Interaction of Lanthanide Ions with Bovine Factor X and Their Use in the Affinity Chromatography of the Venom Coagulant Protein of Vipera russelli

Barbara C. Furie and Bruce Furie

Summary

The substitution of trivalent lanthanide ions for Ca(II) in the Ca(II)-dependent activation of bovine Factor X by the coagulant protein of Russell's viper venom was studied at pH 6.8. Factor X contains two high affinity metal binding sites which bind Gd(III), Sm(III), and Yb(III) with a $K_d$ of about $4 \times 10^{-12}$ M and four to six lower affinity metal binding sites which bind Gd(III), Sm(III), and Yb(III) with a $K_d$ of about $1.5 \times 10^{-3}$ M. In comparison, 1 mol of Factor X binds 2 mol of Ca(II) with a $K_d$ of $3 \times 10^{-4}$ M and weakly binds many additional Ca(II) ions. No binding of Gd(III), Gd(III), or La(III), the coagulant protein was purified lo-fold in 40% yield from crude venom and migrated as a single band on gel electrophoresis in sodium dodecyl sulfate.

By substituting lanthanide ions for Ca(II) in the coagulant protein of Russell's viper venom, we have demonstrated that lanthanide ions may be used to inhibit Ca(II)-dependent catalysis and coagulant protein interactions. This method may have general application to the affinity purification of proteins involved in Ca(II)-dependent protein interactions such as those participating in blood coagulation.

Methods and Materials

Bovine Factor X, purified from fresh bovine plasma by BaSO$_4$ adsorption and DEAE-Sephadex chromatography (22), appeared homogeneous by disc and sodium dodecyl sulfate gel electrophoresis. Factor X and the coagulant protein of Russell's viper venom activities were assayed as previously described (8). Protein concentration was estimated from the absorbance at 280 nm using an $E_{1\text{cm}}^{1\text{gm}}$ of 9.5 for Factor X (22) and 13.4 for the venom protein (5).

Lanthanide ions, obtained as the anhydrous chloride salts from K & K Laboratories, were prepared as 0.1 M solutions acidified with nitric acid to pH 2 and stored at 4°C. Boiling solutions of lanthanide ions in nitric acid were prepared as 0.1 M solutions for use in kinetic experiments.
Dialysis method of Colowick and Womack (25) using radioactive lanthanide ions was evaluated at 25°C, at pH 6.8, by the steady state kinetics of lanthanide ions and calcium to Factor X or the venom coagulant protein. A 0.1 ml aliquot of the reaction mixture was diluted into 0.4 ml of 25 mM imidazole, pH 6.8. After 5 or 10 min at 37°C, a 0.3 ml of 25 mM imidazole, pH 6.8, was added to the activated Sepharose suspension and the mixture was stirred for 18 hours at 4°C. The Sepharose-Factor X conjugate was washed sequentially with 0.1 M ammonium acetate, pH 7.1; 1 M NaCl; 6 M guanidine HCl, 25 mM imidazole, 0.15 M NaCl, 10 mM NiCl₂, pH 6.8; and 25 mM imidazole, 0.15 M NaCl, 10 mM EDTA, pH 6.8, and stored at 4°C. Preparations of Coagulant Protein of Russell's Viper by Affinity Chromatography—The venom coagulant protein was purified from crude Russell's viper venom on columns of Sepharose-Factor X. Crude Russell's viper venom (Sigma), 20 mg, was dissolved in 1 ml of 25 mM imidazole, 0.5 M NaCl, pH 6.8, and dialyzed at 4°C for 3 hours against 500 ml of the same buffer. The solution, after clarification by centrifugation at 4,000 x g for 10 min in a Sorvall RC-2B refrigerated centrifuge, was adjusted to 10 mM NiCl₂ by the addition of 0.1 M NaCl and incubated for 2 hours at 4°C. A fine white precipitate that formed was removed by centrifugation.

