes are observed to be identical, consistent with a Förster-type mechanism in both cases.

Parvalbumin is a small (M_r 12000) protein commonly found in the muscle tissue of vertebrates which is prototypical of calcium-modulated proteins as a class (Kretsinger, 1980). Its X-ray structure has been determined (Moews & Kretsinger, 1975) and shows that it contains six α-helical regions, labeled A–F. The loops between the C and D helixes and between the E and F helixes each form metal ion binding sites which are referred to as CD and EF, respectively. Since metal ion binding is central to the function of calcium-modulated proteins, it is important that it be understood in a fundamental way. Parvalbumin, being structurally well characterized and simpler than calmodulin or troponin C, each of which contains four metal ion binding sites, is the most suitable candidate for definitive studies in this area. Previous studies (Benzonana et al., 1972; Donato & Martin, 1974; Nelson et al., 1977; Sowadski et al., 1978; Cavé et al., 1979; Haiech et al., 1979a; Horrocks & Collier, 1981; Rhee et al., 1981; Lee & Sykes, 1981; Horrocks et al., 1983; Corson et al., 1983a,b; Williams et al., 1984) have resulted in a controversy regarding whether the filling of the CD and EF metal ion binding sites occurs in a simultaneous or sequential fashion during metal ion titration experiments and whether there is any cooperativity between the sites. The present paper and the following one (Breen et al., 1985) provide a variety of spectroscopic and kinetic data which address these important questions.

The present pair of papers exploits the fluorescence and unique absorption spectroscopic properties of the single tryptophan of a parvalbumin isotype from codfish (pI = 4.75). These spectroscopic parameters are extremely sensitive to the degree of metal ion binding and provide a convenient means of monitoring titration experiments. In addition, we have made use of the laser-excited luminescence of the Eu(III) ion and the tryptophan-sensitized luminescence of Tb(III) to probe metal ion binding to parvalbumin. A considerable body of evidence (Horrocks, 1982; Martin, 1983) indicates that trivalent lanthanide ions [Ln(III)][1] are valid probes for the calcium binding sites in proteins and that their luminescence properties constitute an extremely useful means of studying them (Horrocks & Sudnick, 1981; Horrocks & Albin, 1984).

Materials and Methods

The codfish (*Gadus callarius L*) parvalbumin isotype (pI = 4.75) was isolated and purified in a preparative sequence similar to that described by Haeich et al. (1979b), involving heat treatment of the centrifuged supernatant followed by separations on DEAE-Sephacel ion-exchange columns at pH 7.6 and 5.7. The final component was pure as judged by SDS nondenaturing polyacrylamide gel electrophoresis. The tendency for the protein to dimerize by forming disulfide linkages was suppressed by storing the protein frozen in solution in the presence of dithiothreitol (Cleland, 1984) at 10 times the protein concentration.

Metal-free (apo) parvalbumin was prepared by stirring a solution of the protein with Chelex chelating resin, obtained from Bio-Rad, at pH 9 for about 12 h. Figure 1 illustrates the UV absorption spectra of parvalbumin after treatment with Chelex and after the addition of 2 equiv of a metal ion. Spectra of parvalbumin with 2 equiv of Ca(II), Cd(II), or any Ln(III) ion are identical. The spectrum of apo cod (pI = 4.75) parvalbumin is identical with that of apo European whiting (*Gadus merlangus*) parvalbumin reported by Permyakov et al. (1980).

Protein concentrations were determined from the measured absorbance by using ϵ_{280} = 7180 M^{-1} cm^{-1} (Closset & Gerday, 1976). No change in protein concentration was observed after

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[1] Abbreviations: Ln(III), trivalent lanthanide ions; Par, parvalbumin; SDS, sodium dodecyl sulfate.

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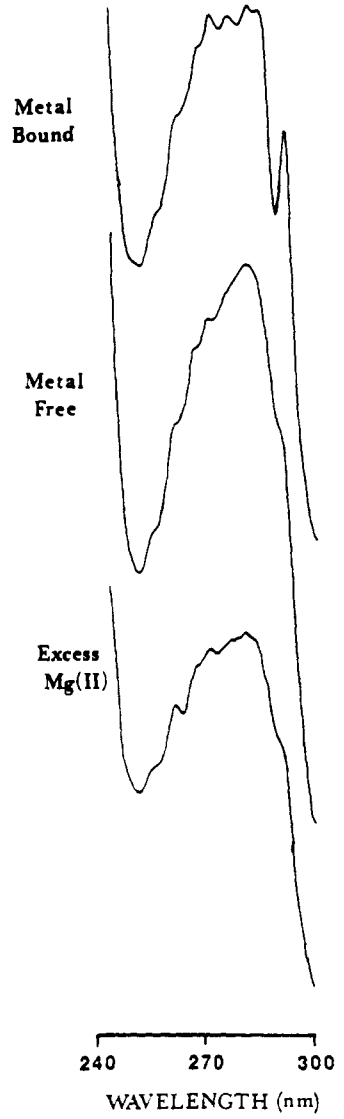

FIGURE 1: UV absorption spectra of codfish parvalbumin with and without Ca(II) or Ln(III) ions bound and in the presence of an excess of Mg(II). Conditions: pH 5.8, 1 mM piperazine, 0.5 M KCl.

