30. Bovine α-Lactalbumin: Identification of Two Metal-Ion-Binding Sites Using the Europium(III) Luminescent Probe

by **Jean-Claude G. Bünzli*** and **Jean-Marc Pfefferlé**

Université de Lausanne, Institut de chimie minérale et analytique, 3, place du Château, CH–1005 Lausanne

(23.VIII.93)

The luminescent Eu^{III} ion has been used to probe the metal-binding sites of bovine α-lactalbumin (BLA) in D_2O. Upon addition of apo-BLA to an Eu^{III}-containing solution, the intrinsic luminescence of the protein is quenched, and the Eu^{III} luminescence is enhanced. Luminescent titrations point to there being at least two different metal-binding sites in the apo-protein. Curve analysis of the high resolution $^5D_0 \leftarrow {}^7F_0$ excitation spectra reveals the existence of three different environments for the bonded Eu^{III} ions. Two environments, labelled I_a and I_b, give $^5D_0 \leftarrow {}^7F_0$ bands very close in energy; they contain four negatively charged groups and are assigned to one site we identify as the calcium-binding site. Site I is protected from solvent influences and is somewhat rigid, since it displays selectivity towards lanthanide ions. The origin of the two similar environments I_a and I_b could not be determined unambiguously. The third environment is ascribed to a nonspecific metal-binding site in which the Eu^{III} ion is more exposed to the solvent (site II). It is sequentially populated after saturation of site I, and its population is pH-dependent. The affinity constant of Eu^{III} for this site was estimated from the excitation spectra: $\log K_2^{app} = 3.5(1)$. Assignment of the metal binding sites has been facilitated by comparison with model compounds, $[Eu(dota)]^-$ (dota = 1,4,7,10-tetraazacyclododecane *N,N′,N″,N‴*-tetraacetate), $[Eu(dtpa)]^{2-}$ (dtpa = diethylenetriamine tetraacetate), and [Eu(bsa)] (bsa = bovine serum albumin). The usefulness and limits of the use of curve-analysis procedures to unravel the various components of $^5D_0 \leftarrow {}^7F_0$ excitation spectra in biological materials are also discussed.

Introduction. – Bovine α-lactalbumin (BLA) is a small protein containing 123 amino-acids and having a molecular weight of 14 177 which is present in cow milk at a concentration of *ca.* 1 g/l. It is known as the modifier protein of the lactose synthetase complex. BLA associates with the enzyme galactosyltransferase to form a complex which catalyzes the biosynthesis of lactose from UDP-galactose and glucose in the mammary gland [1]. The A-conformer of apo-BLA binds strongly and reversibly one Ca^{II} ion; this induces a conformational change into the native N-conformer in which the exposition of the hydrophobic region of the protein surface to the solvent is strongly reduced [2] [3]. Binding of Zn^{II} seems to occur at a specific site which promotes an A-like conformation [4] [5]. It is believed that Zn^{II}, together with Ca^{II}, plays a specific role in the control of BLA conformation, henceforth on the action of BLA in lactose synthesis [5]. BLA also binds several other cations including Mn^{II}, Tb^{III} [6] [7], Cd^{II}, and Gd^{III} [8] [9], but both the nature and the number of metal binding sites remain ill-defined. Previous studies of the BLA metal sites [4–7] have been based on indirect methods, mainly the measurement of intrinsic fluorescence parameters. Direct methods such as ^{113}Cd-NMR or EPR of Cd^{II}- and Mn^{II}-substituted BLA have only demonstrated the similarity between the calcium-binding sites of BLA and proteins of the calmodulin family [8], while an EPR study of Gd^{III} bound to BLA pointed to there being additional metal-binding sites [9].

The trivalent lanthanide ion Eu^{III} is finding widespread application as a luminescent probe in studies of biological systems. Its use as a replacement probe for Ca^{II} is made possible by the analogy between the chemical and physical properties of these two ions: the Eu^{III}-substitution is thought to induce little perturbation in the biological macromolecule [10]. The nondegenerate nature of the $^5D_0$ excited level and of the $^7F_0$ ground level results in a unique $^5D_0 \leftarrow {}^7F_0$ transition for each chemical environment of the Eu^{III} ion. Therefore, measurement of this transition *via* a laser-excitation technique allows one to determine the number of metal binding sites in a given macromolecule (*cf.* oncomodulin [11] or calmodulin [12]).

In this paper, we intend to determine the number of metal-binding sites in BLA, their relative population under various experimental conditions, *e.g.* pH, and their affinity for Eu^{III}. To assign the components of the $^5D_0 \leftarrow {}^7F_0$ transition, we resort to a curve-resolution method, which allows us to separate the various components. All measurements were made in D_2O in order to avoid a too important quenching of the europium luminescence by the O–H vibrations.

Experimental. – *Preparation of the Protein.* The Ca^{II} content of native BLA (*Sigma Chemical Co.*, type *I*) was found to vary upon the lot number. The two batches used in these experiments were lot *14F-8030* and lot *124F-8086*, which contained 2.2 and 1.0 mol Ca^{II} per mol protein, respectively. The purity of commercial BLA was checked by electrophoresis on SDS-acrylamide gel using a 16-cm *Biorad Protean Tm II* system equipped with a *Biorad 500/200* power supply. The protein was loaded in quantities ranging from 5 μg up to 210 μg. Assay for the glycosylated form of the protein was performed as follows: the gel was soaked in a CCl_3COOH soln. for 1 h, washed, and placed in a soln. containing 1% HIO_4 and 3% AcOH for 1 h. It was finally soaked in the *Schiff* reagent during 18 h. Results showed a slight contamination with BSA (1%) and β-lactoglobulin (< 1%), but no glycosylated protein was evidenced. The presence of trace amounts of these proteins does not significantly influence the luminescence measurements. Apo-α-lactalbumin was prepared according to [6]: an acidic soln. of BLA (*Sigma Chemical Co.*, 80 mg/10 ml, pH 1.9) was passed through a *Sephadex G-10* column (*Pharmacia*, 5 $cm^2 \times 95$ cm) previously equilibrated with 0.012M HCl (*Merck, p.a.*). The protein was eluted with 0.012M HCl and stored as a lyophilized powder at –22°. The residual Ca^{II} content, measured by ICP AES (*Perkin-Elmer 6500* spectrometer), was found to be in the range of 0.02 to 0.03 mol Ca^{II} per mol protein. The concentration of the apo-BLA in stock solns. was determined spectrophotometrically (*Perkin-Elmer Lambda-7* spectrometer) at 280 nm using $\varepsilon_{280} = 28500\,M^{-1} \cdot cm^{-1}$, as determined by the *Lowry* method. This value is identical to that reported for Ca^{II}-BLA [3].

