with crown ethers. For most Eu(II)-crown complexes (such as with 15-crown-5), the intensity is great from the beginning and maintains a constant value during the irradiation.

Emission profiles taken during the irradiation periods indicate that uncomplexed, methanol-solvated Eu(II) gives a 487-nm emission peak, which undergoes blue shifting as the complex formation proceeds.

Excitation spectra in Figure 2 for the complex with **1** also show variations before and after irradiation, suggesting that the inner coordination sphere of Eu(II) is subjected to changes with exposure to UV. Another interesting feature in the excitation spectra is that peak intensities around 240 nm increase with irradiation time. The peak is not observed for other crown ethers that do not have any brominated groups in their side chains. This peak probably is a charge-transfer transition, involving the Eu(II) ion and the brominated side chain.

Figure 3 illustrates the variation in absorption spectra of the complex of **1**. The peak around 200 nm increases with the passage of irradiation time, while other peaks[8] due to the 4f/5d configuration of Eu(II) become smaller. This phenomenon is not observed for other crown complexes lacking a bromoalkyl group, which maintain the same absorption intensity during the irradiation.

A discrepancy between the absorption and excitation spectra is due to the fact that the concentration of the complex for excitation spectra measurement was too high. Use of a diluted solution (8×10^{-4} mol dm^{-3}) narrowed the gap, and every peak shifted to lower wavelength. A tail of an excitation peak that should have been seen at 200 nm was observed at around 220 nm, because the effective wavelength range of our spectrofluorometer is from 220 up to 590 nm.

Photochemical decomposition of the bromoalkyl group is not likely, because no free radical was detected in the ESR spectrum taken for the complex irradiated with UV radiation. In addition, there was no change in IR spectra (C-Br stretching 670 cm^{-1}) for the complexes isolated before and after the irradiation.

There is steric hindrance in ligands **1** and **2** exerted by a bromoalkyl side chain, which impedes the ground-state complex formation. The excited Eu(II) ion becomes a strong electron acceptor and is taken into the cavity of the crown ether in spite of the hindrance. Once the complex is formed, it is quite stable and does not dissociate.

Crown ethers having no side chain or having a side chain without a bromoalkyl substituent (such as 2-ethyl-2-methyl or 2-propyl-2-methyl) do not exhibit such changes in emission intensities. The Eu-Br CT must play an important role in the intensity increase.

A mixture of Eu(II) with 2-BrMe-2-Me(12-crown-4) (**3**) in methanol solution showed almost the same emission intensity as that of the $EuCl_2$-methanol solution and gave no increase in emission intensity even after 2-h irradiation. This is because the cavity of **3** is too small to accommodate an Eu(II) ion, and there can be no complex formation for this case.

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Articles

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Lanthanide Complexes of Ionophores. 2. Spectroscopic Characterization of Lanthanide(III) Ion Binding to Lasalocid A and A23187 in Methanol[1]

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Laser luminescence excitation and UV absorption spectroscopy were used to monitor the coordination of the carboxylic ionophores lasalocid A and A23187 to lanthanide(III) ions in methanol. The formation of mono- and bis(ionophore) chelates was demonstrated by monitoring changes in the $^7F_0 \rightarrow {}^5D_0$ excitation spectrum of Eu(III) in the presence of the ligands. Changes in ligand UV absorption spectra in the presence of Tb(III) indicate that coordination occurs at the salicylate group of lasalocid A and at the α-ketopyrrole and benzoxazole moieties of A23187. The high binding constants imply that both ligands coordinate in a multidentate fashion. A 1:1 complex forms between Ca(II) ions and lasalocid A with a dissociation constant at 1.24×10^{-5} M and $\Delta H°$ and $\Delta S°$ values of -4.8 ± 0.8 kcal mol^{-1} and -39 ± 3 cal deg^{-1} mol^{-1}, respectively, for the dissociation reaction.

Introduction

The characterization of metal ion binding sites in biological molecules is relevant to the understanding of their activity, in terms of structural features and the role of the metal ions. Substitution of trivalent lanthanide ions (Ln(III)) for biologically significant, yet spectroscopically "silent", metal ions is a well-documented procedure for probing these sites.[2] The ability of europium(III) to luminesce in solution at room temperature,[3,4] along with its similarity to calcium(II) in ionic radius and coordination number, has made it one of the more popular Ln(III) probe species.