Preparation of Sepharose-Factor X—Bovine Factor X was coupled to Sepharose (Pharmacia) using the method of Cuatrecasas et al. (24). Activated Sepharose was prepared at 25°C using 300 mg of CNBr (Eastman) and 2 ml of washed Sepharose 4B. Three milliliters of Factor X (2.5 mg per ml) in 20 mM phosphate, 0.15 M NaCl, pH 6.8, was added to the activated Sepharose suspension and the mixture was stirred for 18 hours at 4°C. The Sepharose-Factor X conjugate was washed sequentially with 0.1 M ammonium acetate, pH 7.1; 1 M NaCl; 6 M guanidine HCl, 25 mM imidazole, 0.15 M NaCl, 10 mM NiCl₂, pH 6.8; and 25 mM imidazole, 0.15 M NaCl, 10 mM EDTA, pH 6.8, and stored at 4°C.

Preparation of Coagulant Protein of Russell's Viper by Affinity Chromatography—The venom coagulant protein was purified from crude Russell's viper venom on columns of Sepharose-Factor X. Crude Russell's viper venom (Sigma), 20 mg, was dissolved in 1 ml of 25 mM imidazole, 0.5 M NaCl, pH 6.8, and dialyzed at 4°C for 3 hours against 500 ml of the same buffer. The solution, after clarification by centrifugation at 4,000 x g for 10 min in a Sorvall RC-2B refrigerated centrifuge, was adjusted to 10 mM NiCl₂ by the addition of 0.1 M NaCl and incubated for 2 hours at 4°C. A fine white precipitate that formed was removed by centrifugation.

The supernatant (1 ml) was applied to a column of Sepharose-Factor X (0.7 x 3 cm) equilibrated with 25 mM imidazole, 0.5 M NaCl, pH 6.8, at 4°C. The column was washed with the same buffer at a flow rate of 30 ml per hour until no further protein was eluted, as monitored by absorbance of the eluate at 280 nm. Bound protein was eluted from the column with 25 mM imidazole, 0.5 M NaCl, 10 mM EDTA, pH 6.8, and collected in 1-ml fractions. After the removal of EDTA by exhaustive dialysis against 25 mM imidazole, 0.15 M NaCl, pH 6.8, the fractions were analyzed for protein concentration and coagulant protein activity. When necessary, the venom protein was concentrated in an Amicon ultrafiltrator employing a PM 10 membrane and stored at −15°C.

Kinetics of Factor X Activation—Substitution of lanthanide ions for Ca(II) in the activation of Factor X by the venom protein was studied qualitatively using 1 mM, 10 μM, 1 μM, or 10 mM lanthanide ions in place of 8 mM Ca(II) in the Factor X assay (8). In other experiments, the kinetics of the Ca(II)-dependent activation of Factor X by the venom protein in the presence of Nd(III) were studied employing a one-stage assay for activated Factor X (3). Under the conditions employed, the development of activated Factor X from Factor X was linear for 12.5 min in the presence of 8 mM Ca(II). The velocity of the hydrolysis of Factor X by the coagulant protein is expressed in units of activated Factor X activity generated per min. The reaction, containing 57 μg of Factor X, CaCl₂, and NiCl₂, in 0.3 ml of 25 mM imidazole, pH 6.8 at 37°C, was initiated with the addition of crude venom (2 μg) in 0.1 ml of 25 mM imidazole, pH 6.8. After 5 or 10 min at 37°C, a 0.1 ml aliquot of the reaction mixture was diluted into 0.4 ml of 15 mM Tris-HCl, 0.1 M NaCl, pH 7.5, at 37°C, and a 0.1 ml aliquot of this solution was added simultaneously with 0.1 ml of 25 mM CaCl₂, 16 mM Tris-HCl, 0.1 M NaCl, pH 7.3, to a preincubated mixture of 0.1 ml of pooled human plasma and 0.1 ml of phospholipid at 37°C. The clotting time determined Factor X concentration, which was calculated using linear curves constructed from plots of the logarithm of the clotting time (s) versus the logarithm of activated Factor X concentration. The Ca(II) and the lanthanide ion concentrations were varied as indicated.

