Interaction of Lanthanide(III) Ions with Bovine Prothrombin Fragment 1. A Luminescence and Nuclear Magnetic Resonance Study

Mary E. Scott,[1a] Martha M. Sarasua,[1a] Henry C. Marsh,[1a] David L. Harris,[1a] Richard G. Hiskey,*[1a] and Karl A. Koehler[1b]

Contribution from the W. R. Kenan, Jr. Laboratories of Chemistry and the Department of Pathology, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27514. *Received September 12, 1979*

Abstract: Addition of lanthanum(III) chloride to bovine prothrombin fragment 1 at pH values of 5.01, 6.13, or 6.89 leads to quenching of the fragment 1 fluorescence and a blue shift in the wavelength of the emission maximum with apparent dissociation constants of 4×10^{-7} and 3×10^{-6} M for the quenching processes. Different degrees of total quenching are observed in 0.1 M potassium chloride containing buffer compared to buffer containing 0.1 M sodium chloride. At least two types of Eu^{3+} binding sites exist on bovine prothrombin fragment 1 at formal Eu^{3+}:F-1 mole ratios from 1.0 to 30. The highest affinity sites, involving two to three Eu^{3+} ions, involve the displacement of as many as eight water molecules from the ion's inner coordination sphere. Another class of sites exists, above a formal ratio of 6, which involves displacement of six to seven water molecules from the metal ion. At a 4:1 formal metal ion:fragment 1 mole ratio a group on fragment 1 with an apparent pK_a of 3 appears to control coordination involving displacement of five to six metal ion waters, while a pK_a of approximately 5.2 is further required for coordination involving maximal water displacement. These apparent pK_as implicate both single and double ionized (involving pK_a^1 and pK_a^2 of the Gla side chain) γ-carboxyglutamic residues in lanthanide ion binding. Variation of either the prothrombin or fragment 1 concentration in the presence of fixed concentrations of $^{139}La^{3+}$ leads to linear plots of $^{139}La^{3+}$ NMR line width vs. protein concentration with nonzero intercepts. The temperature dependence of the $^{139}La^{3+}$ NMR line widths vs. fragment 1 concentration is interpreted in terms of increasing ion binding at temperature above 25 °C. Fixing both the concentration of protein (prothrombin, 3.9 mg/mL, or fragment 1, 1.1 mg/mL) and lanthanum ion concentrations, variation of the system pH allows, for both proteins, assessment of ionizable protein functional groups involved in the binding of lanthanum. A broad carboxyl group titration curve is observed which extends down to pH 2 and up to near pH 6, perhaps reflecting ionizations of γ-carboxyglutamate residues in these proteins. In the case of prothrombin, a group ionizing above pH 6.0 may be involved. Changing the lanthanum ion concentration in the presence of fixed amounts of fragment 1 allows both the calculation of an apparent dissociation constant for less tight relatively nonspecific La^{3+} ion binding sites of 8.9 mM and the calculation of the line width of fragment 1 bound $^{139}La^{3+}$ ions, 3.1×10^5 Hz. In the presence of 5 mM calcium chloride, these values change to 14 mM and 2.6×10^5 Hz, respectively.

Introduction

The importance of deducing an adequate model which describes the process of calcium ion mediated binding of the vitamin K dependent blood coagulation factors to phospholipid surfaces has led a number of laboratories to investigate the interaction of a wide variety of metal ions with prothrombin and factor X as well as fragments derived from these molecules.[2-8] Although these and other studies frequently involve the divalent group 2A cations Mg^{2+} and Ca^{2+}, an impressive variety of other metals have been employed including Mn^{2+}, $Fe^{2+,3+}$, Zn^{2+}, and the lanthanides. Particularly the latter series of metal ions is considered to be useful for probing metal ion binding sites in proteins and the response of proteins to metal ion binding.[9-11]

Reuben[12] demonstrated the potential utility of the application of lanthanum-139 nuclear magnetic resonance ($^{139}La^{3+}$ NMR) methods to the investigation of some aspects of the hydrodynamic structure of bovine serum albumin. Similarly, changes in the intrinsic fluorescence of a variety of blood coagulation factors have been utilized as indirect measures of metal ion binding.[3,5,13] Finally, Horrocks and Sudnick[14] and Horrocks et al.[15] have elaborated a luminescence method which allows the deduction of lanthanide ion hydration via direct measurement of lanthanide luminescence lifetimes.

Thus, it is ultimately very important to compare and contrast the nature of calcium ion and lanthanide ion interactions with prothrombin and fragment 1. The lanthanide ions, lanthanum(III) and europium(III), were selected for this study. Lanthanum is diamagnetic and will not cause direct paramagnetic quenching of tryptophan fluorescence of fragment 1, possesses a spin of $^7/_2$, a charge of 3+, and an ionic radius of 1.02 Å (compared to Ca^{2+}, 0.99 Å), and shows activity in a considerable variety of calcium ion dependent biological systems.[11] Europium(III) was selected because of its favorable luminescence properties.

We employ in this study lanthanide ion quenching of the intrinsic fluorescence of fragment 1, europium ion luminescence lifetimes, and $^{139}La^{3+}$ ion nuclear magnetic resonance in order to explore the metal ion:protein interaction over a wide range of metal ion concentrations and make some comparisons with known interactions of divalent group 2 cations with fragment 1 and of lanthanide ions with simple Gla-containing peptides. It will be evident from the results presented here that the luminiscence studies involving fragment 1 are reporting specific tight binding processes while, at 25 °C, $^{139}La^{3+}$ NMR studies reflect less tightly and, perhaps, less specifically bound La^{3+} ions. Thus, the interaction of lanthanide ions with fragment 1 is complex and involves a variety of distinct types of binding sites.

Materials and Methods

Bovine prothrombin and prothrombin fragment 1 were prepared and purified according to the method described by Mann.[16] Both the isolated prothrombin and fragment 1 were stored in 50% glycerol at −20 °C until used. Both were found to be single banded on SDS polyacrylamide gel electrophoresis after staining with Comassie Blue and yielded satisfactory amino acid and Gla analyses. Protein concentrations were determined utilizing the extinction coefficients for prothrombin and fragment 1 reported by Mann.[16] The correction for light scattering described by Mann was employed.[16] All dialyses or other manipulations of these proteins were carried out at 4 °C.