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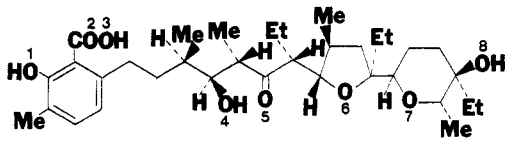
Ionophores are organic sequestering agents that possess the ability to encapsulate a metal ion and transport it across artificial and biological membranes. The structural features of an ionophore that enable it to do this include (1) a polar interior, which interacts with the metal ion in a manner dictated by steric factors and potential coordinating groups, and (2) a nonpolar exterior, which permits the ionophore complex to be soluble in a hydrophobic medium. As a result, these substances serve as carriers of ions through lipid membranes.[5,6]

There are two classes of ionophores. The first, naturally occurring antibiotics, is further divided into neutral ionophores (which contain no dissociable protons) and carboxylic ionophores (which contain a carboxylate group that deprotonates upon complexation). Synthetic ionophores such as crown ethers and cryptands constitute the second class. The present paper focuses on two calcium-binding, carboxylic ionophores, lasalocid A and A23187 (**1** and **2**, respectively), and their



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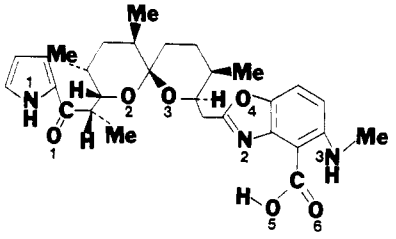

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interaction with metal ions in a relatively polar medium (methanol). The structures of these ionophores show considerable dependence on solvent polarity with an "open", linear-chain form favored in polar solvents and a hydrogen-bonded, cyclic form favored in nonpolar media.[5]

Solid-state work involving lasalocid A and A23187 is rather limited. Structures of sodium, barium, and silver complexes are known for the former, while only calcium complexes of the latter have been elucidated thus far (see Results and Discussion for details).[7-12] Solution studies are in greater abundance, particularly for lasalocid A. Binding constants have been reported for a number of alkali- and alkaline-earth-metal ions with lasalocid A in a variety of solvents[13,14] and for Mg(II) and A23187 in ethanol.[15] In more qualitative terms, the following selectivity sequences have been determined for the two ionophores by radioisotopic extraction from an aqueous solution into a lipid phase: Cs > Rb ~ K > Na > Li and Ba >> Sr > Ca > Mg for lasalocid A[16,17] and Li > Na > K ≃ O and Mn >> Ca ~ Mg >> Sr > Ba for A23187.[15] A23187 is more specific for divalent ions than is lasalocid A, since the interaction of A23187 with monovalent cations is negligible.

No general conclusions regarding the stoichiometry of these metal ion/ionophore complexes can be drawn from results in the literature, as the nature of the complexes formed depends on experimental conditions. Species having 1:1, 1:2, and 2:1 stoichiometries have been reported.[13,15,18] A23187 has been shown to be a more effective calcium transporter than lasalocid A,[19,20] probably due to its greater tendency to form neutral 1:2 metal:ligand species.[20]

Only fragmentary information is available regarding the interaction of lanthanide ions with lasalocid A and A23187.[16,21-26] We report here laser-excited luminescence results on the europium(III)/lasalocid A and europium(III)/A23187 systems in methanol. In addition, absorption spectroscopy was used to examine the mode of lasalocid A and A23187 coordination to terbium(III), as well as for quantitation of terbium(III) and calcium(II) binding to lasalocid A.

Experimental Section

The chemicals used in this study, along with their sources and purities, are as follows: $EuCl_3 \cdot 6H_2O$ and $TbCl_3 \cdot 6H_2O$, Aldrich, 99.9%; $CaCl_2 \cdot 2H_2O$, Aldrich, 98+%; lasalocid A (sodium salt = NaLas), Aldrich, 99+%; A23187 (free acid), Calbiochem-Behring Corp., lot no. 203065; methanol (spectral grade), Fisher Scientific, 0.1% H_2O. All chemicals were used without further purification. The metal and ligand solutions were prepared with freshly opened methanol, filtered under a nitrogen atmosphere, and used immediately. Concentrations of the metal stock solutions were determined by titration with EDTA, using arsenazo indicator.[27] Concentrations of the ligand stock solutions were determined by measuring the absorbance at a wavelength of known extinction coefficient (Las^- ϵ_{305} = 4170 M^{-1} cm^{-1}; A23187 ϵ_{290} = 17070 M^{-1} cm^{-1}). All dilutions were carried out with use of standard volumetric glassware and the appropriate microliter syringe (Hamilton Co., accuracy ≤±1%).

All spectroscopic studies were performed by using a Teflon-capped quartz cuvette with a 1-cm path length, except for the binding constant determinations in which a 5-cm cylindrical cell was employed. Experiments were carried out at ambient temperature, unless otherwise indicated. Constant-temperature experiments were conducted with use of a Haake D3 water bath (±0.1 °C).