Reagents. All reagents were of anal. grade. The $LnCl_3$-hydrated salts were obtained from the corresponding oxides (*Apache Chemicals*, 99.99%). Stock solns. of lanthanide chlorides were standardized by titration with EDTA at pH 6.0 using xylenol orange as indicator and urotropin as buffer. Protein and reagent solns. were prepared with quartz-bidistilled water. Luminescence measurements were performed in D_2O 99.95% (*Ciba-Geigy*) in presence of Tris buffer (*Fluka, puriss.*) and NaCl or KCl (*Merck, p.a.*). The solns. were prepared by addition of the required volume of protein and $EuCl_3$ stock solns., delivered by a micropipette (*Socorex 841*), into 1-cm quartz cells filled with 3.00 ml D_2O or H_2O. The pH measurements were recorded with a *Metrohm 6.0204* micro-electrode and a *Metrohm E-632* pH-meter. The pD is obtained by adding 0.41 to the pH-meter reading [13].

Luminescence Measurements. Luminescence titrations were carried out at r.t. (*Perkin-Elmer LS-3* spectrofluorimeter), with 1-cm quartz cells containing 3.00 ml of protein soln. Emission intensities were averaged over a 16-s period and read 1–2 min after the addition of titrant, to allow for equilibration. High-resolution excitation spectra were recorded between 577 and 581 nm using a tunable *Rhodamin-6G* dye laser (*Coherent CR-599*, bandpass 0.03 nm) pumped with a 8-W Ar laser (*Coherent CR-8*). The spectra were digitalized by a *Datalab-DL-4000* signal averager (12-bit resolution) and transferred into a *Norsk-560* computer.

Curve Analysis of the Excitation Spectra. Calculations were performed by a least-squares procedure on spectra defined by 300 points and using *Gaussian* functions, with the help of a computer program based on a *Gauss-Newton* algorithm. The quality of the curve fitting is expressed by the residual index '*R*' defined by: $R = \Sigma \| Y_{obs}| - |Y_{calc}\| / \Sigma |Y_{obs}|$. Decompositions of a series of $^5D_0 \leftarrow {}^7F_0$ excitation spectra has been performed with pure *Gaussian* functions, pure *Lorentzian* functions as well as with linear combinations of these functions. Pure *Lorentzian*

functions lead to rapid divergence. The goodness of fit obtained with linear combinations is identical to that obtained with pure *Gaussian* functions, but the *Lorentzian* fraction fluctuates widely from one component to the other (between 5 and 40%) and cannot been constrained without divergence. Moreover, the estimated errors on the curve parameters are 50% larger than with pure *Gaussian* functions.

Results and Discussion. – *Luminescence Spectra.* The binding of Eu^{III} ions to apo-BLA is evidenced by changes in both the intrinsic luminescence of the protein, arising from tryptophan groups, and the luminescence of the Eu^{III} ion. The intensity of the intrinsic luminescence, decreases up to a ratio $R_{Eu} = [Eu^{III}]_t/[BLA]_t$ of 1.0–1.2 and then remains approximately constant (*Fig. 1*). This is similar to what was observed for Tb^{III}; in the latter case, the binding of one equivalent of Tb^{III} to apo-BLA produces an A→N conformational change, accompanied by a decrease of the intrinsic luminescence [7].

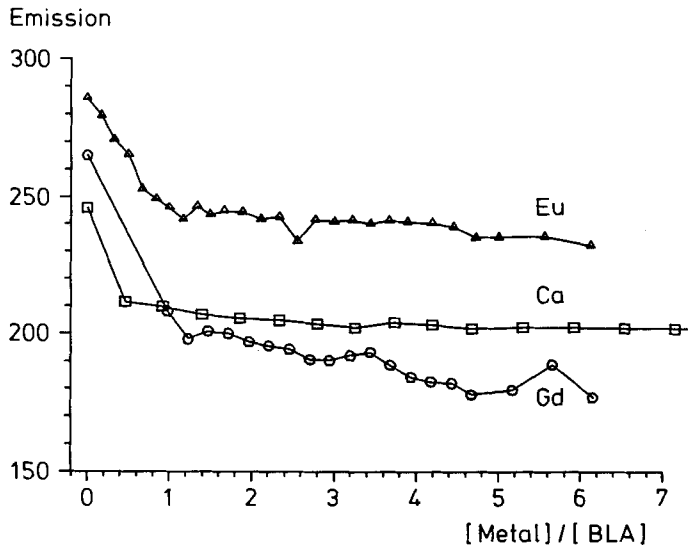

Fig. 1. *Intrinsic luminescence of BLA monitored during titration with various metal ions in D_2O.* Conditions: $\lambda_{exc} = 280$ nm, $\lambda_{an} = 330$ nm. (Δ): Eu, [BLA] = $2.6 \cdot 10^{-5}$ M, [*Tris*] = 0.02M, [NaCl] = 0.01M, pD 6.7; (○): Gd, [BLA] = $6.0 \cdot 10^{-5}$M, [*Tris*] = 0.1M, [NaCl] = 0.01M, pD 6.4; (□): Ca, [BLA] = $7.0 \cdot 10^{-5}$ M, [*Tris*] 0.1M, [NaCl] = 0.01M, pD 6.7.