Salts employed were reagent grade or better. Ultrapure calcium chloride and lanthanum chloride were obtained from Alfa Inorganics. Ethylenediaminetetraacetic acid disodium salt (EDTA) was Fisher Certified grade, tris(hydroxymethyl)aminomethane (Tris) was Ultrol

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grade from Calbiochem, and N-mopholinoethanesulfonic acid (MES) was obtained from both Sigma Chemical Co. and Calbiochem. Water used was distilled and then deionized. pH values were measured with an Orion 601A digital pH meter equipped with an Orion semimicro combination pH electrode. The meter was calibrated with two buffers. Triethylamine was obtained from Sigma Chemical Co. and used without further purification.

Sample Preparation. Bovine prothrombin fragment 1 was dialyzed once against 1 M EDTA to remove any possible metal ion contaminants. For the La^{3+} titrations, the fragment 1 was then exhaustively dialyzed into either 0.1 M KCl and 5 mM MES, pH 6.20, or 0.1 M KCl in 5 mM Tris, pH 7.4. After dialysis, the fragment 1 was diluted with either 0.1 M KCl or 0.15 M NaCl in 0.01 M MES of the desired pH such that the absorbance of the protein at 290 nm was 0.05. For the Eu^{3+} titrations, the fragment 1 was exhaustively dialyzed into 0.05 M KCl and 0.01 M Tris, pH 7.4. The fragment 1 was then diluted with this same buffer such that the absorbance at 280 nm was 0.76. In some experiments, the pH of the fragment 1 solution was adjusted directly with HCl or NaOH solutions.

La^{3+} Fluorescence Titrations. La^{3+} titrations were performed by addition to the fragment 1 solution of microliter amounts of 10^{-4}, 10^{-3}, or 10^{-2} M $LaCl_3$ stock solutions prepared in 5 mM MES, 0.15 M NaCl, pH 6.1. After each addition of $LaCl_3$, a fluorescence emission spectrum of the fragment 1 was recorded. An excitation wavelength of 290 nm was used. Uncorrected fluorescence emission spectra were recorded using a Hitachi Perkin-Elmer MPF-2A fluorescence spectrophotometer equipped with a thermostated cell compartment. Experiments were carried out at 25 °C. Fragment 1 emission occurred at approximately 337 nm, in the absence of La^{3+} ions. Emission maximum wavelengths were obtained for all fluorescence spectra by manually subtracting from each emission spectrum a buffer blank. The Raman emission from the aqueous solvent occurred close enough in wavelength to the fragment 1 emission to slightly distort the shape of the fluorescence peak and prevent accurate direct readings of the emission maximum.

A 3×10^{-6} M solution of N-acetyltryptophanamide was freshly prepared in 10 mM MES buffer, pH 6.0, in 0.15 M sodium chloride. At 25 °C, microliter amounts of a 1 M lanthanum chloride solution in 5 mM buffer, pH 6.0 in 0.15 M sodium chloride, were added to the stirred N-acetyltryptophanamide solution and fluorescence emission spectra recorded employing an excitation wavelength of 290 nm. The λ_{max} for N-acetyltryptophanamide emission was 353 nm and it did not shift on addition of La^{3+} ions. In the presence of up to 0.2 M $LaCl_3$, the fluorescence intensity of N-acetyltryptophanamide did not decrease by more than 2%, after corrections for dilution were made. Over the concentration range employed for the La^{3+} titrations of fragment 1, described above, the fluorescence intensity of N-acetyltryptophanamide was, within our estimated experimental error, unquenched.

Luminescence Lifetime Measurements. The apparatus used for measurements of the luminescence lifetimes was described in a previous paper.[17] Briefly, the excitation source was a Molectron UV 400 nitrogen laser pumping a Molectron DL 200 tunable dye laser, pulsed at 10/s. The laser dye used for direct excitation of Eu^{3+} was PBBO at 395 nm. The energy output of the dye laser was about 70 μJ per 5–10-ns pulse. The Eu^{3+} emission at 592 nm was monitored at right angles to the excitation path after passing through a JY H-20V 0.2 m monochromator by a Hamamatsu R777 photomultiplier tube. The signal was fed into a Tektronix R7912 transient digitizer for dc amplification and digitization. The signal was then fed into a DEC PDP 11/34 computer where the data was signal averaged and stored on floppy disks. The luminescence decay curves were regressed using a linear least-squares analysis.

Eu^{3+} Titrations. The Eu^{3+} titration of fragment 1 was carried out by addition of microliter quantities of 10^{-3} M $EuCl_3$ to a 0.76 mg/mL fragment 1 sample in 0.05 M KCl, 0.01 M Tris, pH 7.3. The luminescence lifetime of the Eu^{3+} in the fragment 1 solution was measured after each addition of $EuCl_3$. A pH titration was performed on a 0.76 mg/mL fragment 1 solution in the above buffer in the presence of a 4:1 formal ratio of Eu^{3+} to fragment 1. The pH was adjusted by addition of very small quantities of either 1 or 0.1 M NaOH or HCl. After each pH adjustment the luminescence lifetime of the Eu^{3+} in the fragment 1 solution was measured. In order to test for Eu^{3+} binding to the buffer employed for fragment 1, luminescence lifetimes were measured for Eu^{3+} in the presence of 0.05 M KCl and in the presence of 0.01 M Tris. The number of waters lost by Eu^{3+} in solution with 0.5 M KCl was about 0.5, which is the experimental error of the measurement technique. The number of water molecules lost by Eu^{3+} in solution with 0.01 M Tris was negligible. Therefore Tris/KCl was chosen as a suitable buffer system for this study.

$^{139}La^{3+}$ NMR Experiments. Prothrombin and fragment 1 were dialyzed exhaustively against a 50 mM MES buffer whose pH was initially adjusted to 6.2 by addition of triethylamine. pH values were varied by slow addition of microliter amounts of hydrochloric acid solutions to rapidly stirred solutions. The lanthanum chloride concentration was varied by addition of microliter quantities of stock lanthanum chloride solutions. pH values were determined before and after each run. No significant pH drift occurred in any case. Details of experimental organization are included in the appropriate figure captions.