Europium luminescence spectral and lifetime data were collected by using the pulsed-nitrogen laser pumped-dye laser system described previously.[3] Excitation spectra of the $^7F_0 \rightarrow {}^5D_0$ transition were obtained by continuously scanning the laser (577.5–581.0 nm) while the $^5D_0 \rightarrow {}^7F_2$ emission (614 nm) was monitored. In a typical titration an aliquot of a europium(III)/ionophore mixture was added to a known amount of $EuCl_3$ stock solution (the europium(III) concentrations being identical in both solutions to eliminate dilution effects). The intensity of the $^7F_0 \rightarrow {}^5D_0$ excitation band of the $EuCl_3$ solvate in methanol is directly proportional to its concentration; consequently, the intensity of this band in solutions containing different Eu(III)/ionophore ratios (all else being equal) was used to monitor the

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relative amounts of free (and therefore bound) europium(III) present in each solution. Excitation was at a single wavelength, λ_{ex} = 578.80 nm (Las^-) or 579.00 nm (A23187). The fraction of free solvated $EuCl_3$, given by the intensity ratio, $I(EuCl_3 + \text{ionophore})/I(EuCl_3)$, was plotted vs. the number of equivalents of ionophore added to yield the titration curve.

The absorption spectra were recorded on a Cary 210 spectrophotometer. Titrations were carried out by adding aliquots of a $LnCl_3$/ionophore mixture to an ionophore stock solution with the ionophore concentrations identical in the two solutions to eliminate dilution effects. The stoichiometries of the complexes formed were obtained from plots of absorbance (or ratio of absorbances) vs. equivalents of metal ion added (λ = 248/310 nm (Las^-), 320 nm (A23187)).

Calculation of Dissociation Constants. Dissociation constants (K_d) were determined from UV absorption by using a computer program, developed in this laboratory, that optimizes the fit of calculated absorption data (from standard free metal ion, free ligand, and complex spectra) to the observed absorption spectra obtained during a metal ion/ligand titration. The algorithm is based upon eq 1, which assumes

$$K_d = [M][L]/[ML] \quad (1)$$

that a 1:1 species (ML) is the only complex present in solution. Substitution of the expressions for the metal (M) and ligand (L) concentrations at equilibrium (eq 2 and 3) into the K_d formula and rearranging to solve for [ML] yield eq 4, where $B = K_d + [M]_i +$

$$[M] = [M]_i - [ML] \quad (2)$$

$$[L] = [L]_i - [ML] \quad (3)$$

$$[ML] = \tfrac{1}{2}(B - (B^2 - 4[M]_i[L]_i)^{1/2}) \quad (4)$$

$[L]_i$ and the subscript i indicates the initial concentrations. [ML], [M], and [L] are calculated for each K_d, and these values are substituted into eq 5, to yield the calculated absorbances for the set of

$$A_{\lambda,[M]_i} = \epsilon_M[M] + \epsilon_L[L] + \epsilon_{ML}[ML] \quad (5)$$

metal concentrations and wavelengths (molar extinction coefficient, ϵ's, are known from the standard curves). These absorbances are then compared to the observed data through eq 6. The reported value of K_d is the one which minimizes Φ.

$$\Phi = \sum_{\lambda,[M]_i} (A_{calcd} - A_{obsd})^2 \quad (6)$$

In the case where a "good" fit was not obtained (e.g. $TbCl_3$/Las^-—vide infra), a slightly modified version of the program was employed, which treates the ϵ's of the fully formed complex at the various wavelengths and K_d as parameters and uses a nonlinear regression analysis to yield the best value of K_d.

Results

All results with lasalocid A were obtained with its sodium salt. Under the conditions employed (0.1 mM Las^-), complexation of the anion of lasalocid A (Las^-) by Na^+ is minimal due to its weak association constant (370 M^{-1}).[12] In essence, we are observing the interaction of the anionic form of the ionophore with various cations. Work with A23187 was performed with the free-acid form of the ligand, as the proton does not appear to inhibit metal ion coordination for the metals examined in this study.

Excitation Spectroscopy. In Figure 1 are shown the $^7F_0 \rightarrow {}^5D_0$ excitation spectra of $EuCl_3 \cdot 6H_2O$ in methanol (A) and its 1:1 (B) and 1:2 (C) complexes with lasalocid A. This electronic transition occurs between nondegenerate states and results in a single excitation maximum for each Eu(III)-containing species.

The spectrum of $EuCl_3 \cdot 6H_2O$ in methanol (0.1 mM) consists of a single peak with a maximum at 579.05 nm (17 269 cm^{-1}). This peak is attributed to the solvated species $Eu(MeOH)_x^{3+}$, on the basis of the following observations: (1) a band at 579.50 nm grows in (with a concomitant decrease in the 579.05-nm band) as Et_4NCl is added to a methanol solution of $EuCl_3$; (2) a second peak at 579.50 nm appears at higher $EuCl_3$ concentrations. Furthermore, the lower wavelength peak occurs at approximately the same energy as that of $Eu(H_2O)_x^{3+}$ (17 274 cm^{-1}). The peak at higher wavelength (present at higher Cl^- concentrations) is attributed to an inner-sphere chloro complex, $EuCl(MeOH)_y^{2+}$. These assignments are consistent with our finding that a shift to lower energy in the $^7F_0 \rightarrow {}^5D_0$ transition occurs as the charge on a Eu(III) complex becomes more negative.[28]