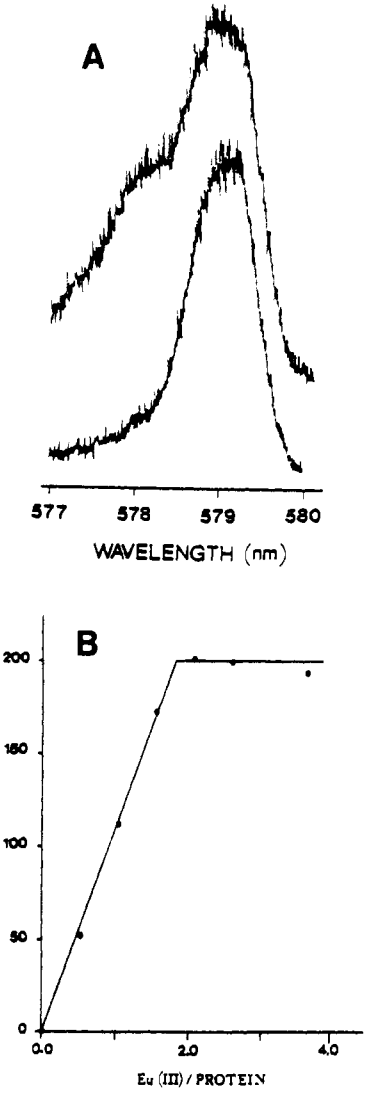

FIGURE 2: (A) $^7F_0 \rightarrow {}^5D_0$ excitation spectra of Eu(III) bound to codfish parvalbumin at pH 5.5 and 6.5 (lower trace and upper trace, respectively). (B) Intensity of the $^7F_0 \rightarrow {}^5D_0$ peak at 579.3 nm, pH 5.8, as a function of added Eu(III) ion.

the protein was treated with Chelex, indicating that parvalbumin has no significant affinity for Chelex resin.

All Ln(III) salts were obtained from either Aldrich or Alfa/Ventron. All metal ion, protein, and other stock solutions were prepared from doubly distilled water that was then run down a 1 × 40 cm column of Chelex resin. All metal ion solutions were standardized by using complexometric methods (Fritz et al., 1958). Dynatech precision sampling syringes were used to add metal ion containing solutions during titration experiments.

To avoid complications due to a subsidiary metal ion binding site on parvalbumin (Horrocks & Collier, 1981; Rhee et al., 1981) which is not present at pH values less than 6.0, all experiments in this study were performed in the pH range 5.6–6.0 unless otherwise specified. Permyakov et al. (1980, 1982) have studied the effects of pH on parvalbumin by monitoring the intrinsic protein fluorescence of the tryptophan-containing parvalbumin from European whiting. They observed no changes in peak position, half-width, or quantum yield over the pH range of ~5.3–10, indicating that there is little structural change in parvalbumin over this range. It is reasonable to expect, therefore, that results obtained from binding studies done in the pH range 5.6–6.0 are equally valid at physiological pH values.

The tendency for the protein to precipitate slowly at pH values less than 6.0 was overcome by having KCl present at a concentration of 0.5 M in all protein solutions except as otherwise noted. All experiments were carried out at ~24 °C.

Absorption and fluorescence spectroscopic measurements were made on Cary-Varian 210 and Perkin-Elmer MPF 44A instruments, respectively.

RESULTS

Laser Experiments. The $^7F_0 \rightarrow {}^5D_0$ excitation spectra of Eu(III) bound to codfish parvalbumin at pH 5.5 and 6.5 are shown in Figure 2A. The single peak present at the lower pH value corresponds to Eu(III) occupying both the CD and EF sites. The two sites are apparently too similar to be resolved in this experiment. In the structure of carp parvalbumin (Moews & Kretsinger, 1975), four carboxylate ligands are involved in Ca(II) ion coordination at each site. Were the fifth carboxylate group, which is present in the CD binding loop of the codfish protein, coordinated to the Eu(III), one might expect a better resolution of the two sites on the basis of the correlation between total ligand charge and the $^7F_0 \rightarrow {}^5D_0$ transition frequency recently noted by us (Albin & Horrocks, 1985). The excitation spectrum taken at pH 6.5 reveals a