$^{139}La^{3+}$ NMR spectra were obtained at 14.13 MHz on a Varian XL-100 FT spectrometer in spinning 10- or 18-mm sample tubes. The instrument was modified for multinuclear operation in the manner described by Marshall et al.[18] The 18-mm probe employed was made by Nicolet Technology, Inc. Since the observed line widths indicated the presence of short T_1 values, rapid pulsing was employed. Typically, a 200-μs pulse followed by a 12-ms acquisition time were employed. No pulse delay was used. Depending on the line widths encountered and the concentration of lanthanum employed, from 10 000 to 200 000 transients were accumulated per spectrum.

Results and Discussion

Fluorescence Quenching by Lanthanum Chloride. The interaction of lanthanum chloride with fragment 1 was examined via the observed quenching of the intrinsic fluorescence of fragment 1 as a function of lanthanum(III) ion concentration. The resulting titration curves at pH values of 5.01, 6.13, and 6.89 are plotted in Figure 1.

$\bar{\nu}$, defined as $(F_0 - F_{obsd})/(F_0 - F_\infty)$, where F_0 is the fluorescence emission intensity of the fragment 1 solution in the absence of lanthanum ions, F_{obsd} is the fluorescence emission intensity of fragment 1 at a given lanthanum ion concentration, and F_∞ is the limiting fragment 1 emission intensity at high lanthanum ion concentrations, plotted against the logarithm of the total lanthanum ion concentration, reveals two distinct classes of metal ion binding sites. The inflection points of the plots suggest that the dissociation constants for the lanthanum:fragment 1 complexes involved are approximately 4×10^{-7} and 3×10^{-6} M. pH appears to have little effect on the quenching process over the pH range investigated. The final degree of quenching of fragment 1 fluoresence was essentially the same in all three runs. A run identical with those presented in Figure 1 was carried out at pH 6.01 in 0.1 M potassium chloride, 5 mM MES. In that case it was observed that, although the shape of the quenching curve was virtually identical with those presented in Figure 1, the final degree of quenching at a La^{3+} concentration of 7×10^{-6} M was about 30% less than the corresponding experiment run in the presence of *sodium* chloride. Additional lanthanum ion titrations of fragment 1 were carried out at pH values of 4.00 and 6.20. In these latter two runs, the sample pH was adjusted down from neutral pH by addition of appropriate amounts of 0.1 or 1.0 M hydrochloric acid. The run at pH 6.2 was essentially identical with that presented in Figure 1 for pH 6.13. At pH 4.00, the observed quenching at a lanthanum ion concentration of 9.0×10^{-6} M was 20% less than observed at a pH value of 6.2. The hypothesis of the existence of two tight lanthanum ion binding sites on bovine prothrombin fragment 1 is strengthened by the fact that these data cannot be fit to a simple linear Hill plot. $^{153}Gd^{3+}$ binding to bovine prothrombin fragment 1 has been investigated by Furie et al.,[19] who note the existence of two tight (K_{diss} average = 0.2 μM) binding sites. This concentration corresponds to that determined for Gd^{3+} binding to bovine prothrombin fragment 1 via fluorescence quenching.[3] Brittain et al., utilizing terbium(III) ion fluorescence, conclude that approximately seven terbium ions bind to bovine prothrombin fragment 1.[20]

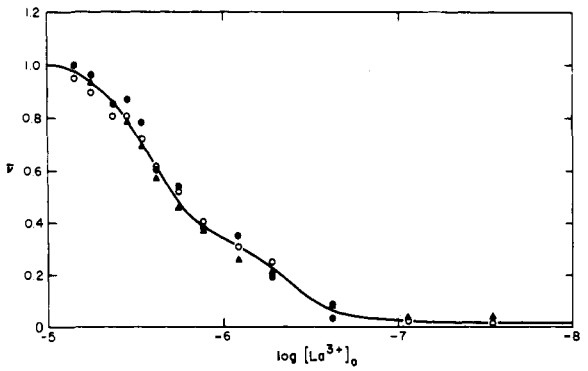

Figure 1. Lanthanum(III) ion quenching of the fluorescence emission of bovine prothrombin fragment 1. The fragment 1 concentration employed was 2.1×10^{-6} M. $\bar{\gamma}$ is defined as $(F_0 - F_{obsd})/(F_0 - F)$, where F_0 is the fluorescence emission of the fragment 1 solution in the absence of lanthanum ions, F_{obsd} is the fragment 1 fluorescence observed at any given concentration of lanthanum ions, and F is the limiting fragment 1 emission intensity at high lanthanum ion concentrations. The buffer consisted of 0.15 M sodium chloride, 5 mM potassium chloride, 10 mM MES buffer. The buffer pH was adjusted by addition of triethylamine. Runs were carried out at 25 °C. Lanthanum titrations were carried out at three pH values: (●), 6.89; (○), 6.13; (▲), 5.01. The pH of the fragment 1 samples was adjusted by dilution of an appropriate concentrated sample of fragment 1 into a buffer solution of such pH that the mixture yielded the reported pH value.

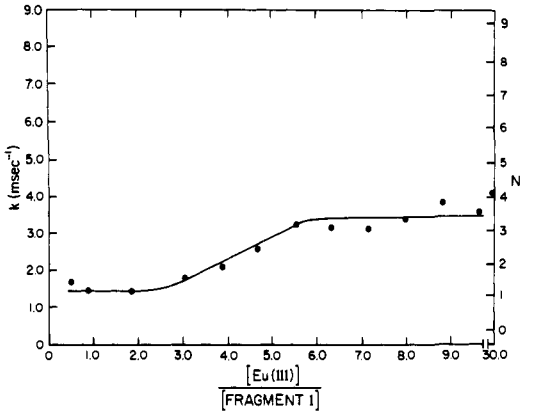

Figure 2. Eu^{3+} luminescence lifetime (k) and hydration (N) as a function of the formal ratio of Eu(III):fragment 1. See Materials and Methods section for details of the method and experiment.