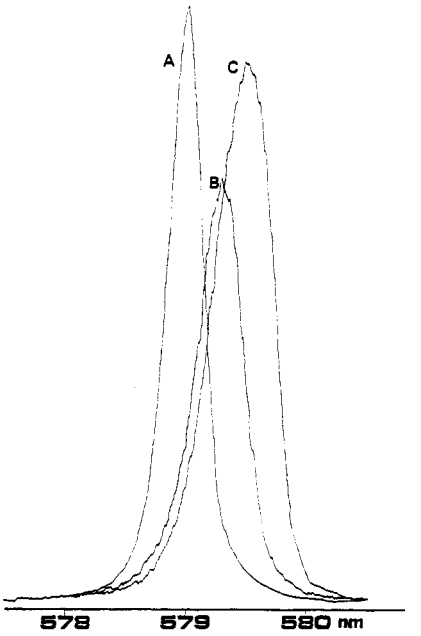

Figure 1. $^7F_0 \rightarrow {}^5D_0$ excitation spectra ([Eu] = 0.10 mM, λ_{em} = 614 nm): (A) $Eu(MeOH)_x^{3+}$; (B) $Eu(MeOH)_x^{3+}$ + 0.11 mM Las^-; (C) $Eu(MeOH)_x^{3+}$ + 0.30 mM Las^-.

Figure 1B,C shows the $^7F_0 \rightarrow {}^5D_0$ excitation spectrum of $EuCl_3$ in the presence of varying amounts of Las^-. The red shift of the peak maximum is consistent with the formation of mono- and bis(lasalocid A) complexes. The bis complex exhibits a band at 579.5 nm, while the signal observed at intermediate metal:ligand mole ratios peaks at a point between those of the free $EuCl_3$ solvate and the bis complex.

Upon complexation of A23187 to $EuCl_3$ in methanol, the excitation signal ($^7F_0 \rightarrow {}^5D_0$) due to $Eu(MeOH)_x^{3+}$ decreases in intensity; however, no signal due to any complex of A23187 is observed. This implies the presence of an efficient, nonradiative deexcitation pathway upon complexation of Eu(III) to this ionophore. Following excitation to the Eu(III) $^5D_0$ level, intramolecular energy transfer to an excited ligand level and subsequent thermal deexcitation to the ground state would account for the absence of observable emission from complexes of A23187. For this process to be energetically favorable, the chelate must have a state in close proximity to (or below) the $^5D_0$ level of Eu(III). Triplet levels of a number of benzoxazole derivatives are known to lie in the region of the $^7F_0 \rightarrow {}^5D_0$ transition.[29,30]

Previous work in this laboratory has shown that the number of solvent molecules coordinated to Eu(III) can be determined from a comparison of lifetime measurements in protonated and deuterated solvents.[31] For reasons indicated below, we were unable to determine the number of solvent molecules that are displaced from Eu(III) upon complexation to lasalocid A. Two experiments indicate that this ionophore provides a pathway

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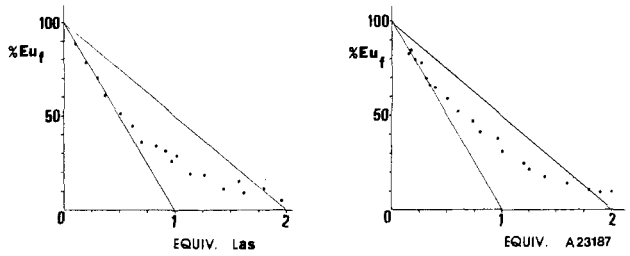

Figure 2. Eu–ionophore titration curves monitored by excitation spectroscopy ([Eu] = 0.10 mM, λ_{em} = 614 nm, λ_{ex} = 578.80 (Las^-), 579.00 (A23187) nm).

for radiationless deactivation: (1) no substantial increase in lifetime is observed upon complexation of Las^- to $Eu(MeOH)_x^{3+}$; (2) a marked decrease in lifetime is observed when Las^- (in MeOD) coordinates to $Eu(MeOD)_x^{3+}$.

By monitoring the intensity of the $^7F_0 \rightarrow {}^5D_0$ excitation band due to $Eu(MeOH)_x^{3+}$ as a function of ionophore concentration, we determined the stoichiometries of the complexes formed. The excitation wavelength (λ_{ex} = 578.80 nm) for the lasalocid A experiment was chosen so that contributions to the measured intensity from $EuLas^{2+}$ or $Eu(Las)_2^+$ were negligible (identical titration curves were obtained by exciting further away from the absorption of the ionophore complexes at 578.60 nm). For A23187, the excitation wavelength was 579.00 nm, the peak maximum for solvated $EuCl_3$, since there are no interferences (i.e. no signal from Eu–A23187 complexes). The titration experiments were run at a constant Eu(III) concentration of 0.1 mM. Several experiments indicate that this value is well above the dissociation constants for all of the Eu(III)–ionophore complexes. At a [ionophore]:[$EuCl_3$] ratio of 0.3, the signal due to $Eu(MeOH)_x^{3+}$ decreases 50% upon a 50% dilution with methanol (dilution from 0.150 to 0.075 mM). This implies that no dissociation of the complex occurs upon going to the lower concentration. Additionally, background intensity is reached when 2 equiv of ionophore has been added (see Figure 2), implying quantitative binding. Further evidence for complete ligand binding at 0.1 mM Eu(III) ion concentrations is presented below.