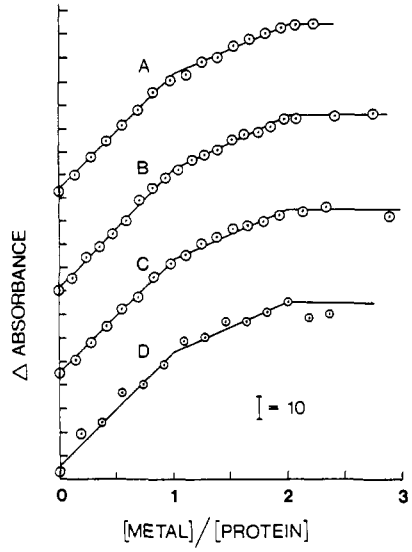

FIGURE 3: Change in the difference in absorbance between 288.2 and 290.4 nm of parvalbumin during the addition of (A) Ca(II) ion, pH 7.4, (B) Eu(III) ion, pH 5.8, (C) Lu(III) ion, pH 7.4, and (D) Lu(III) ion, pH 5.7.

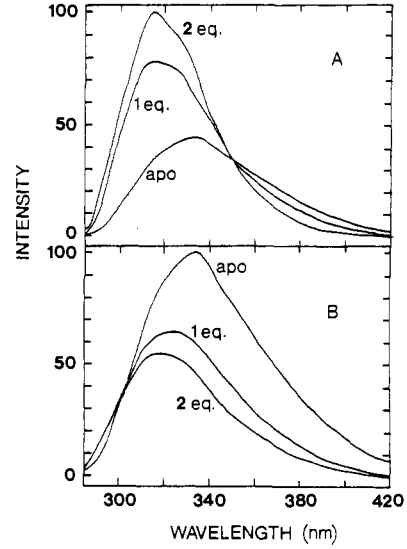

FIGURE 4: Emission spectra of parvalbumin (λ_{ex} = 280 nm) as a function of added (A) Gd(III) ion and (B) Eu(III) ion.

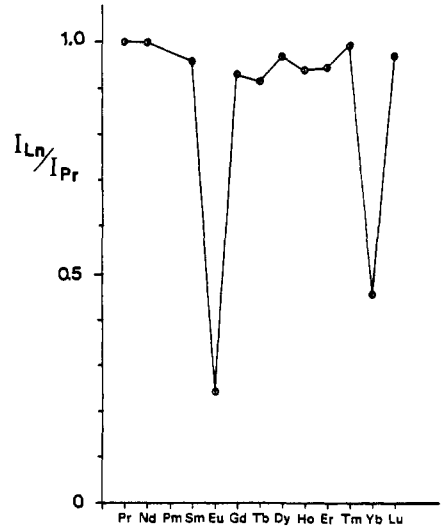

FIGURE 5: Fluorescence intensities at 320 nm of parvalbumin bound with 2 equiv of different Ln(III) ions relative to Pr(III). Conditions: λ_{ex} = 280 nm, pH 5.8, 1 mM piperazine, 0.4 M KCl.

feature to shorter wavelength due to binding of Eu(III) to a subsidiary site as noted earlier (Horrocks & Collier, 1981; Rhee et al., 1981). A titration carried out at pH 5.8 based on the intensity of the excitation peak at 579.3 nm (Figure 2B) shows absolute linearity until 2.0 equiv of Eu(III) has been added. The absence of a break in the curve at 1.0 equiv could be due to a high degree of positive cooperativity in binding, to equal and random binding to the two sites, or to the inability of the laser experiment to distinguish the individual sites owing to the near-equality in absorption properties, quantum yields, and frequencies associated with the two ion binding environments. The last of these three possibilities is the only one consistent with the remaining data in this paper.

Absorption Spectra. It is clear from Figure 1 that the sharp tryptophan spectral peak at 291 nm is diagnostic of the metal-bound form of the protein. In addition to providing a convenient means for determining whether or not the protein is calcium free, the change in the difference in absorbancy between the trough at 288.2 nm and the peak at 290.4 nm, ΔA, is useful for monitoring the binding of metal ions in titration experiments. Figure 3 shows the results of titrations of apoparvalbumin with Ca(II) at pH 7.4, with Eu(III) at pH 5.8, and with Lu(III) at pH 7.4 and 5.7. The changes observed in ΔA are all virtually complete after 2 equiv of metal ion has been added, with the plots exhibiting a distinct change in slope after about 1 equiv of metal ion has been added. In no case can the dependence of ΔA on metal ion added be described as linear up to the addition of 2 equiv.

Interestingly, although Mg(II) has been reported (Robertson et al., 1981) to bind to parvalbumin (K_d = 91 μM), albeit more weakly than the other ions studied, the absorption spectrum of the apo form of parvalbumin to which a large excess of Mg(II) ions has been added (Figure 1) does not show the sharp tryptophan feature at 290 nm characteristic of the other metal ion bound species. If, indeed, Mg(II) binds to the CD and EF sites, it does so in a manner different from Ca(II) and does not induce the same sort of conformational change in the protein that Ca(II) or the Ln(III) ions do.