Interaction of Lanthanide Ions with Factor X and Venom Protein—The binding of lanthanide ions and calcium to Factor X or the venom coagulant protein was examined at 25°C, at pH 6.8, by the steady state dialysis method of Colowick and Womack (25) using radioactive ¹⁵³Gd(III), ¹⁵²Sm(III), ¹⁴¹Yb(III), or ⁴⁴Ca(II). The upper chamber of a siliconized dialysis cell (Technilab model 226) contained 0.75 ml of the protein solution in 25 mM imidazole, 0.15 M NaCl, pH 6.8, and radioactive metal ions. Six fractions of 4 ml each were collected after each addition of the unlabeled metal ion. Imidazole (25 mM), 0.15 M NaCl, pH 6.8, was pumped through the lower chamber at a flow rate of 0.5 ml per min using a Sigmamotor peristaltic pump. Fractions containing ¹⁵⁸Gd(III) or ¹⁴¹Yb(III) were counted in a Packard gamma scintillation spectrometer (model 5375) through a 40 to 240 k.e.v. window. Fractions containing ¹⁵²Sm(III) or ⁴⁴Ca(II) were counted in Bray's solution (26) using a Packard Tri-Carb (model 3390) liquid scintillation spectrometer. Data were interpreted using a Scatchard plot (27). In the naphthalene analysis is the number of moles of Gd(III) bound per mol of Factor X. c is the molar concentration of unbound Gd(III). Linear plots of data describing the upper and lower limits of the slope were obtained by linear regression analysis using a Wang 500 calculator.

Fluorescence spectra were obtained on a Perkin Elmer model MPF-2A fluorescence spectrophotometer equipped with a constant temperature block maintained at 25°C using a Forma circulating constant temperature bath. A 310-nm filter in the emission light path was employed for recording emission spectra to minimize the second order scatter peak of the excitation beam. A 340-nm filter in the emission light path was similarly employed for the excitation spectra. Prior to each experiment protein solutions were filtered through a Swiney Millipore filter (25-μm pore size) to remove small quantities of insoluble material, and placed in a 1-cm square quartz fluorescence cell (Hellma No. 101).

Ultraviolet absorption spectra were obtained at 29°C on a Cary 15 spectrophotometer. Ultraviolet absorption difference spectra were obtained by the method of Yankeelov (28) employing the partitioned cell technique (Hellma No. 228).

RESULTS

Interaction of Lanthanide Ions with Factor X—The binding of lanthanide ions to Factor X was examined by the rate dialysis method (22) using radioactive trivalent lanthanide ions. In experiments with ¹⁵³Gd(III), the rate of dialysis of 1.6 μM ¹⁵³Gd(III) across the dialysis membrane was 1 × 10⁻⁵ cm per min in the absence of Factor X and 746 cm per min in the presence of 19 μM Factor X. The subsequent stepwise addition of unlabeled GdCl₃ to concentrations ranging from 3.2 μM to 91 μM was associated with a stepwise increase in the rate of ¹⁵³Gd(III) dialysis. At Gd(III) concentrations greater than 29 μM, turbidity of the protein solution was noted in the dialysis cell. Using the analysis of Colowick and Womack (25) to determine the concentration of Gd(III) free in solution and the concentration of Gd(III) bound to Factor X, these data were interpreted using a Scatchard plot (27) (Fig. 1A). The results suggest that there are two high affinity metal binding sites on Factor X to which Gd(III) ions bind with an average dissociation constant, Kd, of about 4 × 10⁻¹⁵ M. Although not well illustrated in this experiment, other experiments using higher Gd(III) concentrations showed that four to six lower affinity metal binding sites on Factor X bind Gd(III) with an average Kd of 1.5 × 10⁻⁷ M.

To determine whether the binding properties of Gd(III) to Factor X are characteristic of other lanthanide ions as well, identical experiments were performed using ¹⁵²Sm(III) and ¹⁴¹Yb(III). These results, summarized in Table 1, indicate that Sm(III) and Yb(III) also have an affinity for two high affinity metal binding sites and four to six lower affinity metal binding sites of Factor X similar to Gd(III). The variation of the experimental Kd determined for different lanthanide ions is within the estimated uncertainty of the data.

Interaction of Lanthanide Ions with Venom Coagulant Protein—The interaction of Gd(III) with the venom coagulant protein was examined by the rate dialysis method using ¹⁵³Gd(III). Despite the use of protein solutions of 5 mg per ml (8.3 × 10⁻⁵ M)
Fig. 1. A, Scatchard plot of the binding of Gd(III) to Factor X. Steady state dialysis was performed at 25°C using 19 μM Factor X, 1.6 mM GdCl₃, 1.6 to 91 μM GdCl₃ in 25 mM imidazole-0.1 M NaCl, pH 6.8. B, Scatchard plot of the binding of Gd(III) to Factor X and the venom coagulant protein. Steady state dialysis was performed as above except that 20 μM venom coagulant protein was also added to the dialysis chamber.