Comparison experiments were performed with Gd^{III} and Ca^{II} to prove that the observed quenching is not produced by an energy-transfer process from the tryptophan groups to the Ln^{III} ion. The Gd^{III} ion induces a quenching similar to that generated by Eu^{III}, while the effect of the calcium ion ceases at 0.5 equiv. of Ca^{II}, indicating only partial binding of this ion to BLA, probably because of the presence of Na^{I} cations in the supporting electrolyte (*ca.* 400-fold excess with respect to BLA) [14].

The luminescence of Eu^{III} in D_2O is strongly modified upon addition of apo-BLA (*Fig. 2*). The intensity of the $^5D_0 \rightarrow {}^7F_0$ and $^5D_0 \rightarrow {}^7F_2$ transitions is enhanced, indicating a lowering in symmetry around the lanthanide ion. The intensity of the $^5D_0 \rightarrow {}^7F_1$ transition, which is insensitive to environmental changes around the metal ion, can be used as an

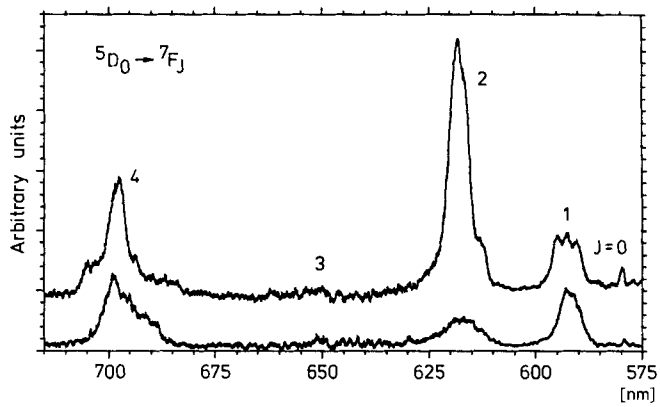

Fig. 2. *Luminescence spectra of* Eu^{III} $1.70 \cdot 10^{-4}$ M *in* D_2O. Conditions: [*Tris*] = 0.02M, [KCl] = 0.01M, pD 6.95(2), λ_{exc} = 395 nm. *Bottom:* without apo-BLA, *top*: in presence of apo-BLA $1.69 \cdot 10^{-4}$ M.

internal standard [10]. The ratio of the integrated transitions $^5D_0 \rightarrow {}^7F_2/{}^5D_0 \rightarrow {}^7F_1$ varies from 0.75 to 4.05 upon addition of an equimolar quantity of BLA, pointing to a strong complexation of Eu^{III} by the protein. The luminescence intensity, taken as the sum of the integrated areas of the $^5D_0 \rightarrow {}^7F_J$ (J = 1, 2, 4) transitions, is enhanced by a factor 2.4 upon complexation, due to a decrease in the number of quenching solvent molecules coordinated to the Eu^{III} ion.

The number of Eu^{III} ions bound per molecule of BLA was determined by monitoring the intensity of the hypersensitive $^5D_0 \rightarrow {}^7F_2$ transition while titrating the apo-protein with Eu^{III}. Two different excitation modes can be used; *i*) energy transfer from aromatic groups to the metal ion, and *ii*) direct excitation to the $^5L_6$ level of Eu^{III}. Although method *i*) has been widely used, it is not appropriate in our case for the following reasons. The A→N conformational change upon complexation may change the distance between the aromatic donor groups and the Eu^{III} ion which, in turn, may modify the efficiency of the energy-transfer process. Moreover, the strong second-order emission of the tryptophan groups interferes with the emission bands of the Eu^{III} ion. Direct excitation was, therefore, preferred. The graph of *Fig. 3* (top) may be divided into three regions. The first extends up to a molar ratio $R_{Eu} = 1.0$ and shows very little enhancement of the Eu^{III} luminescence. The second region, up to $R_{Eu} = 2.0$–3.0 corresponds to a very steep increase in luminescence intensity. In the third region, the luminescence intensity levels out up to $R_{Eu} = 8$, and remains approximately constant. These data point to the presence of at least two metal ion sites in apo-BLA.

The turbidity of the solutions gives information on the conformational changes occurring during the titration. No turbidity is detected for $R_{Eu} < 1.0$. If the binding of one Eu^{III} ion induces the same conformational changes as Tb^{III} does, as inferred from the quenching of the intrinsic luminescence, the Eu^{III}-bonded protein should be in its N-form and cannot self-associate. When more Eu^{III} is added, a second site starts to be populated and the solution becomes slightly milky, but no precipitation occurs. This suggests a second conformational change, maybe towards an 'expanded A' form [7], which allows self-association of the protein. The absorbance of the solution has been measured at both