Intrinsic Protein Fluorescence. Variations in the fluorescence of the single tryptophan residue of cod parvalbumin provide a sensitive means of monitoring conformational changes in the protein caused by metal ion binding. Energy transfer from the fluorescent tryptophan to the energy-acceptor ions Eu(III) and Yb(III) may also be followed using fluorometry. Figure 4A shows the change in fluorescence emission as the non-energy-acceptor ion Gd(III) is added to apoparvalbumin. The quantum yield for emission increases, and the emission maximum moves to shorter wavelengths as the metal ion bound species is formed. A similar behavior is observed for Ca(II) and all the Ln(III) ions except Eu(III) and Yb(III). Figure 4B shows the behavior of the tryptophan emission as Eu(III) is added to the apoprotein. The emission maximum moves to shorter wavelengths, as is the case for the other metal ion bound forms, but the intensity is severely attenuated. Yb(III) behaves similarly although the intensity is decreased to a somewhat lesser extent. The dramatic difference between Eu(III) and Yb(III) and the remaining Ln(III) ions in this regard is shown graphically in Figure 5 where the relative fluorescence intensities of the fully metal ion loaded forms of cod parvalbumin, taken under identical conditions, are compared across the Ln(III) ion series.

These changes in quantum yield can be used to monitor metal ion titrations as illustrated in Figure 6 for Eu(III) and Gd(III), where the relative quantum yields decrease and increase, respectively, as the metal ion is bound. Again, definite

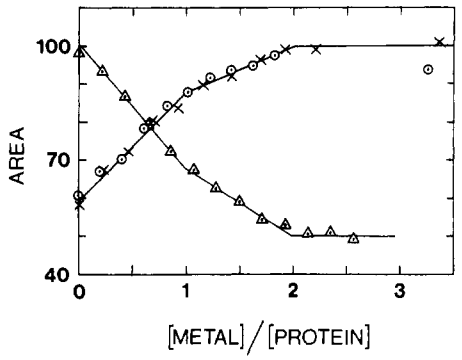

FIGURE 6: Relative quantum yield (area under emission spectrum) as a function of added Eu(III) ion (Δ), pH 5.7, and Gd(III) ion, pH 7.6 (×) and pH 5.7 (○).

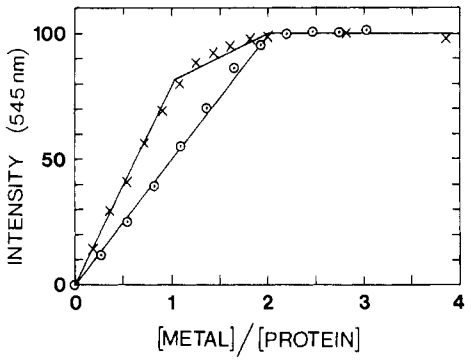

FIGURE 7: Tryptophan-sensitized Tb(III) emission at 545 nm (λ_{ex} = 295 nm) as a function of total equivalents of Tb(III) added to apoparvalbumin (×) and Ca(II)-bound parvalbumin (○), pH 5.7.

breaks in the slopes occur after 1 equiv has been added, and the change is complete after 2 equiv of metal ion is bound.

Sensitized Tb(III) Emission. When Tb(III) is bound to a protein containing fluorescent amino acid residues (Phe, Tyr, or Trp), emission from the $^5D_4$ level of Tb(III) is often observed upon irradiating the aromatic residue in its UV absorption band (Brittain et al., 1976). Tb(III) emission exhibits maximum intensity at about 545 nm. This phenomenon arises because of a nonradiative energy transfer between the fluorescent residue and the bound Tb(III) ion and can be used to monitor the binding of this ion in a highly specific manner. Figure 7 shows a plot of the tryptophan-sensitized Tb(III) emission at 545 nm as a function of equivalents of Tb(III) added to apoparvalbumin. A break in the slope of the curve occurs at about 1 equiv of added Tb(III), and maximal sensitized emission is achieved upon the addition of about 2 equiv. Also shown in Figure 7 is a plot of data for an analogous titration carried out in the presence of 2 equiv of Ca(II). In this case, there is no change in slope at 1 equiv, in accord with our previous results (Horrocks & Collier, 1981).

Metal Ion Competition Experiments. In a recent study (Albin et al., 1984), the relative binding constants for the entire series of Ln(III) ions to a multidentate chelating agent were determined by means of a simple method developed by us. In these experiments, a known amount of Eu(III) is added to a known concentration of ligand in solution, and the intensity of the $^7F_0 \rightarrow {}^5D_0$ excitation peak due to the Eu(III)–ligand complex is recorded. A known amount of competing Ln(III) ion is added, and the Eu(III)–ligand excitation peak is again recorded. The amount by which this signal is reduced in intensity is directly proportional to the amount of Eu(III) ion that has been displaced by the competing ion, thus yielding relative binding constants. If the absolute binding constant for Eu(III) or the competing ion is known, then the absolute binding constant for the partner ion can be determined from these data.