Interaction of Lanthanide Ions with Factor X in Presence of Venom Coagulant Protein—The binding of Gd(III) to Factor X and coagulant protein in equimolar concentrations was evaluated by rate dialysis at pH 6.8 and 25°C. These data, analyzed using a Scatchard plot, are presented in Fig. 1B. The solution of Factor X and coagulant protein contained two high affinity binding sites per mol of Factor X-coagulant protein complex which bind Gd(III) with a K_d of 3.2 × 10⁻⁷ M and four or five lower affinity binding sites which bind Gd(III) with an average K_d of 1.8 × 10⁻⁷ M.

Interaction of Calcium(II) with Factor X—The interaction of calcium(II) with Factor X was studied at pH 6.8 and 25°C by rate dialysis using ³⁵Ca(II) in a solution containing 10 mg per ml of Factor X (1.6 × 10⁻⁴ M). Although considerable scatter of the data points was noted in multiple experiments, the extrapolation of a line representing the best least mean square fit of the data suggested that 2 mol of Ca(II) bind to 1 mol of Factor X with a K_d of 3.1 × 10⁻⁸ M. Additionally, many Ca(II) ions bind to the protein at higher Ca(II) concentrations but measurement of these weak interactions was beyond the technical limits of the method.

A summary of the interaction of metals with Factor X is shown in Table I.

| Dissociation constants, K_d, describing interaction of trivalent lanthanide ions and divalent calcium with Factor X at pH 6.8 |
| --- |
|  | High affinity sites | Lower affinity sites |
| Gd(III) | 4.2 × 10⁻⁷ | 1.5 × 10⁻⁵ |
| Gd(II) | 2.4 × 10⁻⁷ | 3.2 × 10⁻⁵ |
| Sm(III) | 5.1 × 10⁻⁷ | 6.0 × 10⁻⁸ |
| Yb(III) | 4.0 × 10⁻⁷ | 5.0 × 10⁻⁸ |
| Gd(III)* | 3.2 × 10⁻⁷ | 1.8 × 10⁻⁶ |
| Ca(II) | 3.1 × 10⁻⁴ | >1 × 10⁻³ |

* Determined in the presence of venom coagulant protein.

Kinetics—Substitution of lanthanide ions for the Ca(II) ions required for the activation of Factor X by the venom protein was examined. The presence of Dy(III), Yb(III), Tb(III), Gd(III), Eu(III), La(III), or Nd(III) in a reaction mixture at 37°C containing Factor X and the venom protein in 25 mM imidazole, pH 6.8, in the absence of Ca(II) was not associated with the development of activated Factor X activity or with the alteration of the electrophoretic mobility of Factor X upon gel electrophoresis in sodium dodecyl sulfate. In control experiments, lanthanide ions added to activated Factor X to the same final concentration did not modify the activated Factor X assay.

Lanthanide ions inhibit the activation of Factor X by the venom coagulant protein in the presence of Ca(II). The effect of NdCl₃ on the rate of the activation of Factor X by the venom protein was measured in a reaction mixture containing 2, 5, or 10 mM CaCl₂. These data were interpreted with the graphical analysis of Dixon (29) using an approach formally analogous to that presented previously (18). Employing any of three possible kinetic models to derive rate expressions, these data are

1 Three formal models were arbitrarily employed for a detailed kinetic analysis of the interaction of Factor X, coagulant protein, lanthanide ions, and Ca(II): in one model, presented elsewhere (18), lanthanide ions, L, compete with calcium, C, for the occupancy of a single metal binding site on the enzyme, E. EC then interacts with the substrate, S, to form a productive complex ESUS which goes to product formation; ELS is nonproductive. In a second model, E and S interact weakly and nonproductively to form ES. In the presence of metals, L and C compete for a single metal binding site on ES whose occupancy by C is critical to catalytic ac-
consistent with the models of competitive inhibition of Ca(II)-dependent catalysis by Nd(III). The $K_i$ may be obtained graphically from the intersection of the lines obtained at different Ca(II) concentrations (Fig. 2). An inhibition constant, $K_i$, of $4 \times 10^{-6}$ M was estimated. In another experiment, a $K_i$ of $1 \times 10^{-4}$ M was determined. These results must be considered tentative because of the technical difficulties encountered performing kinetic experiments using a biological clotting assay. However, within the context of assumptions of the models and the models themselves, these results were reproducible under a limited range of conditions.