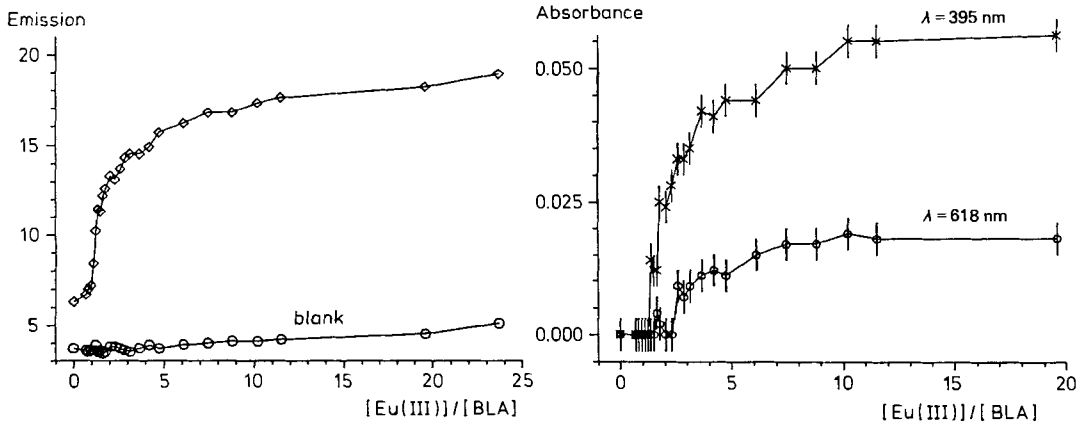

Fig. 3. Left: *Luminescent titration of apo-BLA with* Eu^{III}. Conditions: $\lambda_{exc} = 395$ nm, $\lambda_{an} = 615$ nm. (◇): [BLA] = $4 \cdot 10^{-5}$ M, [*Tris*] = 0.1 M, [NaCl] = 0.01 M, pD 6.6; (○): blank, same conditions, without BLA. Right: Changes in absorbance occurring during the titration of BLA, $\lambda = 395$ nm (*X*) and 618 nm (○); reference: D_2O, [Eu^{III}] = $6.58 \cdot 10^{-4}$ M, [*Tris*] 0.02 M, [NaCl] = 0.01 M, pD 6.6.

the excitation and emission wavelengths in order to quantify the inner-filter effect [15] (*Fig. 3*, bottom). The calculated correction amounted to less than 3%, indicating that turbidity does not affect luminescence markedly and data were, therefore, not corrected. Subsequent measurements were carried out under experimental conditions minimizing turbidity.

Analysis of the $^5D_0 \leftarrow {}^7F_0$ *Excitation Spectra.* To gain further insight into the number and the nature of the Eu^{III}-binding sites, we have resorted to laser-excited $^5D_0 \leftarrow {}^7F_0$ excitation spectra recorded under various experimental conditions. Each specific chemical environment of Eu^{III} produces a distinct excitation band which may be evidenced by high-resolution scanning of the excitation spectrum. However, since biological ligands have fluctional structures, the resulting excitation bands are often broad and unresolved, which necessitates a curve analysis procedure.

Typical $^5D_0 \leftarrow {}^7F_0$ excitation spectra of Eu^{III} ions bound to apo-BLA are presented in *Fig. 4*. When R_{Eu} is small (0.53), the spectra display only one, relatively narrow (full width at half height, *fwhh* = 7–8 cm^{-1}), but asymmetrical, band. This band cannot be fitted with one *Gaussian* curve (*Fig. 4, a*) and its mathematical analysis requires two components, labelled I_a and I_b (*Fig. 4, b*), separated by only 2.5 cm^{-1} (17251.2 ± 0.1 and 17253.7 ± 0.4 cm^{-1}), and with *fwhh* = 7.2 ± 0.2 and 12.9 ± 0.5 cm^{-1}, respectively. Using *Horrocks'* empirical relationship [10], which relates the energy of the $^5D_0 \leftarrow {}^7F_0$ transition to the total formal charge of the ligands coordinated to Eu^{III}, we find that coordination of the metal ion occurs through four negatively charged groups (charge = −4.0 ± 0.5 and −3.7 ± 0.5 for I_a and I_b, respectively). Since Ln^{III} ions are known to bind strongly to the Ca sites of proteins, we assign site I to the Ca^{II} site of BLA. The calculated formal charge of this site is indeed in line with the coordination environment of Ca found in the crystallographic structure of baboon milk α-lactalbumin [16] [17]. The latter is comprised of four COO, one CO, and one OH groups and includes Asp-82, Asp-83, Asp-84, Asp-87, Asp-88, and Thr-86. Moreover, this region of the protein being somewhat rigid and protected from