Figure 2 shows graphs of percent free $EuCl_3$ solvate ($Eu(MeOH)_x^{3+}$) as a function of added Las^- or A23187 with the total Eu(III) concentration held constant at 0.1 mM. The curves clearly indicate the presence of both mono- and bis(ionophore) complexes. At less than 0.6 and 0.4 equiv of Las^- and A23187, respectively, 1:1 complexes predominate (i.e. at 0.30 equiv of ionophore, 70% free Eu(III) remains). At higher ligand to metal ratios, mixtures exist with the $Eu(ionophore)_2^+$ complex predominating as one approaches the 2-equiv point. Comparison of the curves for the two ionophores indicates that A23187 forms the bis complex more readily than lasalocid A (the free Eu(III) signal drops off more slowly).

Absorption Spectroscopy. The absorption spectra of metal-saturated solutions contain information pertaining to the groups on the ionophore that are coordinated to the metal ion. The spectrum of Las^- in methanol (Figure 3 (Las-trace A)) consists of a maximum at 305 nm (ϵ = 4170 M^{-1} cm^{-1}) and a shoulder at ~240 nm (this compares to HLas in which there are maxima at 317 nm (ϵ = 4100 M^{-1} cm^{-1}) and 247 nm).[13] Both of these features have been attributed to the salicylate chromophore of lasalocid A.[13] Upon complexation to Ca^{2+}, the band at 305 nm shifts to 308 nm and the shoulder at 240 nm becomes the peak at 244 nm (intensity changes are also noted—see Figure 3 (Las-trace B)). Larger changes in peak maxima (to 316 and 246 nm) are observed for the Tb(III)–Las complex (Figure 3 (Las-trace C)). The red shifts (relative to Las^-) imply that both metal ions are bound to the salicylate group of lasalocid A. Upon coordination of Las^- to Ca^{2+} or Tb^{3+}, peak positions shift toward those observed for HLas.

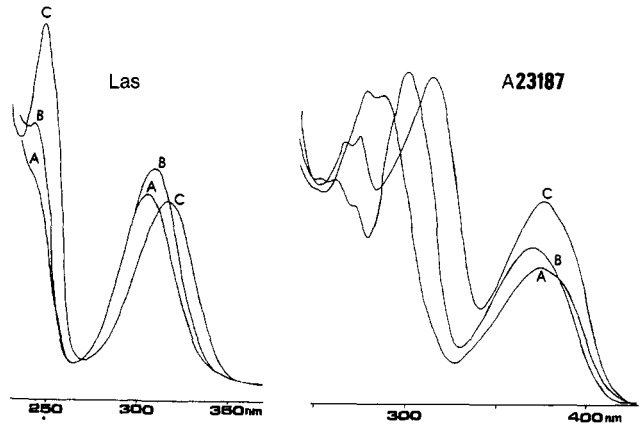

Figure 3. Absorption spectra ([ionophore] = 0.10 mM): (A) lasalocid A—anionic form, A23187—acid form; (B) Ca(ionophore)$^+$; (C) Tb(ionophore)$^{2+}$.

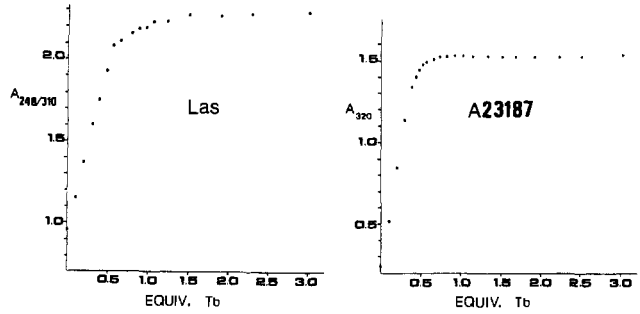

Figure 4. Tb–ionophore titration curves ([ionophore] = 0.10 mM).