Affinity Purification of Venom Protein from Crude Russell’s Viper Venom—The demonstration that lanthanide ions bind tightly to Factor X and competitively inhibit the binding of a Ca(II) ion(s) essential for the activation of Factor X by the venom protein suggested that Factor X and coagulant protein might form a ternary complex of Factor X-coagulant protein-lanthanide ion which is both stable and nonproductive. When crude Russell’s viper venom in 10 mM NdCl$_3$ was applied to a column of Sepharose-Factor X, most of the crude venom protein did not adhere to the derivatized Sepharose (Fig. 3, upper panel). The bound protein, eluted with 10 mM EDTA, exhibited a 10-fold increase (range was 8- to 15-fold) in the specific activity of the coagulant protein compared to crude venom. About 75% of the original coagulant protein activity applied to the column was recovered in either the bound or the unbound material; one-half of the coagulant protein activity was associated with the bound protein fraction. Sodium dodecyl sulfate gel electrophoresis of this fraction yielded a major band representing greater than 90% purity and corresponding to a molecular weight of 62,000 (Fig. 4). This is in good agreement with the molecular weight of 60,000 for the venom coagulant protein obtained by sodium dodecyl sulfate gel electrophoresis for protein purified by DEAE-cellulose chromatography and gel filtration on Sephadex G-200 (30). When large quantities of protein were applied to the gels, some low molecular weight material could be identified which was thought to be due to nonspecific binding of protein to the Sepharose-Factor X column. In control experiments, no protein adhered to the Sepharose-Factor X conjugate in the absence of metal ions (Fig. 3; lower panel). Substitution of Tb(III), Gd(III), or Tb(III) for Nd(III) to promote nonproductive protein complex formation yielded similar elution profiles. We would conclude that any of the trivalent metal ions of the lanthanide series will facilitate satisfactory affinity purification.

Examination of the temperature and metal concentration dependence for optimal binding led to the employment of 10 mM
Fro. 4. Sodium dodecyl sulfate gel electrophoresis of venom coagulant protein prepared by affinity chromatography. Left, crude Russell's viper venom (200 μg). Right, affinity purified coagulant protein (21 μg). Gels were stained with Coomassie blue.

lanthanide solutions in columns at 4°. The use of 1 mM NdCl₃ yielded smaller quantities of bound protein representing 28% of the applied coagulant protein activity. No protein was bound to the Sepharose-Factor X conjugate in the presence of 0.1 mM NdCl₃. Affinity chromatography performed with 10 mM NdCl₃ at 25° consistently resulted in the leaching of bound coagulant protein from the Sepharose-Factor X column prior to elution with EDTA; at 4°, recovery of the coagulant protein in the bound fraction was optimized.

Maximal binding of the venom coagulant protein was observed when a large excess of crude venom was placed onto the column. When smaller quantities of venom were applied, the protein content of the bound fraction and the specific coagulant protein activity were decreased. Presumably, the binding constant describing the interaction of Factor X and coagulant protein in the presence of Nd(III) is such that, given the fixed concentration of Factor X bound to the Sepharose, higher concentrations of venom coagulant protein increase the amount of Factor X-Nd(III)-venom coagulant protein complex. The specific removal of the venom protein from the Sepharose-Factor X conjugate was facilitated by the chelation of lanthanide ions by EDTA. EDTA forms very tight complexes with lanthanide ions (11) and competes favorably with the metal binding sites of the protein for the metal ions.

The specificity of the interaction of the venom coagulant protein with Factor X covalently bound to Sepharose was evaluated using columns of Sepharose. In the presence of 10 mM NdCl₃, no detectable fraction of crude venom bound to the unconjugated Sepharose column which could be eluted with EDTA. These results suggest that the interaction of the venom coagulant protein with Factor X covalently bound to Sepharose is specific and probably simulates the metal-dependent ternary complex formed in solution.