Table I: K_d^{Eu}/K_d^{Ln} Ratios for Various Ln(III) Ions[a]

Ln(III)	K_d^{Eu}/K_d^{Ln} ratio	Ln(III)	K_d^{Eu}/K_d^{Ln} ratio
Ce	1.91 ± 0.17	Dy	0.47 ± 0.06
Pr	2.01 ± 0.41	Ho	0.57 ± 0.18
Nd	2.25 ± 0.47	Er	0.42 ± 0.06
Sm	1.60 ± 0.42	Tm	0.49 ± 0.02
Eu	1.00	Yb	0.46 ± 0.09
Gd	0.50 ± 0.08	Lu	0.31 ± 0.12
Tb	0.64 ± 0.05		

[a] See the text for the definition of K_d ratios.

Analogous experiments on parvalbumin were carried out, although since there are two nonequivalent binding sites, a rigorous analysis of the results in terms of the actual relative binding constants is not possible. Since it has been established that Eu(III) binds sequentially to the EF and CD sites in that order, the titration experiment, based on the $^7F_0 \rightarrow {}^5D_0$ excitation intensity (Figure 2), shows that the CD and EF sites produce identical contributions to the intensity when they are filled by Eu(III). An approximate treatment was undertaken wherein the two sites on parvalbumin were assumed to be equal and independent, such that the metal ion exchange reaction can be represented simply as involving a single site (present at twice the actual parvalbumin concentration) according to

$$Ln^{3+} + ParEu \rightleftarrows Eu^{3+} + ParLn \qquad (1)$$

where the equilibrium constant, K, of reaction 1 is given by

$$K = \frac{K_{Eu}}{K_{Ln}} = \frac{[Eu^{3+}][ParLn]}{[Ln^{3+}][ParEu]} = \frac{([Eu^{3+}]_i - \alpha I')(I - I')}{[[Ln^{3+}]_i - \alpha(I - I')]I'} \qquad (2)$$

where I and I' are the $^7F_0 \rightarrow {}^5D_0$ intensities before and after addition of the competing Ln(III) ion, α is the known proportionality constant between the intensity and the concentration of bound Eu(III), and the subscript i denotes the initial concentration of each metal ion. The results of the competition experiments analyzed in this manner are given in Table I. Values for "K_d^{Eu}/K_d^{Ln}" greater than 1.0 indicate that a particular Ln(III) ion binds more tightly than Eu(III). While it is not possible to achieve a rigorous determination of the K_d ratios for the individual sites from these data, we were able to show, by computer simulation, using a version of the complex equilibrium programs LIGAND and TRANSPORT (Biomedical Computing Technology Information Center, Vanderbilt Medical Center), that the calculated "K_d^{Eu}/K_d^{Ln}" values are a reasonable approximation of the average of $(K_d^{Eu}/K_d^{Ln})^{CD}$ and $(K_d^{Eu}/K_d^{Ln})^{EF}$ when these ratios, individually, lie in the range 0.2–5.0. It is clear from the results that the early members of the Ln(III) ion series show a greater affinity for the binding sites of parvalbumin than those at the end. It may or may not be significant that the early Ln(III) ions more nearly match the ionic radius of Ca(II) than the later members. The observed trend, which may be characteristic of calcium-modulated proteins as a class, can be understood in terms of variations in the on rates of the Ln(III) ions across the series. Further discussion of this point is reserved for the following paper which is concerned with kinetic measurements on these systems (Breen et al., 1985).

Discussion

Metal Ion Binding Models. For a protein such as parvalbumin, which contains two tight binding sites, there are a number of possible metal ion binding scenerios: (1) the two sites bind the metal ions independently with identical or nearly identical binding constants; (2) the two sites are independent,