This again indicates interaction between the metal ion and the carboxylate group of the salicylic acid moiety. This is expected behavior considering the high affinity of lanthanides (and Ca^{2+}) for oxygen donor ligands, particularly carboxylate groups.[32]

The spectrum of the acid form of A23187 contains more detail than that of lasalocid A. Maxima are observed at 378, 290, and 278 nm, with an ϵ_{290} of 17070 M^{-1} cm^{-1} in methanol (Figure 3 (A23187-trace A)). The band at 378 nm has been attributed to the benzoxazole group and the 290-nm absorption to the α-ketopyrrole substituent.[15] Upon deprotonation of the carboxylic acid (with NaOMe), the 378-nm band shifts to 364 nm, while the band at 290 nm is red shifted to 293 nm. For the Ca^{2+} and Tb^{3+} complexes, respectively, the shifts relative to the free acid are as follows: 378 → 370, 378 nm; 290 → 302, 316 nm. The fact that both bands are shifted relative to A23187 suggests the involvement of both the benzoxazole and α-ketopyrrole chromophores in metal coordination.

Information pertaining to the stoichiometric composition of lanthanide complexes with the ionophores is also provided by UV absorption measurements. Owing to the fact that spectral changes observed upon complexation of Las^- were small compared with those for A23187, the ratio provides a more sensitive measure of the stoichiometry of the complexation process. A plot of the ratio of absorbances at 248 and 310 nm (Figure 4) for a 0.1 mM Las^- solution vs. equivalents of $TbCl_3$ (Tb(III) was used instead of Eu(III) due to the presence of a charge-transfer band for Eu(III) in methanol that contributes to the absorption spectrum in the UV region) exhibits three regions of interest: (1) a linear relation from 0 to 0.5 equiv of Tb(III), (2) a change in the absorbance ratio from this point to approximately 1 equiv of Tb(III), and (3) no further change beyond 1 equiv. The first region suggests the formation of a single complex ($Tb(Las)_2^+$) and the second implies the presence of both $TbLas^{2+}$ and $Tb(Las)_2^+$. The fact

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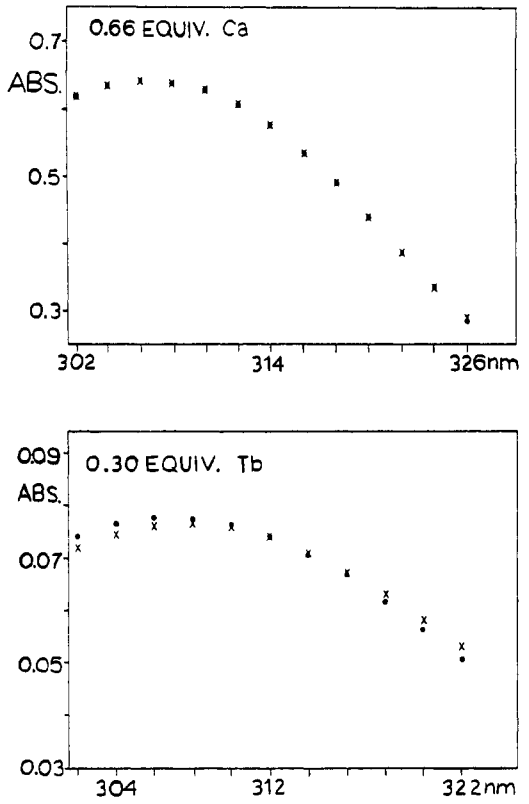

Figure 5. Computer fit of absorption titration curves ([Las⁻] = 3.98 × 10^{-6} M (Tb), 3.01 × 10^{-5} M (Ca); K_d = 1.24 × 10^{-5} M (Ca), 2.1 × 10^{-6} M (Tb)): (•) experimental, (×) calculated.

that no spectral changes occur after 1 equiv of Tb(III) has been added is also consistent with the formation of tight complexes.

The analogous experiment with A23187 (Figure 4) results in a similar plot of absorbance (320 nm) vs. concentration of terbium(III) up to the point at which 0.5 equiv of Tb(III) has been added. Beyond this point, curvature is noted with no absorbance change occurring after the addition of 1 equiv. This suggests the presence of 1:1 and 1:2 (Tb(III):A23187) complexes, in accord with the laser findings in the Eu(III) case.

Binding constants can be obtained from absorption spectra by working at lower concentrations and/or with more weakly binding metal ions. Our computer program (see Experimental Section), which assumes the presence of only a 1:1 species, was able to fit the measured changes in absorbance of lasalocid A ($\sim 10^{-5}$ M) in the presence of varying amounts of Ca^{2+} (0.1–10 equiv) at 24 °C. In a typical experiment, absorbance readings were taken at 10 wavelengths in the region of maximal spectral change for 15 different Ca^{2+} concentrations. The 150 data points obtained were best fit with a value of 1.24 × 10^{-5} M for K_d with $\Phi = 0.005$ ($\Phi = \sum(A_{calcd} - A_{obsd})^2$). This result is in reasonable agreement with the value (2.69 × 10^{-5} M) found for the same system by using CD spectra.[13] The summation is taken over all wavelengths and metal concentrations with absorbances ranging from 0.2 to 0.8. The high quality of the fits (shown for a particular metal ion concentration in Figure 5A) and the reproducibility of K_d over a range of ligand concentrations indicate the formation of only a 1:1 species at the concentrations employed ((1–3) × 10^{-5} M Las⁻). The dissociation constant was observed to decrease with increasing temperature, being 9.66 × 10^{-6} and 7.68 × 10^{-6} M at 34 and 42 °C, respectively.