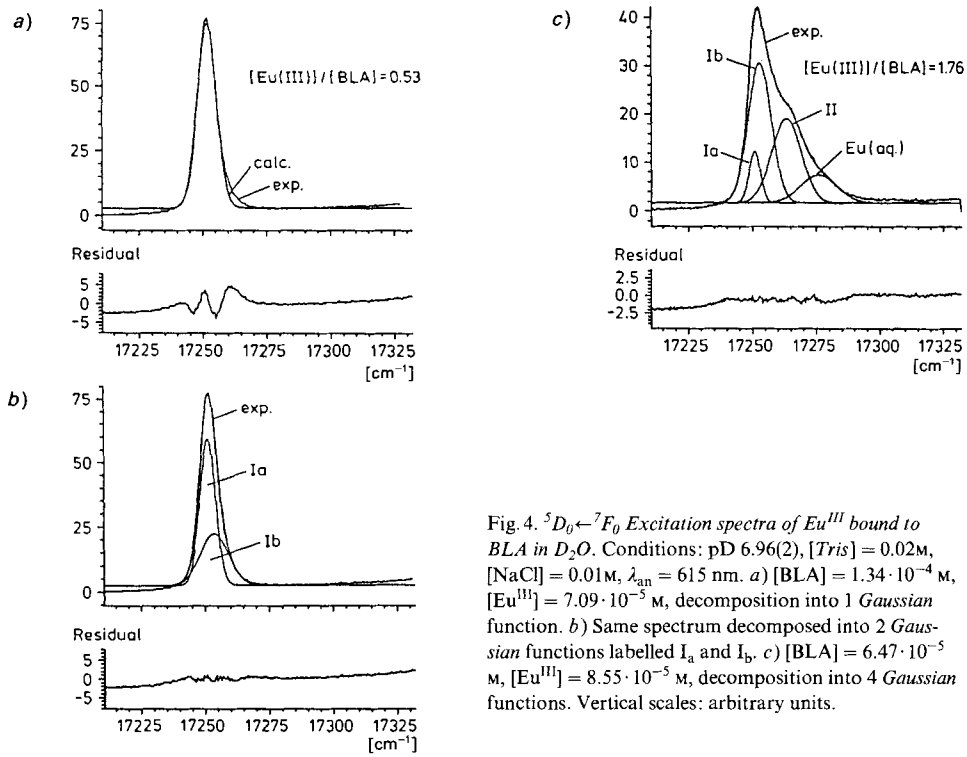

Fig. 4. *$^5D_0 \leftarrow {}^7F_0$ Excitation spectra of Eu^{III} bound to BLA in D_2O.* Conditions: pD 6.96(2), [*Tris*] = 0.02M, [NaCl] = 0.01M, λ_{an} = 615 nm. *a*) [BLA] = $1.34 \cdot 10^{-4}$ M, [Eu^{III}] = $7.09 \cdot 10^{-5}$ M, decomposition into 1 *Gaussian* function. *b*) Same spectrum decomposed into 2 *Gaussian* functions labelled I_a and I_b. *c*) [BLA] = $6.47 \cdot 10^{-5}$ M, [Eu^{III}] = $8.55 \cdot 10^{-5}$ M, decomposition into 4 *Gaussian* functions. Vertical scales: arbitrary units.

external influences, Eu^{III} binding in this site would account for the relative sharpness of band I.

When R_{Eu} is increased, the excitation profile becomes more complicated. An example is given in *Fig. 4, c*, for a ratio equal to 1.76. Curve-resolution of the spectrum requires now four components. Two of them at 17250.9 ± 0.2 cm^{-1} (*fwhh* = 5.2 ± 0.6 cm^{-1}) and 17252.9 ± 0.7 cm^{-1} (*fwhh* = 10.6 ± 0.6 cm^{-1}) correspond to bands I_a and I_b of the previous spectrum. Another one, labelled II, is broader (*fwhh* = 13 ± 2 cm^{-1}), occurs at an energy 10 cm^{-1} higher than I_b, and is assigned as arising from the population of a second metal-binding site of BLA. The fourth band, at 17275.8 ± 0.1 cm^{-1} (*fwhh* = 16.7 ± 0.2 cm^{-1}) is readily ascribed to the Eu^{III} aquo-ion by comparison with control experiments on similar solutions without BLA. The observation of free Eu^{III} ions in the solution (even at R_{Eu} = 1.1) implies that site II has a smaller affinity for metal ions than environments I_a and I_b. Moreover, the corresponding excitation band being broad, this site probably resides in a more flexible region of the protein, possibly nearer the surface of the macro-molecule. If this were correct, binding at site II would be less selective and more pH-sensitive. To substantiate this hypothesis, we have studied the $^5D_0 \leftarrow {}^7F_0$ excitation spectra of solutions containing bovine serum albumin (BSA). The latter protein binds several metal ions without specificity, for instance four Gd^{III} ions with the same affinity [18]. The $^5D_0 \leftarrow {}^7F_0$ excitation spectrum of Eu^{III} bound to BSA displays a single broad band with characteristic parameters similar to those of band II: maximum at 17267.9 ± 0.3 cm^{-1} and *fwhh* = 17.8 ± 0.1 cm^{-1}. The band is broader than band II of Eu^{III}-bonded BLA,

which may be explained by the presence of four distinct, but nonspecific, sites in BSA. The total formal ligand charges, calculated from the above-mentioned relationship amount to -2.2 ± 0.5 for band II and -1.6 ± 0.5 for BSA. This result confirms the assumption made about the second metal-binding site of BLA.

At this point, it is necessary to bring additional evidences supporting the correctness of the mathematical decomposition of the excitation spectra. Care should indeed be exercised in performing such an analysis, particularly, if the number of components or the bandshapes are not known precisely: an analytical solution is never unique and the number of components can be either overestimated or underestimated [19]. In our case, we have only taken into account the components generating a visible maximum or a shoulder in the spectra. Moreover, the parameters for the Eu^{III} aquo-ion were fixed and the bandwidths, which depend to which extent the Eu^{III} environment is structurally rigid, were allowed to vary within a preset range of 6–19 cm^{-1} only. The lower value holds in cases where motional narrowing has a strong influence [20], and the upper value corresponds to the less structurally defined aquo-ion. The mean difference in energy between components I_a and I_b is small but reproducible (2–2.5 cm^{-1}) and significantly larger than the laser bandwidth (1.2 cm^{-1}). Despite this, it is not obvious that these components do not arise from an instrumental artefact. We have, therefore, tried to find model complexes containing one coordination site, and whose excitation spectra could be used as reference for the bandshape of the $^5D_0 \leftarrow {}^7F_0$ transition. The $[Eu(dota)]^-$ complex (dota = 1,4,7,10-tetraazacyclododecane *N,N′,N″,N‴*-tetraacetate) was selected first, because it is rigid [21], highly stable [22], and it exhibits a strong luminescence with a unique $^5D_0 \rightarrow {}^7F_0$ transition [23]. The $^5D_0 \leftarrow {}^7F_0$ excitation spectrum is indeed perfectly symmetrical, which demonstrates the absence of instrumental artefact in our experiments. The curve analysis reveals that its bandshape is neither a pure *Gaussian*, nor a *Lorentzian* curve, but a linear combination containing 36.5% of *Lorentzian* function, in agreement with published results [24]. The latter value reflects the exceptional rigidity of the $[Eu(dota)]^-$ molecule, resulting in a lower contribution of the inhomogenous broadening to the bandshape. A second model chosen was $[Eu(dtpa)]^{2-}$ (dtpa = diethylenetriamine pentaacetate), which provides a more flexible environment to the lanthanide ion. The $^5D_0 \leftarrow {}^7F_0$ spectrum of this complex contains a main band and a small component, which increases at low pH, reflecting the protonation and exposure to the solvent of one of the COO^- groups [22]. The main band exhibits a 100% *Gaussian* bandshape, with a *fwhh* (11.2 cm^{-1}) similar to that of the $[Eu(dota)]^-$ complex (11.5 cm^{-1}). The $[Eu(dtpa)]^{2-}$ complex being certainly a better model for metal-binding sites of proteins than the rigid $[Eu(dota)]^-$ moiety, we have used pure *Gaussian* functions in the mathematical decomposition of the excitation spectra of BLA (*cf.* also *Experimental*).