but one site is considerably tighter than the other; (3) the two sites exhibit cooperativity in their binding. Cooperativity has been considered in the past because the two sites are separated by only 11.8 Å. In the present discussion, we will specify metal ion binding to parvalbumin by the following shorthand notation: Par[Ca,Eu] indicates that a Ca(II) ion occupies the CD site and Eu(III) the EF site; Par[–,–] denotes apoparvalbumin. Taking the physiologically important Ca(II) ion to illustrate, we consider the consequences of the above three types of metal ion binding behavior on the titration of apoparvalbumin by this ion. If type 1 behavior obtains, Par[–,Ca], Par[Ca,–], and eventually Par[Ca,Ca] will form statistically during the course of the titration for experiments carried out at protein concentrations well above the dissociation constants, as they have been here. Even if the changes in absorbancy or fluorescence elicited by the conformational change upon binding to the CD or EF sites were quite different, the fact that the two sites are filled at the same rate would prevent any break from appearing in the titration curve after 1 equiv of Ca(II) has been added. In situation 2 with one binding constant, e.g., that for the EF site, an order of magnitude larger than the other, the first equivalent of added Ca(II) will go largely to forming species Par[–,Ca] with Par[Ca,Ca] formed as the second equivalent is added. Provided the absorption or fluorescence change being monitored is significantly different for the two species, a break in the titration curve will occur at 1 equiv of added Ca(II). This model is consistent with all of our data. For strong positive cooperativity (situation 3 above), the binding of an ion at one site markedly increases the strength of binding at the other site. In such a case, the only species present in significant quantities during the course of a titration would be Par[–,–] and Par[Ca,Ca], and no break in the titration curve would occur at the 1 equiv point. Likewise, a strong negative cooperativity (the two sites being initially about equal in affinity) would lead to the formation of equal quantities of Par[Ca,–] and Par[–,Ca] after the addition of 1 equiv of metal ion, followed by the formation of the fully loaded form. The break at the 1 equiv point in the titration curve based on sensitized Tb(III) emission (Figure 7) is clearly inconsistent with this model. Strong negative cooperativity with one site initially of higher affinity would have consequences in a titration experiment identical with the unequal affinity, independent sites situation (model 2 above); however, cooperativity of any sort is inconsistent with the results of the following paper (Breen et al., 1985).

Titrations Based on Tryptophan Absorbance Changes. Inspection of the titration curves based on changes in tryptophan absorbance (Figure 3) reveals that all of them exhibit a more or less distinct break at the point of 1 equiv of added metal ion and none can be described in terms of a simple linear change to the 2 equiv point. In terms of the models described above, these results are consistent only with the second situation wherein there is sequential binding of the metal ions, first to the tighter site and then to the weaker one. This conclusion is consistent with some, but not all, reports in the literature and with the kinetic results of the following paper (Breen et al., 1985). The experiments with Lu(III) at both pH 5.7 and 7.4 suggest that the observed behavior is independent of pH, at least over a modest range. The finding that metal ion binding is sequential both for Ln(III) ions and also for Ca(II) has significant implications in the use of the former as probes for the latter.

Sensitized Tb(III) Luminescence. Since the nonradiative energy transfer between tryptophan and Tb(III) occurs only when the latter is bound to the tryptophan-containing protein, sensitized Tb(III) emission provides an ideal means of monitoring the binding of this ion to a protein. The titration of apo cod parvalbumin with Tb(III) based on the sensitized luminescence of the latter (λ_{ex} = 295 nm; λ_{em} = 545 nm) exhibits a decrease in slope after 1 equiv of Tb(III) has been added (Figure 7). This result implies a sequential binding of Tb(III) to parvalbumin with about 80% of the total sensitization going to the first-bound Tb(III). This observation provides a clue to the order of binding of Tb(III) to the CD and EF metal ion binding sites. The single tryptophan of cod parvalbumin occupies position 109 in the sequence (Elsayed & Bennick, 1975). Because of the fact that the structurally well-characterized carp parvalbumin contains only 108 residues, the geometrical position of Trp-109 cannot be accurately estimated. Nevertheless, on the basis of the fact that the α-carbon of residue 108 of carp parvalbumin is 16.1 Å from the EF site and 21.5 Å from the CD site, it is reasonable that the tighter Tb(III) site is the EF site. This conclusion is consistent with crystallographic results which show the EF site to be more readily occupied by Tb(III) in the crystalline state (Moews & Kretsinger, 1974; Sowadsky et al., 1978). Our earlier suggestion that Tb(III) binds simultaneously to the CD and EF sites (Horrocks & Collier, 1981) was made on the basis of experiments carried out in the presence of Ca(II). Under these conditions, no break in the titration curve occurs at 1 equiv (Figure 7), presumably because the competition of Tb(III) and Ca(II) for the two sites obliterates the site selectivity for Tb(III).

Protein Fluorescence. When metal ions bind to apoparvalbumin, they cause the molecule to undergo a significant conformational change as evidenced by an increase in intensity and a shift to shorter wavelengths of the tryptophan fluorescence (Figure 4). Eu(III) and Yb(III) ions affect the fluorescence differently for reasons discussed in the next section. Using the area under the tryptophan emission curve to follow the titration with Gd(III) reveals that the largest increase (54%) in quantum yield occurs upon binding of this ion to the EF site, with the remaining (22%) change occurring with the occupation of the CD site. Analogous results were observed for Ca(II) (data not shown), suggesting that similar conformational changes occur upon the binding of the different classes of ions. The present finding of sequential metal ion binding are in good agreement with those of Permyakov et al. (1980) on Ca(II) binding to the tryptophan-containing parvalbumin isotype from European whiting wherein the tryptophan residue occupies position 102 in the amino acid sequence.