Using the same program, we attempted to obtain the dissociation constant for the 1:1 complex of terbium(III) with lasalocid A. The higher association constants for lanthanide(III) complexes as compared to those for calcium (II)[2] made

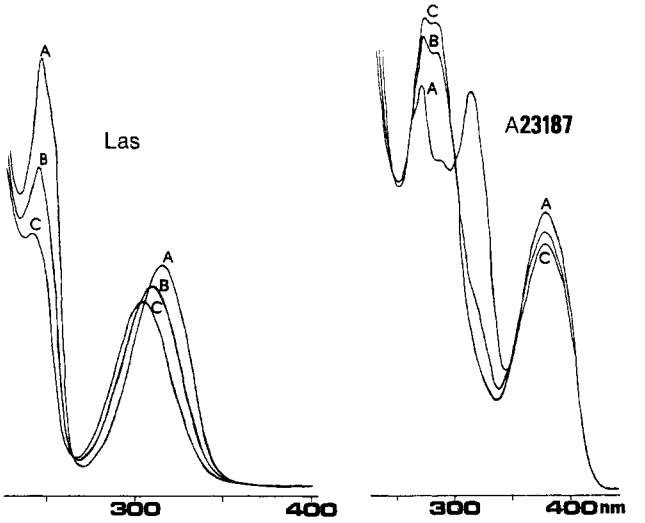

Figure 6. Absorption spectra of Tb–ionophore in methanol/H_2O ([Tb] = 0.20 mM, [ionophore] = 0.10 mM). Methanol:H_2O: (A) 100:0; (B) 80:20; (C) 60:40.

it necessary to work at a lower concentration of Las⁻. The minimum lasalocid A concentration for which reproducible results could be obtained was 4 × 10^{-6} M (with a range of 0.1–3.5 equiv of Tb(III)) for a 5-cm cell. At this concentration, Tb(III) did not appear to bind quantitatively. Nevertheless, it was impossible to obtain a good fit using the computer program with the assumption of only a 1:1 stoichiometry. Exclusion of points corresponding to less than 1 equiv of Tb(III) did not improve the fit. Systematic patterns in the difference between calculated and observed absorbances suggest that more than one species exists (1:1 and 1:2) (see Figure 5B), however attempts to improve the quality of the fit by treating the extinction coefficients of the $TbLas^{2+}$ standard spectrum as parameters resulted in only a small improvement. Moreover, this "adjusted" standard spectrum was significantly different from the initial guess for $TbLas^{2+}$ (obtained from a 2:1 Tb(III):Las⁻ solution, [Las⁻] = 0.1 mM). This again implies a more complex set of equilibria than the computer model presumes (the best obtainable fit gave a K_d value of 2.1 × 10^{-6} M for $TbLas^{2+}$).

The effect of the addition of water on lanthanide–ionophore coordination (in methanol) was examined by following changes in the UV absorption spectra. Figure 6 illustrates the effect on a 1:2 mole ratio of Tb(III):ionophore on going from 100% methanol to 60:40 methanol:water. The spectral features shift toward those of free ligand as the percent water increases. This implies weaker ligand–metal interactions in the more polar solvent. A likely cause is a shift in the equilibrium (eq 7) to

$$M(\text{ionophore})_y^{(3-y)+} + x(\text{solvent}) \rightarrow M(\text{solvent})_x^{3+} + y(\text{ionophore}^-) \quad (7)$$

the right as the amount of water present increases, owing to a greater affinity of the metal ion for water over ionophore and/or an "opening up" of the ionophore resulting from a decrease in intramolecular hydrogen bonding.

The absorption spectra of Tb(III)–ionophore complexes were measured over the temperature range of 24–47 °C to see if any changes in structure or affinity occur at higher temperatures (e.g. due to unfolding of the ionophore). No significant changes were observed over this range for either ionophore at concentrations well above their respective K_d's.

Discussion

Lasalocid A. On the basis of fluorescence, CD, and NMR studies, Chen and Springer[22] suggested that Pr(III) forms 1:1, 1:2, and 1:3 complexes with lasalocid A in methanol and that the lanthanide ion is bound only to the salicylate moiety. They

also reported stepwise formation constants for $PrLas^{2+}$, $Pr(Las)_2^+$, and $Pr(Las)_3$: $K_1 = 10^7$, $K_2 = 10^6$, and $K_3 = 10^5$ M^{-1}. The nature of their experiments and their computer modeling do not, however, clearly implicate the presence of $Pr(Las)_3$, nor were the binding constants determined with any accuracy (the authors point out that they could be in error by an order of magnitude). Richardson and DasGupta,[21] using absorption and emission techniques, concluded that the lanthanides form 1:1 and 1:2 complexes ($Ln(III):Las^-$) in methanol and that coordination occurs through the salicylate group and several other oxygen donor atoms, but quantitative estimates of binding constants were not made. Hanna et al.[26] employed ^{13}C NMR spin–lattice relaxation measurements to study the interaction of Gd(III) with lasalocid A. They concluded that, in the polar solvent *N*,*N*-dimethylformamide, Gd(III) binds only to the anionic carboxylate moiety, while in chloroform solution oxygen atoms 4, 7, and 8 are involved in coordinating the metal ion as well. No information regarding stoichiometry or binding constants was obtained in this study.