Finally, to better demonstrate the reality of the two components I_a and I_b, series of excitation spectra were recorded *vs.* the analyzing wavelength. The latter was scanned through both the $^5D_0 \rightarrow {}^7F_1$ and $^5D_0 \rightarrow {}^7F_2$ transitions (*Fig. 5*). All the spectra are identical in both cases, except for a small, but significant shift of the maximum upon scanning the analyzing wavelength through the hypersensitive $^5D_0 \rightarrow {}^7F_2$ transition. This is indicative of the presence of two different environments for the Eu^{III} ion, one of which emitting at lower wavelength than the other.

To assess the pD influence on the Eu^{III} binding to apo-BLA, four titrations were performed at different pD values. The parameters of the $^5D_0 \leftarrow {}^7F_0$ components and the

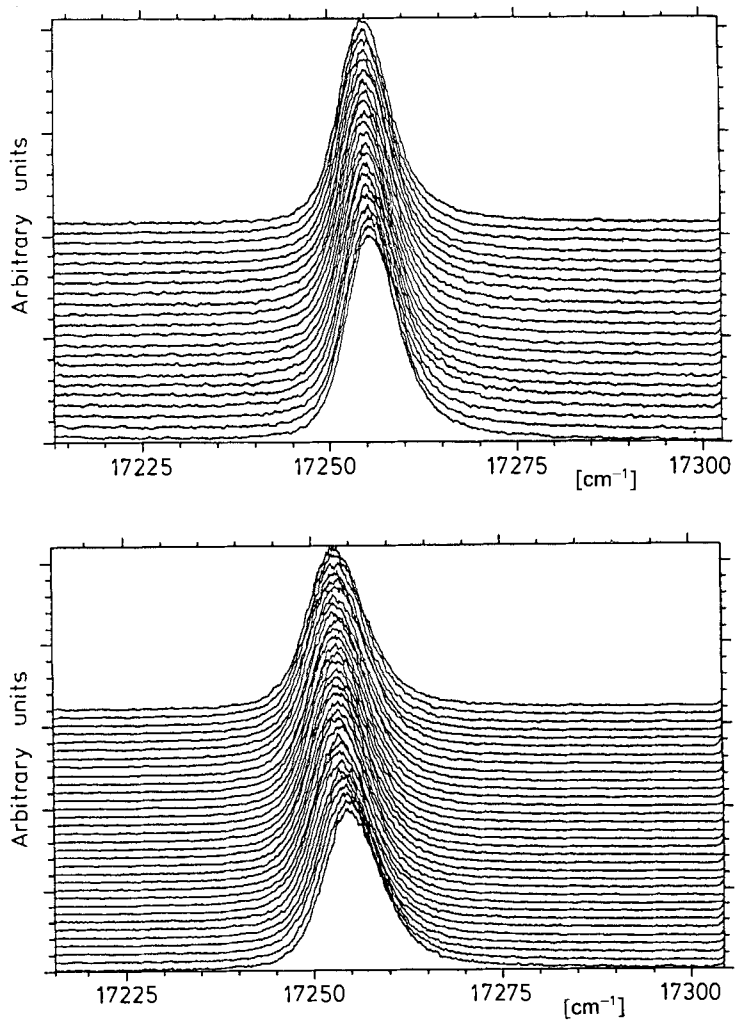

Fig. 5. $^5D_0 \leftarrow {}^7F_0$ *Excitation spectra of* Eu^{III} *bound to environments* I_a *and* I_b *of BLA plotted* vs. *the analyzing wavelength.* Conditions: $[Eu^{III}] = 7.8 \cdot 10^{-5}$ M, [BLA] = $8.56 \cdot 10^{-5}$ M in D_2O, [*Tris*] = 0.02M, [KCl] = 0.01M, pD 6.94(2). *Top*: λ_{an} varied from 590 to 595.25 nm by 0.25-nm increments. *Bottom*: λ_{an} varied from 615 to 620 nm by 0.15-nm increments.

total formal charges of the ligands coordinated to the metal ion are reported in the *Table*. The only significant pD dependance is a substantial increase of the intensity of component II with increasing pD (*Fig. 6*). These data confirm that the binding groups of the corresponding environment in BLA are exposed to the solvent and probably protonated at low pD. On the other hand, the successive population of sites I and II is little affected by the pD change: population of site II always starts around $R_{Eu} = 1$, indicating that no important conformational change occurs within the investigated pD range.

Table. *Mean Energies and Bandwidths (fwhh* [cm^{-1}]) *of the Four Components of the* $^5D_0 \leftarrow {}^7F_0$ *Transition Recorded at Different pD's and Concentrations.* Standard deviations on the last digit are given in parentheses, except for the parameters kept constant during calculation.