Binding of Terbium(III) to Factor X—The fluorescence properties of Tb(III) were studied in the presence of Factor X. Excitation at 280 nm of a solution of Factor X (2.8 μM) and TbCl₃ (2.2 μM) in 0.1 M NaCl at pH 6.8 and 25° produced emission maxima at 490 and 545 nm (Fig. 5) as well as intrinsic tryptophan emission centered at about 344 nm. A small maximum at 560 nm represents the second order scatter peak; variation of the excitation wavelength predictably altered the wavelength of this peak. Solutions of Factor X in the absence of Tb(III) showed intrinsic tryptophan fluorescence but no emission at 490 or 545 nm. The emission spectrum of 2.2 μM Tb(III) in 0.1 M NaCl at pH 6.8 in the absence of Factor X was not observable while excitation at 280 nm of solutions containing 10 mM Tb(III) in 0.1 M NaCl at pH 6.8 produced emission spectra with maxima which were about one-half of the amplitude of those obtained for 2.2 μM Tb(III) in the presence of Factor X. From these data it would appear that Tb(III) exhibits about a 10,000-fold fluorescence enhancement when bound to Factor X. The uncorrected fluorescence excitation spectrum of the Tb(III)-Factor X complex, monitored at 490 nm, had a maximum at 283 nm; this spectrum was similar to the ultraviolet absorption spectrum of Factor X in the aromatic region. The ultraviolet absorption difference spectrum between Factor X-Tb(III) versus Factor X and Tb(III) was minimal, indicating that the increased Tb(III) fluorescence in the presence of Factor X is not due to an in-
increased absorption at 280 nm, but due to energy transfer from Factor X to Tb(III). These results would suggest that a tyrosine or tryptophan residue in Factor X, in or near a terbium binding site(s), is an energy donor and that the protein-bound Tb(III) is an energy acceptor. An increase in fluorescence emission at 490 and 545 nm (Fig. 6) and a 10% quenching of intrinsic Factor X fluorescence was associated with the titration of Factor X with Tb(III) in 0.1 mM NaCl at pH 6.8. Turbidity associated with protein precipitation was observed in solutions containing TbCl₃ in excess of 20 μM. Although the absence of a plateau precluded the complete analysis of the titration experiments, a dissociation constant, K_d, describing the interaction of Tb(III) and Factor X was roughly estimated to be about 2 × 10⁻⁵ M per n, where n is the number of Tb(III) ions bound to 1 mol of Factor X which participates in significant energy transfer (31). Because there are four to six lower affinity metal binding sites determined by the rate dialysis experiments, n is an integer between 1 and 6. A K_d between 3 and 19 μM may be estimated from the fluorescence titration; these values correspond favorably to the dissociation constant determined by rate dialysis describing the interaction of other lanthanide ions with the lower affinity binding sites of Factor X.

**DISCUSSION**

The mechanism of the activation of Factor X by the venom coagulant protein has been shown to be enzymatic (9), involving proteolytic cleavage of a single bond on Factor X (3-5). The products of this reaction include polypeptide fragments of 44,000 and 11,000 molecular weight (4) whose structures appear to be highly complementary and bind to each other with high affinity (5).

The formation of binary and ternary complexes between lanthanide ions and bovine Factor X or the coagulant protein of Russell's viper venom (or both) was investigated with the objective of defining the metal binding properties of Factor X in the presence and absence of the venom protein and the effect of the substitution of lanthanide ions for calcium on the Ca(II)-dependent activation of Factor X by the venom protein. The similarity between the ionic radii and the electrostatic binding of trivalent lanthanide ions and calcium(II) to oxygen ligands originally led to the suggestion that lanthanide ions, with interesting magnetic and electronic properties, might facilitate the physical and biological characterization of Ca(II) binding proteins (11, 12). These ions have subsequently proved useful in characterizing metal binding sites of Ca(II) binding proteins (14, 16, 18, 20), in examining three-dimensional structures of proteins and the active site of proteins by x-ray crystallography and nuclear magnetic resonance relaxation techniques (15, 16, 19, 21), and in evaluating the role of metal ions in the catalytic mechanism of hydrolases (13, 14, 17, 18).