Our results clearly indicate the presence of two lanthanide ion complexes of lasalocid A, namely, $LnLas^{2+}$ and $Ln(Las)_2^+$. Spectral shifts upon Ln(III) complexation of bands due to the salicylic acid group demonstrate that this group is involved in the coordination. Several other observations indicate that other oxygen sites on lasalocid A are involved in lanthanide ion coordination as well. The magnitude of the affinity constants of Ln(III) and Las^- compared to that of simple organic ligands (e.g. acetate) suggests that lasalocid A coordinates in a multidentate fashion.[33] No crystal structures are available for Ca(II) or Ln(III) complexes with lasalocid A, but structures of the Ba(II), Ag(I), and Na(I) complexes are known. The Na(I) and Ag(I) complexes are 1:1 species with metal coordination through oxygen atoms 4–8.[7,8,10] The barium salt is a 1:2 complex with one Las^- bound through oxygen atoms 3–8 and the second through oxygen atoms 3 and 8 (in addition, one water molecule is coordinated, giving a total coordination number of 9).[9] CD studies[13] of Las^- and its metal ion complexes in polar solvents indicate that the multidentate coordination observed in the solid state persists in solution, consistent with our results.

From the temperature dependence of the dissociation constant of the 1:1 Ca(II):lasalocid A complex we estimate a $\Delta H°$ of -4.8 ± 0.8 kcal mol^{-1} and $\Delta S°$ of -39 ± 3 cal deg^{-1} mol^{-1}. The association reaction is clearly entropy driven, requiring the expulsion of several solvent molecules from the first coordination sphere of Ca(II) upon sequestration by a multidentate lasalocid A ligand. This entropy change is somewhat larger than that due to complexation of Ni(II) by this ionophore in methanol (26 cal deg^{-1} mol^{-1}),[13] consistent with the greater cordination number of Ca(II) and hence the potentially larger number of methanol molecules to be displaced.

A23187. Little work has been reported concerning the interaction of A23187 with lanthanide ions. Pfeiffer et al.,[15] using absorption and fluorescence spectroscopy, suggested the presence of $La_2(A23187)_3^{3+}$ or mixtures of $La(A23187)^{2+}$ and $La(A23187)_2^+$ in ethanol. Their studies also indicated the formation of 1:2 (M(II):A23187) complexes[15] or mixtures of 1:1 and 1:2 complexes[18] with Mn^{2+} and alkaline-earth cations in organic solvents. Our findings demonstrate the existence of both $Ln(A23187)^{2+}$ and $Ln(A23187)_2^+$ in methanol.

The crystal structure of $Ca(A23187)_2(H_2O)$ shows the Ca(II) ion to be seven-coordinate with binding to O1, O5, and N2 of each ionophore ligand and to a water molecule.[11,12] Our absorption data for Tb(III) and Ca(II) complexes of A23187 are consistent with a similar bonding scheme in methanol. Changes in the absorption spectrum upon complexation of Tb(III) to the free acid form of A23187 imply coordination to the α-ketopyrrole and benzoxazole groups. The likely ligating atoms of these groups are the carbonyl (O1) of the α-ketopyrrole moiety and the nitrogen (N2) of the benzoxazole group. This conclusion is based on an examination of space-filling models and the structure of the calcium salt.[11,12] The involvement of the carboxylate moiety in binding is also likely, considering the high affinity of lanthanide ions for this group.

Summary. We have demonstrated that both lasalocid A and A23187 interact strongly with lanthanide ions in methanol. The formation of mono and bis complexes was detected by using laser-excitation spectroscopy. A23187 tends to form the bis complex more readily than does lasalocid A. This may contribute to the greater effectiveness of A23187 in the transport of Ca^{2+} across membranes, since neutral species are needed to transport Ca^{2+} through an organic phase.[19,20] The binding of both ionophores to lanthanides is best described in terms of multidentate coordination involving the carboxylates and additional ligand donor atoms on the ionophore molecules. The large negative entropy of dissociation of the Ca(II)–lasalocid A complex implicates a multidentate mode of metal ion chelation.

Acknowledgment. This research was supported by Grant CHE-82-05127 from the National Science Foundation.

(33) Martell, A. E. "Stability Constants of Metal-Ion Complexes"; The Chemical Society: London, 1971.