	I_a	I_b	II	Aquo-ion
pD 5.87(2), 0.67 < [Eu^{III}]/[BLA] < 9.10 (16 spectra)				
Energy	17253.9(1)	17254.8(4)	17264(2)	17271.6
fwhh	6.0(8)	11(2)	18.7(4)	16.6
Charge[a])	−3.8	−3.7	−2.2	
pD 6.46(2), 0.88 < [Eu^{III}]/[BLA] < 10.6 (11 spectra)				
Energy	17253.8(7)	17255.8(1)	17267(2)	17278.5
fwhh	6.15(3)	11.6(7)	14.5(3)	17.8
Charge[a])	−3.8	−3.5	−1.5	
pD 6.96(2), 0.53 < [Eu^{III}]/[BLA] < 4.23 (9 spectra)				
Energy	17250.9(2)	17253.0(6)	17264(2)	17275.8
fwhh	6.1(5)	11.4(8)	15(2)	16.7
Charge[a])	−4.2	−3.9	−2.2	
pD 7.36(2), 1.32 < [Eu^{III}]/[BLA] < 4.23 (6 spectra)				
Energy	17252.1(2)	17255.0(9)	17263(5)	17275.9
fwhh	6.9(2)	12.5(2)	17(4)	17.4
Charge[a])	−4.0	−3.6	−2.4	

[a]) Calculated from *Horrocks* relationship; accepted uncertainty: ± 0.5 unit [10].

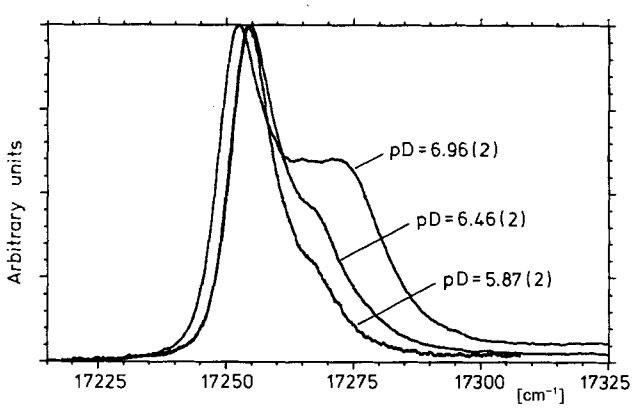

Fig. 6. $^5D_0 \leftarrow {}^7F_0$ *Excitation spectra of* Eu^{III} *bound to BLA at various pD values.* [BLA] = $3 \cdot 10^{-5}$ M, [Eu^{III}] = $9 \cdot 10^{-5}$ M in D_2O, [*Tris*] = 0.02M, [NaCl] = 0.01M.

Population Analysis of the Metal Ion Sites. After identification of the various $^5D_0 \leftarrow {}^7F_0$ components, a titration of BLA by Eu^{III} has been performed to gain information on the relative population of the two metal ion sites. The changes of area of the $^5D_0 \leftarrow {}^7F_0$ components *vs.* R_{Eu} are depicted in *Fig. 7.* We notice the successive population of sites I (Ia + Ib) and II, reflecting a large difference of affinity between the corresponding environments. Most significant is the decrease of band I ($I_a + I_b$), when environment II

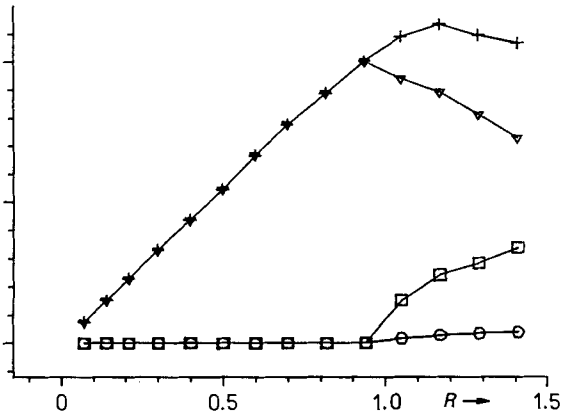

Fig. 7. *Area of the* $^5D_0 \leftarrow {}^7F_0$ *components* vs. $R = [Eu^{III}][BLA]$. Conditions: [BLA] = $1.35 \cdot 10^{-4}$ M in D_2O, [*Tris*] = 0.02M, [KCl] = 0.01M, pD 6.96(2). +: Total area ($I_a + I_b + II$), ▽: band I ($I_a + I_b$), □: band II, ○: Eu(aq). Vertical scale: arbitrary units.

starts to be populated. Similar results are obtained when Ca^{II}-BLA is titrated with Eu^{III} [25]. Since the ratio of the binding constants K_1/K_2 is very large, a change in the relative population of sites I and II can only be explained, if conformational changes are taken into account. The binding of Eu^{III} to site II probably promotes an A-like ('expanded') conformation [7] reducing the affinity for site I. However, we cannot exclude that the change from a N to an A-like conformation does not modify the coordination around the Eu^{III} ion in site I and, consequently, its quantum yield.

The apparent affinity constant K_2^{app} for site II, defined by the following equilibrium

$$[Eu^1(bla)] + [Eu(aq)] \rightleftharpoons [Eu^1Eu^2(bla)],$$

can be roughly estimated by use of the intensity data for band II for $R_{Eu} > 1.0$ and pD = 6.96 assuming that the affinity for site I is not modified upon populating site II. After a minor correction for the interaction between the *Tris* buffer and the Eu^{III} ion [26], we calculate log $K_2^{app} = 3.5(1)$. This value is quite similar to the affinity constant log $K_1 = 3.89$ determined for the binding of Gd^{III} to bovine serum albumin [18], in agreement with our assignment of site II to a nonspecific metal-binding site. Attempts to calculate K_2^{app} at pD's lower than 6.96 failed, indicating that K_2^{app} is smaller in more acidic medium, due to the protonation of the ligating groups.

The binding constant K_1^{app} of Eu^{III} to site I is too large to be evaluated from the excitation spectra (estimates for Ca^{II}-binding range between 10^6 and 10^9 M^{-1} [1]). Nevertheless, the intensity of components I in the $^5D_0 \leftarrow {}^7F_0$ excitation spectra can be used to determine the relative affinity of the different lanthanide ions for site I. Indeed, adding 1 equiv. of Ln^{III} ions to a solution containing Eu^{III} and apo-BLA in a 2:1 molar ratio causes a drop in the intensity of component I ($I_a + I_b$), reflecting the Eu^{III} substitution. The drop, which reflects the affinity of the Ln^{III} ion for BLA, amounts to 17% for La and to 29% for Lu. This points to a sufficient rigidity of site I to exhibit a selectivity towards similar metal ions with varying ionic radii.