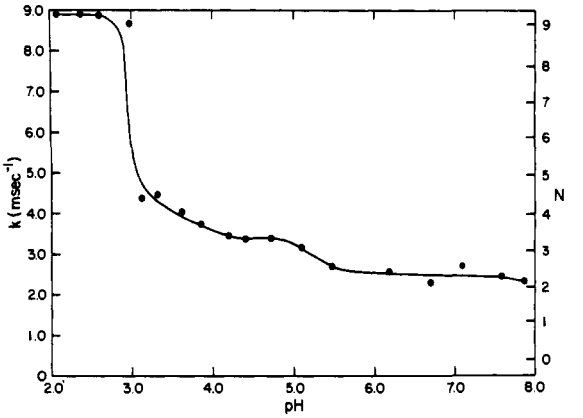

Figure 3. The effect of pH on the Eu^{3+} luminescence lifetimes (k) and Eu^{3+} hydration (N) at 4:1 Eu(III):F-1 formal ratio. See Materials and Methods section for details of the method and experiment.

As has been hypothesized for calcium and magnesium ion induced quenching of the intrinsic fluorescence of fragment 1, the observed La^{3+} ion induced quenching may be the result of a conformational change in fragment 1 occasioned by metal ion binding. Although La^{3+} is a very weak direct quencher of *N*-acetyl-L-tryptophanamide fluorescence, we cannot rule out, on the basis of these data, the existence of direct quenching of fragment 1 tryptophan fluorescence due to the presence of locally high concentrations of La^{3+} on the surface of fragment 1.

In addition to the fluorescence intensity changes reported above, addition of lanthanum ions to fragment 1 leads to a blue shift in the fragment 1 fluorescence emission spectrum. At pH values of 6.89 and 6.13 in the absence of lanthanum ions, the fragment 1 emission λ_{max} occurs at 337 nm. Addition of lanthanum ions leads to a decrease in emission λ_{max} to 334 nm. This latter wavelength is reached at a total lanthanum ion concentration of 2.4×10^{-6} M and remains constant up to the final total concentration employed, 1.9×10^{-5} M. A similar experiment carried out at pH 5.01 yielded similar results except that the fragment 1 emission λ_{max} was 336 nm in the absence of added lanthanum ions and the emission λ_{max} leveled off at 334 nm initially at a 1.3×10^{-6} M total lanthanum ion concentration. The magnitude and direction of the λ_{max} shifts observed in these lanthanum ion titrations are very similar to those observed for fragment 1 titrations carried out by varying calcium ion concentration.[21] However, approximately a 1000-fold increase in the calcium ion concentration, relative to the concentration of lanthanum employed here, was required to achieve the same degree of emission λ_{max} shift. Addition of calcium chloride to a final concentration of 30 mM results in no further quenching of the fluorescence of a fragment 1 solution at pH 6.1 to which 10^{-4} M lanthanum chloride had been previously added.

The observed blue shift in the fluorescence emission maximum of fragment 1 suggests either that the Franck-Condon tryptophan excited state involved is less available to stabilization by the environment during the lifetime of the excited state in the presence of lanthanum than in its absence or that the distribution of emitting tryptophans is changing. It is instructive to note that, compared with the emission of *N*-acetyltryptophanamide free in solution, the emission of fragment 1 at neutral pH is itself substantially blue shifted. The La^{3+} concentrations at which maximal blue shifts are observed are 2.4×10^{-6} and 1.3×10^{-6} M at pH values of 6.89 and 6.13 for the former and at a pH value of 5.01 for the latter.

The observed effect of potassium ion containing buffers on the apparent extent of the observed metal ion induced fluorescence change in fragment 1, although not explained here, is consistent with the observation reported by Henriksen and Jackson[2] on the buffer dependence of calcium ion binding to bovine prothrombin and fragment 1 and observations of Madar et al.[22] regarding monovalent and divalent ion binding to Gla-containing peptides.

Eu^{3+} Titrations of Fragment 1. For the titration of fragment 1 with Eu^{3+}, a plot of k, the rate constant of Eu^{3+} luminescence decay, vs. the ratio of the concentration of Eu^{3+} ions to fragment 1 was constructed (Figure 2). A plot of k vs. pH was made for the pH titration of fragment 1 in the presence of a 4:1 formal ratio of Eu^{3+} to fragment 1 (Figure 3). The rate constant for decay of lanthanide ion luminescence has been shown to be proportional to the number of water molecules, N, in the primary coordination sphere of the ion.[14,15] Therefore, the right-hand ordinate of the above plots was established as an N axis in order to ascertain the number of water molecules coordinated to the Eu^{3+} complexed to fragment 1. There are assumed to be nine water molecules coordinated to aquo-Eu^{3+} [14,15] A quantity ΔN can then be calculated as the number of water molecules displaced from Eu^{3+} by binding ligands ($\Delta N = N_{aquo\text{-}Eu^{3+}} - N_{Eu^{3+}\ ligand\ complex}$). For a rapidly exchanging system, the observed rate constant is a weighted average of all Eu^{3+} species, bound and free, present in solution. For a complex molecule such as fragment 1, which has multiple

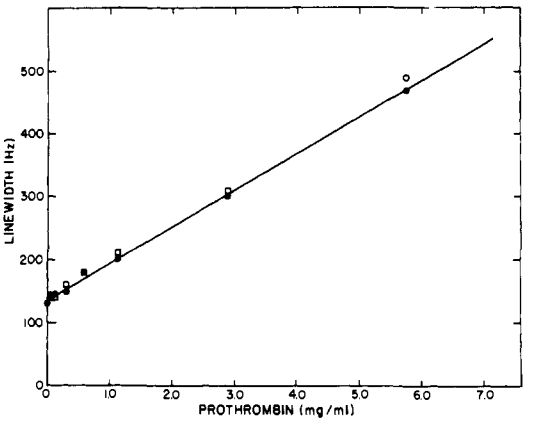

Figure 4. The dependence of the observed $^{139}La^{3+}$ line width on the concentration of bovine prothrombin in the presence and absence of added calcium ions. The total lanthanum ion concentration was 0.18 M in 0.15 M sodium chloride, 50 mM MES buffer pH adjusted to 6.2 by addition of triethylamine. The prothrombin concentration was varied from 0.058 to 5.76 mg/mL. The symbol (●) indicates runs carried out as described above. The symbol (□) indicates calcium chloride from a 2 M stock solution in the buffer described above such that the final total calcium ion concentration was 16 mM. Runs were carried out at ambient temperature (ca. 27 °C).

binding sites, the observed decay constant of Eu^{3+} luminescence is a composite of Eu^{3+} bound at the different possible protein sites. Our decay curves for the Eu^{3+} fragment 1 complexes were treated as single exponential decays and yielded single straight lines in the linear least-squares analysis. However, it is possible that the observed decay curves could actually be multiexponential with the characteristic decay constants very close in value and thus unresolved.