Quenching of Protein Fluorescence by Eu(III) and Yb(III) Ions. In marked contrast to the other ions studied, Eu(III) and Yb(III) cause a sharp decrease in the fluorescence quantum yield as they bind to apoparvalbumin, although the blue shift of the fluorescence maximum is still observed as they coordinate to the protein (Figures 4 and 5). The titration carried out by monitoring tryptophan emission as Eu(III) is added to the apoprotein (Figure 6) shows a distinct break after the addition of 1 equiv with the emission quenched 32% by the Eu(III) ion in the EF site and a total of 49% upon occupancy of both sites, when compared to the emission of the apoprotein. The sizable quenching of tryptophan fluorescence by Eu(III) and Tb(III) is due to a nonradiative energy-transfer process. In the case of Eu(III), we attributed this to a Förster-type energy transfer to a charge-transfer level in Eu(III) (Horrocks & Collier, 1981). Quite recently, Abusaleh & Meares (1984) have offered an alternative suggestion, namely, that protein fluorescence quenching occurs via electron

transfer from an excited-state tryptophan to the readily reducible Eu(III) or Yb(III) ions when these are bound to proteins. This idea, while attractive, does not withstand detailed scrutiny, at least as far as parvalbumin is concerned. The quantum yield of the tryptophan in the calcium-loaded form of cod parvalbumin is 0.16 (Horrocks & Collier, 1981) which is decreased to 0.04 when fully bound by Eu(III) (Figure 5). The observed excited-state lifetime of tryptophan in this protein is 3.3 ns (Eftink & Ghiron, 1984). This information is sufficient to establish a radiative rate constant (k_{rad}) of 4.8×10^7 s^{-1}, a nonradiative deexcitation constant (k_{nonrad}) of 2.5×10^8 s^{-1} in the absence of Eu(III), and a rate constant for nonradiative energy transfer to Eu(III) (k_{Eu}) of 9.0×10^8 s^{-1}. Were k_{Eu} to correspond to an electron transfer, this would imply that electron transfer can occur over a distance of more than 15 Å in parvalbumin at a rate about 9 orders of magnitude greater than it does over a distance of 11.8 Å between a ruthenium-labeled His-83 and the Cu(II) center of the protein azurin (Kostič et al., 1983). Further support for our original explanation is provided by the fact that we observe the long-wavelength tail of a charge-transfer band in both Eu(III) (ϵ_{310} = 118 M^{-1} cm^{-1}) and Yb(III) (ϵ_{310} = 84 M^{-1} cm^{-1}) loaded forms of cod parvalbumin, whereas no such absorption is present in parvalbumin bound to Tb(III) or the other ions. It is perhaps significant that the ratio of ϵ_{380} values for Eu(III) and Yb(III) is virtually identical with the degree of fluorescence quenching produced by these two ions (Figure 5). As discussed elsewhere (Horrocks & Collier, 1981), the considerable energy transfer into a charge-transfer state of Eu(III) does not result in significant amounts of sensitized Eu(III) emission in the visible region of the spectrum, whereas the minimal energy transfer to f electronic excited states of Tb(III) in parvalbumin with an efficiency of about 5×10^{-4} results in measurable Tb(III) luminescence, with insignificant quenching of the tryptophan fluorescence.

Additional evidence for the idea that a Förster-type nonradiative energy-transfer mechanism is operative, both in sensitizing the Tb(III) emission and in the Eu(III)-induced quenching of tryptophan fluorescence, is provided by the following quantitative comparison of the two experiments. The data in Figure 7 show that Tb(III) ions in the EF and CD sites produce 80% and 20% of the sensitized luminescence, respectively. If the Eu(III) quenching of fluorescence is analyzed by comparing the fluorescence observed in the presence of 1 and 2 equiv of Eu(III) (Figure 6) with the fluorescence expected for the protein bound by 1 and 2 equiv of a nonquenching ion, then one finds that the first equivalent of added Eu(III) produces 79% of the total quenching observed when 2 equiv of this ion is bound. This result agrees well with the relative amounts of energy transfer to the sensitized Tb(III) ions in the two sites, suggesting that the distance dependencies of the two energy-transfer processes are identical. This strongly argues against an electron-transfer mechanism being involved in the case of Eu(III).

Finally, it should be noted that our earlier quantitative analysis of the tryptophan to Tb(III) energy transfer in cod parvalbumin (Horrocks & Collier, 1981) was flawed by our erroneous assumption that the single tryptophan residue in the codfish isotype occupies the same position as in the European whiting parvalbumin. A detailed comparison of the cod and whiting parvalbumins with regard to their metal ion binding and energy-transfer properties is currently in progress in this laboratory.

Registry No. Ce, 7440-45-1; Pr, 7440-10-0; Nd, 7440-00-8; Sm, 7440-19-9; Eu, 7440-53-1; Gd, 7440-54-2; Tb, 7440-27-9; Dy, 7429-91-6; Ho, 7440-60-0; Er, 7440-52-0; Tm, 7440-30-4; Yb, 7440-64-4; Lu, 7439-94-3; Ca, 7440-70-2.