Conclusions. – The data reported in this paper demonstrate the existence of at least two metal-binding sites in BLA. The site labelled I has a high affinity for Eu^{III}. It is relatively rigid and may be identified as the Ca site reported for baboon milk α-lactalbumin (Asp-82, Asp-83, Asp-84, Asp-87, Asp-88, and Thr-86) [17]. The presence of the

COO^- groups Asp-98 and Asp-88 in a Ca site is unusual for a Ca-binding protein. This Ca^{II} site is somewhat similar to an EF-loop, with two differences, namely a more compact structure due to the absence of two amino acids and a different coordination polyhedron, which is a pentagonal bipyramid instead of a distorted octahedron [17]. A similar coordination polyhedron was also observed for human α-lactalbumin [27]. EF-Loops are known to have a preference for binding the smallest lanthanide ions [28], which explains the larger affinity of BLA for Lu with respect to La found in this study. We have also shown that site I is comprised of two different environments with similar affinities for the metal ion. These environments are too similar to allow a more detailed characterization. They could be related to the presence of COO^- groups which may vary their denticity, depending upon steric interactions [25].

The second site is sequentially populated after saturation of site I. Its affinity is low, and its population increases with increasing pD, indicating that this site is more exposed to the solvent and less specific. We tentatively assign it to the distinct Zn^{II} site evidenced by other authors [4] [9]. The two metal-binding sites we identified in BLA by direct evidence are being studied in more detail, both by luminescence and potentiometric techniques.

This research is supported by grants from the *Swiss National Science Foundation*. We thank the *Fondation Herbette* (Lausanne) for the gift of two lasers.

REFERENCES

[1] H.A. McKenzie, F.H. White, Jr., *Adv. Protein Chem.* **1991,** *41,* 173.
[2] Y. Hiraoka, T. Segawa, K. Kuwajima, S. Sugai, *Biochem. Biophys. Res. Commun.* **1980,** *95,* 1098.
[3] J.-C. Van Ceunebroeck, I. Hanssens, M. Joniau, F. Van Cauwelaert, *J. Biol. Chem.* **1985,** *260,* 10944.
[4] K. Murakami, L.J. Berliner, *Biochemistry* **1983,** *22,* 3370.
[5] G. Musci, L.J. Berliner, *Biochemistry* **1985,** *24,* 6945.
[6] S.C. Bratcher, M.J. Kronman, *J. Biol. Chem.* **1984,** *259,* 10875.
[7] M.J. Kronman, S.C. Bratcher, *J. Biol. Chem.* **1984,** *259,* 10887.
[8] L.J. Berliner, P.D. Ellis, K. Murakami, *Biochemistry* **1983,** *22,* 5061.
[9] G. Musci, G.H. Reed, L.J. Berliner, *J. Inorg. Biochem.* **1986,** *26,* 229.
[10] J.-C.G. Bünzli, in 'Lanthanide Probes in Life, Medical, and Earth Sciences', Eds. J.-C.G. Bünzli and G.R. Choppin, Elsevier Science Publ. B.V., Amsterdam, 1989, Chapt. 7.
[11] M.T. Henzl, E.R. Birnbaum, *J. Biol. Chem.* **1988,** *263,* 10674.
[12] P.M. Mulqueen, J.M. Tingey, W. de W. Horrocks, Jr., *Biochemistry* **1985,** *24,* 6639.
[13] A.K. Covington, M. Paabo, R.A. Robinson, R.G. Bates, *Anal. Chem.* **1968,** *40,* 700.
[14] M. Hamano, K. Nitta, K. Kuwajima, S. Sugai, *J. Biol. Chem.* **1986,** *100,* 1617.
[15] K.J. Wiechelmann, *Internat. Lab.* **1986,** *March,* 82.
[16] D.I. Stuart, K.R. Acharya, N.P.C. Walker, S.G. Smith, M. Lewis, D.C. Phillips, *Nature (London)* **1986,** *324,* 84.
[17] K.R. Acharya, D.I. Stuart, N.P.C. Walker, M. Lewis, D.C. Phillips, *J. Mol. Biol.* **1989,** *208,* 99.
[18] J. Reuben, *Biochemistry* **1971,** *10,* 2834; F. Friedberg, *FEBS Lett.* **1975,** *59,* 140.
[19] G.C. Allen, R.F. McMeeking, *Anal. Chim. Acta* **1978,** *102,* 73.
[20] S.J. Brauer, *J. Phys. Chem.* **1982,** *86,* 3759.
[21] M.-R. Spirlet, J. Rebizant, J.-F. Desreux, M.-F. Loncin, *Inorg. Chem.* **1984,** *23,* 359.
[22] M.F. Loncin, J.-F. Desreux, E. Merciny, *Inorg. Chem.* **1986,** *25,* 2646.
[23] C.C. Bryden, C.N. Reilly, *Anal. Chem.* **1982,** *25,* 2646.
[24] W. De W. Horrocks, Jr., C.W. Mc Nemar, *Appl. Spectrosc.* **1989,** *43,* 816.
[25] J.-M. Pfefferlé, Ph. D. Dissertation, Université de Lausanne, 1989.
[26] J.-M. Pfefferlé, J.-C.G. Bünzli, *Helv. Chim. Acta* **1989,** *72,* 1487.
[27] K. Harata, M. Muraki, *J. Biol. Chem.* **1992,** *267,* 1419.
[28] T. Drakenberg, M. Swärd, A. Cavé, J. Parello, *Biochem. J.* **1985,** *227,* 711.