In the titration curve of fragment 1 with Eu^{3+} from 1:1 to 2:1 formal ratio of Eu^{3+} to fragment 1, a ΔN of about eight water molecules was observed to be displaced from Eu^{3+}, indicating possible eight-coordinate fragment 1 Eu^{3+} binding which is not unlike the eight-coordinate binding typically observed for Ca^{2+} ions. A ΔN value of 8 for fragment 1 bound europium(III) ions stands in striking contrast to ΔN values obtained with model Gla-containing peptides.[17] Such studies suggest that a single peptide-bound Gla residue will interact with Eu^{3+} with an overall loss of approximately two water molecules from the metal ion. Furthermore, in the absence of steric or other constraints, a peptide:Eu^{3+} complex of 2:1 is preferred for peptides containing single Gla residues. Peptides with adjacent Gla residues form 1:1 complexes with Eu^{3+} with a total displacement of four waters from the metal ion primary coordination sphere. The first break in the titration curve after the initial plateau occurred between 2:1 and 3:1 formal ratio of Eu^{3+} to fragment 1 indicating that there are two to three tight Eu^{+3} binding sites on fragment 1. Thus, tight binding of approximately 2 mol of Eu(III)/mol of fragment 1 is observed via metal ion luminescence lifetime measurements. At a formal Eu(III):fragment 1 ratio of 2:1 approximately eight water molecules had been displaced from each of the Eu(III) ion's inner coordination spheres by fragment 1 ligands. Consistent with such multidentate complexation, the corresponding affinities, judged from fragment 1 fluorescence quenching by La(III) ion and the work of others,[19,20] are high. The general affinity of lanthanide ions for fragment 1 is on the same order of magnitude as that observed for Eu^{3+} ion binding to simple Gla-containing peptides.[17] The equilibrium dialysis studies of Bajaj et al.[4] for Ca^{2+} binding to fragment 1 indicate that there are about three tight calcium binding sites on fragment 1. It is conceivable that such tight binding sites are actually the same as those for Eu^{3+} since the two ions have very similar ionic radii and binding properties. As more Eu^{3+} ions are added it appears likely that less tight fragment 1-Eu^{3+} binding sites are occupied by Eu^{3+} with more water molecules remaining attached to the Eu^{3+} ion than at the tight Eu^{3+} binding sites. The Eu^{3+} ions at these weaker sites would raise the observed weighted average value of k and, indeed, the observed k value increased from 1.45 ms^{-1} in the plateau region below a 2:1 formal ratio of Eu^{3+} to fragment 1 to 3.45 ms^{-1} above 6:1 formal ratio Eu^{3+} to fragment 1. The titration curve was level from about a 6:1 through about a 30:1 formal ratio of Eu^{3+} to fragment 1 corresponding to a ΔN of about 5.5 water molecules. The second break in the curve at 6:1 Eu^{3+}:fragment 1 could indicate three more binding sites which are not as tight as the first three.

The pH titration curve for fragment 1 in the presence of 4:1 Eu^{3+}:fragment 1 was flat from pH 7.8 to 6.0. This pH region is above the normally expected pK_a values of Gla carboxyls. Almost seven (6.8) water molecules are displaced from Eu^{3+} by fragment 1 in this pH range. From pH 6.0 down to 4.7, k increased (ΔN decreased) and leveled out to a plateau from pH 4.7 to 4.4. The ΔN value at this plateau was about 5.6 water molecules. From pH 4.4 down to 3.1, ΔN decreased gradually to a value of 4.5 water molecules. From pH 3.1 down to 2.5 a dramatic decrease in ΔN down to almost zero waters displaced was observed. This value of $\Delta N = 0$ was maintained down to pH 2.1, indicating almost complete loss of Eu^{3+} binding to fragment 1 at very low pH. The region of the titration curve from 6.0 down to 2.0 resembles an ionization curve for multiple carboxyl groups. As the carboxyls are protonated they lose their ability to bind Eu^{3+}. The first ionization, corresponding to a group with a pK of about 2.9-3.0, is very critical to the binding of Eu^{3+} to fragment 1. The pH region (Figure 3) over which the value of k remains effectively constant as pH is varied has as its midpoint a pH of 4.0. This value is similar to inflection points observed in titrations of Eu^{3+} complexes with Gla-containing peptides.[17]

Studies Involving $^{139}La^{3+}$ NMR. The dependence of the observed $^{139}La^{3+}$ line width on the concentration of prothrombin is linear (Figure 4) with a nonzero intercept at the ordinate equal to the observed line width of lanthanum ion (0.18 M) in buffer in the absence of prothrombin. This observation indicates that fast exchange is occurring between La^{3+} solvated in aqueous buffer and protein-bound La^{3+}. The prothrombin concentration was varied from 0.058 to 5.76 mg/mL in the presence of 0.18 M lanthanum chloride at pH 6.2 in 50 mM MES and 0.15 M sodium chloride. At this relatively high concentration of lanthanum ions the presence of 16 mM calcium ions has no appreciable effect on the slope of the line, consistent with the fluorescence observations noted above.