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Stopped-Flow Kinetic Studies of Metal Ion Dissociation or Exchange in a Tryptophan-Containing Parvalbumin†

Patrick J. Breen,‡ Kenneth A. Johnson,§ and William DeW. Horrocks, Jr.*,‡
Department of Chemistry and Biochemistry Program, The Pennsylvania State University, University Park, Pennsylvania 16802
Received December 27, 1984

ABSTRACT: The rates of dissociation of 2 equiv of various metal ions [Ca(II), Cd(II), Pr(III), Nd(III), Sm(III), Eu(III), Gd(III), Tb(III), Dy(III), Ho(III), Er(III), Yb(III), and Lu(III)] from the primary CD and EF metal ion binding sites of parvalbumin (isotype pI = 4.75) from codfish (*Gadus callarius L*) were measured by stopped-flow techniques. The removal or replacement of metal ions was monitored by changes in sensitized Tb(III) luminescence or in intrinsic protein tryptophan fluorescence as quenching ions [Eu(III) or Yb(III)] were bound or removed or as the apoprotein was formed. In experiments wherein the bound metal ions were removed by mixing the parvalbumin with an excess of 1,2-diaminocyclohexanetetraacetic acid (DCTA), the kinetic traces were best fit by a double exponential with k_{off} rate constants of 1.07 and 5.91 s^{-1} for Ca(II), 1.54 and 10.5 s^{-1} for Cd(II), and ~0.05 and ~0.5 s^{-1} for all of the trivalent lanthanide ions. In experiments wherein the bound metal ions were exchanged with an excess of a different metal ion, pseudo-first-order rate constants were proportional to the concentration of excess attacking metal ion for both the fast and slow processes in most experiments. In these cases, extrapolation of the rate constants to zero concentration of attacking metal ion gave values which agree well with the DCTA scavenging results. This finding demonstrates that the off rate constants do not depend on the occupancy of the neighboring site and therefore implies that there is no significant cooperativity in metal ion binding between the two sites in parvalbumin.

Calcium-modulated proteins function by responding to the increase in cytosolic Ca(II) ion concentration which occurs upon cell stimulation. The binding of Ca(II) to such proteins results in a conformational change such that the calcium-loaded forms are then capable of interacting with target proteins, for example, to trigger muscle contraction in the case of troponin C or to activate a variety of enzymatic processes in the case of calmodulin (Kretsinger, 1980). Knowledge of the kinetic processes by which metal ions, particularly Ca(II), are bound or removed from this class of proteins is vital to an understanding of their mechanisms of action. For many of the same reasons that were outlined in the introduction to the preceding paper (Breen et al., 1985), we have chosen as the initial object of our studies in this area a parvalbumin isotype from codfish (*Gadus callarius L*) which contains a single tryptophan residue. In this study, we exploit the fluorescence properties of the tryptophan and its ability to sensitize the luminescence of bound Tb(III) to measure the kinetics of metal ion replacement or removal from the two principal Ca(II) binding sites in this protein, known as CD and EF. The question of possible cooperativity between the two sites in binding metal ions is addressed in this study.

Materials and Methods

The preparation, purification, and metal ion removal from codfish parvalbumin and the sources of all chemicals used are as given in the preceding paper (Breen et al., 1985). Solutions of metal ion loaded protein were prepared by addition of 2.0–2.2 equiv of metal ion to apoparvalbumin. Stock solutions of the piperazine buffer solution, KCl, and *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid (DCTA)[1] were all stored over Chelex chelating resin to absorb all contaminating di- and trivalent metal ions.

Kinetic experiments were carried out at 24.8 °C on a stopped-flow apparatus described elsewhere (Johnson & Porter, 1983). All protein solutions were buffered to pH 5.8 with 1 mM piperazine unless otherwise indicated and contained 0.5 M KCl. The time course of the kinetic reactions was monitored by means of changes in the intensity of the tryptophan fluorescence (340-nm band-pass filter, 14-nm bandwidth). Excitation was in all cases accomplished with UV radiation into the tryptophan absorption band (280-nm band-pass filter, 11-nm bandwidth). The kinetics of metal ion removal from parvalbumin were measured by mixing the metal ion bound form of the protein (~50 μM or less in concentration) with an excess of the metal ion chelator DCTA (1–10 mM in concentration) and following the decrease in Tb(III) luminescence when that metal ion is the one removed, or changes in the tryptophan fluorescence as the apoprotein is produced from the metal ion bound species [see Figure 4 of Breen et al. (1985)]. Metal ion replacement reactions were carried out by mixing a metal ion bound form of the protein with an excess of the substituting metal ion (1–10 mM in concentration). These reactions were followed either by

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‡ Department of Chemistry.
§ Biochemistry Program.

[1] Abbreviations: DCTA, *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid; Ln(III), trivalent lanthanide ions.

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