The linearity of the independence of ^{139}-La^{3+} line widths on the concentration of bovine prothrombin fragment 1 and the temperature dependence of such plots are illustrated in Figure 5. The lanthanum ion concentration was fixed at 0.010 M and the fragment 1 concentration was varied from 0.046 to 0.93 mg/mL. This experiment was carried out at 10, 25, 40, and 60 °C. The results are plotted as the difference between the observed $^{139}La^{3+}$ line width in the presence of protein and the observed $^{139}La^{3+}$ line width in the absence of protein because of the observed approximately linear dependence of the $^{139}La^{3+}$ line width on the viscosity of water. For example, at 10 °C the viscosity of water is 1.3077 cP and the observed $^{139}La^{3+}$ line width, in the absence of protein, is 210 Hz, while at 60 °C where the viscosity of water is reduced to 0.8688 cP the observed $^{139}La^{3+}$ line width is 112 Hz. The lines drawn in Figure 5 are the results of linear least-squares fits to the data. The statistical data characterizing these lines are collected in the caption to Figure 5. The results from Figure 5 are used to construct Figure 6, which illustrates the temperature dependence of $^{139}La^{3+}$ line width difference (i.e., the difference between the $^{139}La^{3+}$ line width in the presence and absence of

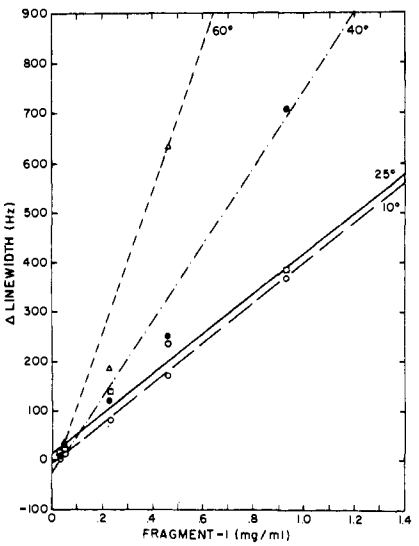

Figure 5. Temperature dependence of the change in $^{139}La^{3+}$ line width as a function of bovine fragment 1 concentration. Fragment 1 concentration varied from 0.046 to 0.93 mg/mL. Points plotted represent the observed line width of $^{139}La^{3+}$ in the presence of fragment 1 minus the observed line width of $^{139}La^{3+}$ at the same concentration (0.010 M) and temperature in the absence of fragment 1. The lines plotted are linear least-squares fits to the data. The following characterizes the calculated lines: at 10 °C (- -) $N = 6$, $R^2 = 1.00$, slope = 400.7, intercept = 7.9 Hz; at 25 °C (—) $J = 6$, $R^2 = 0.98$, slope = 419.3, intercept = 14.1 Hz; at 40 °C (-.-.-) $N = 6$, $R^2 = 0.98$, slope = 745.1, intercept = −24.2 Hz; at 60 °C(- - - -) $N = 4$, $R^2 = 0.96$, slope = 1362, intercept = −37.5 Hz. R^2 = coefficient of correlation, N = number of data points in the correlation (including point 0,0), intercept is on $\Delta\nu_{1/2}$ axis, slope is in terms of increment in $\Delta\nu_{1/2}$/1.0 mg mL^{-1} fragment 1.

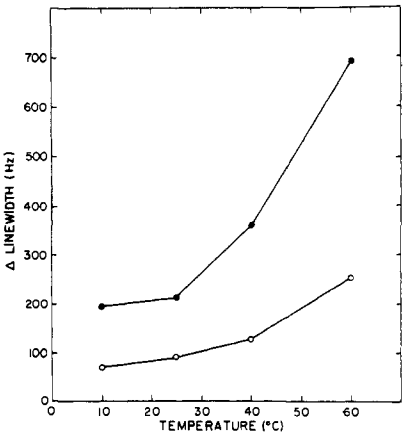

Figure 6. Variation in $\Delta\nu_{1/2}$ with temperature at fixed bovine fragment 1 concentrations (● = 0.5 mg/mL, 0 = 0.2 mg/mL). The points are taken from the least-squares lines drawn in Figure 5.

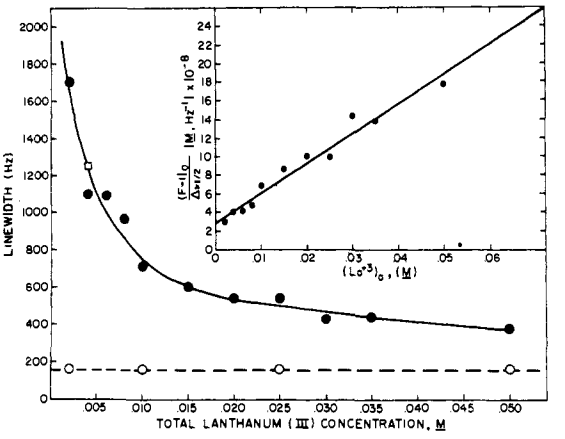

Figure 7. $^{139}La^{3+}$ line width is plotted against total lanthanum ion concentration in the presence of 0.92 mg/mL bovine prothrombin fragment 1, $(F\text{-}1)_0 = 3.8 \times 10^{-5}$ M. The symbols have the following meanings: 0, data from runs in 50 mM MES buffer, pH 6.2 adjusted with triethylamine, 0.15 M sodium chloride; □ indicates an experiment containing lanthanum ion at the indicated concentration and a calcium ion concentration of 10 mM. Insert: a plot $(F\text{-}1)_0/\Delta\nu_{1/2}$ (M, Hz^{-1}) × 10^{-8} vs. total lanthanum ion concentration.[30] The line drawn is the result of a linear least-squares fit to the data ($R^2 = 0.96$). The symbol 0 is used to plot the observed La^{3+} line width in the absence of fragment 1.

protein at a given temperature) on temperature. Although only two concentrations of fragment 1 were included in Figure 6, it is clear that a family of curves can be generated from the data in Figure 5. There is little change in $^{139}La^{3+}$ ion width in the presence of fragment 1 upon increasing the temperature of the system from 10 to 25 °C. Above 25 °C, however, the $^{139}La^{3+}$ line width in the presence of fragment 1 *increases* with temperature, in contrast to expectation based upon correlation time shortening with increasing temperature.

The formalism developed by Reuben and Luz[23] and Hubbard[24,25] for the motional dependence of relaxation parameters in spin 7/2 systems predicts, as we observe, that the line width of hydrated La^{3+} in solution should narrow as the correlation time becomes shorter (as a result of increasing the system temperature). A similar deduction can be made regarding the line width of fragment 1 bound La^{3+} ions. The increasing line width of La^{3+} in the presence of fragment 1 contrasts with this prediction. In an exchanging system, if the apparent association constant for binding of La^{3+} to fragment 1 decreases as temperature is lowered to 25 °C from 60 °C, a decrease in line width would be expected.[26] This result is consistent with the reported endothermic nature of lanthanide interactions with trypsin,[27] bovine serum albumin,[28] and lysozyme.[29] Under conditions where the equilibrium concentration of La^{3+} in the presence of fragment 1 can be approximated by the total La^{3+} concentration, it is possible to obtain an estimate of both the apparent average dissociation constant for the lanthanum: fragment 1 complex and the line width of fragment 1 bound lanthanum ions.[30] The results of such an experiment are plotted in Figure 7. A linear least-squares fit to the data ($R^2 = 0.96$) yields a value of K_s, the apparent dissociation constant for the La^{3+}:fragment 1 complex, of 8.9 mM and 3.1 × 10^5 Hz for the line width of bound $^{139}La^{3+}$. In the presence of 5 mM calcium chloride, these values for the La^{3+}:fragment 1 complex change slightly to 14 mM and 2.6 × 10^5 Hz, respectively.

A calculation based on the Stokes-Einstein equation and a specific volume of 0.74 g/cm^3 for fragment 1, ignoring hydration, a molecular weight of 24 000, and assuming spherical geometry for fragment 1 yields values of τ_c for fragment 1 of 1.17 × 10^{-8} and 1.80 × 10^{-8} s at 25 and 10 °C, respectively. Thus, from the observed bound lanthanum ion line width ($n\nu_{1/2}$) and the equation[31]

$$\pi\nu_{1/2} = \frac{1}{T_2^*} = \frac{1}{98}\left(\frac{e^2qQ}{\hbar}\right)^2\left(1 + \frac{n^2}{3}\right)\tau_c \qquad (1)$$

it is possible to calculate the expected bound line width for a single La^{3+} ion bound to fragment 1 and hence a value for n (the experimental number of La^{3+} ions bound). We have assumed an asymmetry parameter of 1 (in the case of La^{3+} it frequently is 0.8 or greater) and assume that under the conditions of these experiments. Furthermore, we will assume that La^{3+} ions are complexed predominantly to carboxyl groups on the protein. Reuben and Luz[23] have determined a value for the term $e^2qQ/\hbar$ for La^{3+} in simple acetate complexes of 7.5 × 10^6 Hz. Employing the fragment 1 bound line widths we report in this study, a value of approximately 100 is calculated for n, the number of La^{3+} ions bound to fragment 1 under the conditions of the experiment in Figure 7. This result suggests that fragment 1, in the presence of approximately 25 mM

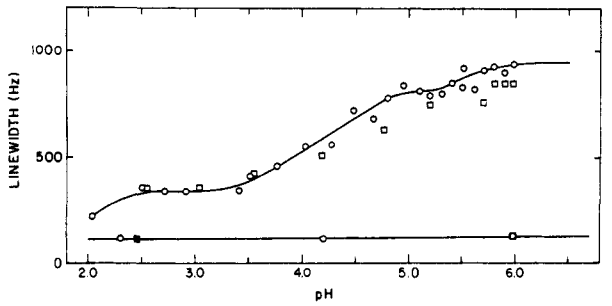

Figure 8. The effect of pH on the $^{139}La^{3+}$ NMR (6 mM) line width in the presence of bovine prothrombin fragment 1. Total fragment 1 concentration was 4.5×10^{-5} M in 0.15 M sodium chloride, 50 mM MES buffer pH initially 6.0 (adjusted with triethylamine). The symbol 0 refers to runs carried out in the presence of a total lanthanum ion concentration of 6 mM. pH was varied by addition of microliter amounts of 1 N hydrochloric acid slowly added to the virorously stirred protein:lanthanum sample. Lanthanum ion line width controls were run under exactly the same conditions, but in the absence of fragment 1. The symbol □ refers to an experiment containing fragment 1 (4.8×10^{-5} M) in 6 mM lanthanum chloride, 5 mM calcium chloride, 0.15 M sodium chloride, 50 mM MES buffer, pH initially 6.0 (adjusted with triethylamine). The symbol ■ corresponds to lanthanum controls run under the same conditions, including calcium ion concentration, but in the absence of fragment 1. In all cases pH was adjusted from high to low values.

lanthanum ions, is globally binding ions. The value of n for fragment 1:La^{3+} ion binding is decreased in the presence of 5 mM Ca^{2+} to approximately 90 making the same assumptions as in the previous calculations.

The effect of pH variation on the observed $^{139}La^{3+}$ line width in the presence of fragment 1 in the presence and absence of 5 mM calcium chloride is illustrated in Figure 8. A small decrease in $^{139}La^{3+}$ line width may occur on decreasing the pH from 6.0 to 5.2. Below pH 5 a broad titration process is significant until a pH value of 3.4 is reached below which the line width plateaus prior to falling off below pH 2.5 to nearly that of unbound $^{139}La^{3+}$. The presence of 5 mM calcium ions may mainfest itself, at higher pH values, in $^{139}La^{3+}$ line widths consistently narrower than those observed in the absence of added calcium ions.

The influence of variation of pH on the binding of a fixed total concentration of lanthanum ions (10 mM) to a fixed amount of prothrombin (3.9 mg/mL) was examined. In contrast to the gradual decrease in $^{139}La^{3+}$ line width near pH 6 observed with fragment 1, the truncated descending limb of a titration process with an apparent pK_a of 5.8 or higher is observed in the pH titration of prothrombin:La^{3+}. Through pH values below approximately 5.5, a broad titration behavior is apparent, with the line width at a pH value of 2.8 approaching essentially the line width of unbound lanthanum.

We have carried out similar pH titrations of fragment 1 in the presence of $^{43}Ca^{2+}$ and $^{25}Mg^{2+}$ by observing the changes in the line widths of these nuclei.[32] Plots of these data according to the Henderson–Hasselbach formalism, log (ionized/protonated) vs. pH, are linear with slopes of 1. The apparent pK_a values for the ionizations involved in metal ion binding are 3.8, 4.3, and 4.3 for calcium (20 mM), magnesium (18 mM), and lanthanum (6 mM), respectively. This difference of 0.5 pH unit between calcium and the other two metal ions may reflect differences in the types and number of carboxylic acid groups involved in the coordination of these metals. Magnesium ion binding to Arg- and Arg(NO_2)-D-Gla-D-Gla-OMe depends on the ionization of groups yielding an apparent pK_a value of approximately 4.6.[33] Thus, although the interactions of Ca^{2+}, Mg^{2+}, La^{3+}, and Eu^{3+} with fragment 1 all appear to involve Gla residues, and perhaps others, these data suggest the existence of apparent differences in the detailed nature of the resulting complexes.

In addition to the ionizations discussed above, it is apparent from Figures 3 and 8 that a low pK_a, corresponding roughly to the first Gla side chain carboxyl ionization,[33,34] is involved in the binding of a significant fraction of La^{3+} to fragment 1. This is in contrast to the observed pH dependence of calcium and magnesium ion binding to fragment 1 and Gla peptides and suggests the potential involvement of special Gla carboxyl group interactions in lanthanide(III) ion complexation. This process is more apparent in the Eu^{3+}:fragment 1 studies reported above, since in that case the observed signal is not so markedly averaged with nonspecific binding contributions.

Conclusion

The principal observations reported here may be summarized as follows. Tight lanthanide ion binding sites are involved in both quenching of fragment 1 intrinsic fluorescence and in the observed blue shift of the emission γ_{max}. Two or three lanthanide ions bind tightly. These tightly bound lanthanide ions are also the most highly coordinated (largest number of water molecules displaced). Furthermore, the pH dependence of complexation suggests that γ-carboxyglutamic acid residues are involved. It is surprising that on the time average only approximately one water molecule remains in the inner coordination sphere of the tightly bound lanthanide ion when complexed with fragment 1. Weaker lanthanide ion binding sites also exists on fragment 1 and prothrombin. As many as 100 mol of La^{3+} bind/mol of fragment 1 at pH 6.2 under conditions of large excess of La^{3+}. The pH dependence of metal ion binding to these sites appears to implicate Gla residues (pK_a^1 of Gla); however, simple carboxyl group ionization dominates the binding behavior of weakly bound ions. The pH dependences of La^{3+} and Mg^{2+} ion binding are similar and different from Ca^{2+} ion binding in that the apparent pK_a of groups affecting Ca^{2+} ion binding is approximately one-half pH unit lower than those involving the former ions.

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(35) Abbreviations: fragment 1 (F-1), amino terminal portion of prothrombin released upon treatment of prothrombin with thrombin; Gla, γ-carboxyglutamic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; MES, *N*-morpholinoethanesulfonic acid; NMR, nuclear magnetic resonance.

Paramagnetic Carbon Monoxide on Magnesium Oxide

R. M. Morris, R. A. Kaba, T. G. Groshens, K. J. Klabunde,*[1] R. J. Baltisberger, N. F. Woolsey, and V. I. Stenberg

Contribution from the Department of Chemistry, University of North Dakota, Grand Forks, North Dakota 58202. *Received July 2, 1979*

Abstract: Upon exposure of thermally activated MgO to CO, a paramagnetic species is produced at room temperature. A variety of experiments have been carried out in order to elucidate the nature of the CO radical: (1) EPR ^{13}CO experiments with computer simulation indicate that the radical is dimeric; (2) infrared experiments indicate that the radical is anion-like rather than cation-like, and reveal the presence of other CO telomeric species; (3) comparisons of CO/MgO with $C_6H_5NO_2$/MgO reveal that similar sites on MgO are necessary to produce the radical-anion-like species in each case.

Introduction

The electron transfer surface chemistry of MgO can be enhanced by two types of activation procedures:[2,3] (1) High-energy irradiation (neutron, X-rays, or ultraviolet irradiation) causes white MgO to turn blue to red-blue. The color is due to the formation of free electrons which are trapped in crystalline defects which are then called paramagnetic F centers (bulk defects) or S centers (surface defects). When small molecules such as O_2,[4] N_2O,[4] CO_2,[5] SO_2,[6] and H_2S[7] are exposed to this activated, colored MgO, stabilized paramagnetic radical anions are formed, such as $O_2^{-}\cdot$, etc. Such radical anions have been probed by ESR techniques.[4-7] (2) Thermal activation by heating under vacuum from 400 to 1000 °C causes the surface of MgO to become susceptible to electron-transfer processes, but no F or S centers are formed. Adsorbed electron-demanding molecules may become paramagnetic, however. For example, aromatic nitro compounds adsorbed on thermally activated MgO surfaces have been reported to yield the corresponding radical anions.[8,9] Tench and Nelson attributed this surface electron transfer activity to the presence of coordinately unsaturated O^{2-} centers. Cordischi and co-workers have reported the formation of two nitrobenzene radicals on MgO, $C_6H_5NO_2^{-}\cdot$ and $C_6H_5\dot{N}O_2H$, the latter species apparently formed by protonation of the radical anion by surface OH groups.[9]

For strongly electron-demanding molecules, such as nitrobenzene, *p*-nitrotoluene, *p*-fluoronitrobenzene, and tetracyanoethylene, there appears to be little correlation between actual electron affinity and concentration of radical anions formed on the MgO surface.[9-11] This finding is most readily explained by noting our previous report showing that nitrobenzene actually forms a *monolayer* of radical anion species on MgO, and steric limitations prevent the addition of more molecules.[12] This is probably the case for all molecules of such high electron affinity.

Molecules of much lower electron affinity exhibit significantly different behavior. Electron-transfer processes do occur, but with apparently much greater selectivity for certain types of surface defects.[12] For example, Lunsford and Jayne[13] reported in 1966 that well-degassed thermally activated MgO interacted with CO at room temperature to produce a paramagnetic species which was believed to be bonded to the surface in a manner similar to the bonding of metal carbonyls.[14] The EPR spectrum of this radical species exhibited an axially symmetric *g* value ($g_\perp = 2.0055$, $g_\parallel = 2.0021$).[13]

In this study three methods have been employed for investigation of the CO radical species: (1) comparisons of activities of a series of thermally activated MgO samples for radical formation (anion radical) with nitrobenzene vs. carbon monoxide; (2) infrared studies of CO adsorbed on thermally activated MgO samples; and (3) studies of adsorbed ^{13}CO on MgO samples employing extensive computer simulation techniques.

Experimental Section

Materials. For most of the results a sample of 99.99% MgO from ROC/RIC (labeled MgO I) was used. Other samples include a second lot of 99.99% MgO from ROC/RIC (MgO II), a 99.9% MgO sample from ROC/RIC (MgO III), and a Fischer reagent grade power (MgO

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