Our results indicate that bovine Factor X has two high affinity metal binding sites which bind Gd(III), Sm(III), and Yb(II1) with an average dissociation constant of 4 × 10⁻⁷ M and four to six lower affinity metal binding sites which bind trivalent lanthanide ions with a dissociation constant of about 1.5 × 10⁻⁵ M. The binding of Gd(III) to the venom protein was not detected. In a solution presumed to contain the ternary complex of Factor X Gd(III)-venom protein, 2 mol of metal binding sites were demonstrated per mol of complex. It is likely that 2 mol of Gd(III) bind to the same sites on Factor X in the presence or absence of the venom protein. Implicit in our investigation is the presumption that the binding properties of the specific lanthanide ions evaluated may be extended to lanthamum and all other lanthanide ions in general. Previous investigations of the interaction of lanthanide ions with trypsinogen (13), trypsin (20), amylase (17), thermolysin (19), and staphylococcal nuclease (18) have emphasized these similarities in studies comparing the binding properties of the lanthanide ions. Minor variations in the dissociation constant of the metal-protein complexes have indicated that lanthanide ions show tighter binding with decreasing ionic size. However, we were unable to detect differences which were significant compared to the experimental error.

The availability of radioactive lanthanide ions facilitated the definition of the number and characteristics of the metal binding sites of Factor X. In particular, dissociation constants of the order of 1 × 10⁻⁴ M could be measured reproducibly and the number of sites per molecule of Factor X defined. On the other hand, the measurement of the dissociation constant describing the interaction of Ca(II) and Factor X required high concentrations of Factor X (1.6 × 10⁻⁴ M) as well as high concentrations of CaCl₂. These are conditions under which Factor X, a highly acidic protein, is known to aggregate, as determined by sedimentation equilibrium centrifugation (22). In our experiments we were consistently limited to a narrow range of Ca(II) concentration where binding was measurable and specific. However, our results are in general agreement with those reported by Yue et al. (32) which suggest that, at pH 8.0, Ca(II) binds to 3 high affinity sites (K_d 2.2 × 10⁻⁴ M) and 25 low affinity sites (K_d 2.5 × 10⁻¹ M).

As in the case with staphylococcal nuclease (18), the absolute Ca(II) requirement for the activation of Factor X by the venom protein may not be substituted by trivalent lanthanide ions. These results may be interpreted to suggest that Ca(II) may be a participant in the catalytic mechanism of zymogen activation by the venom protein, a role distinct from that of stabilization of the metal-dependent enzyme-substrate complex. Alternatively, small but significant distortion of the metal binding

---

Fig. 6. Effect of Tb(III) concentration in the fluorescence emission spectrum of Tb(III)-Factor X. Tb(III) was added to a solution of Factor X (2.8 μM) and 0.1 mM NaCl, pH 6.8 at 25°C. The fluorescence emission at 490 nm (● --- ●) and 545 nm (Δ --- Δ) was monitored. Protein precipitation was noted when the Tb(III) was added in excess of 20 μM.

---

2 B. C. Furie and A. J. Gottlieb, unpublished results.
site by trivalent lanthanides due to the differences in charge density or coordination number may inhibit catalysis, but not substrate binding (19).

From the data presented, it would appear that lanthanide ions compete with Ca(II) for the metal binding sites of Factor X to form a nonproductive complex of Factor X-metal-venom protein. We have employed kinetic models describing the interaction of the venom protein with Factor X in the presence of Ca(II) and lanthanide ions. A salient feature of these models is that a single Ca(II) ion interacts with Factor X and venom protein to facilitate ternary complex formation and Factor X hydrolysis. Furthermore, trivalent lanthanide ions compete with Ca(II) for the occupancy of this essential metal binding site, and enhance the formation of a stable, nonproductive complex of Factor X-metal-venom protein. The linearity of the data at each Ca(II) concentration and the common intercept of all three sets of data are consistent with these models of competitive inhibition. However, because of assumptions in these models, the estimation of the $K_i$ of lanthanide ions in this reaction at pH 6.8 of 1 to 4 $\mu M$ must be considered a first approximation. This $K_i$ may be compared to the $K_{d}$ of 0.4 $\mu M$ and 15 $\mu M$ describing the interaction of lanthanide ions with the high and lower affinity metal binding sites, respectively, as determined by the rate of dialysis method. We suggest that, within the experimental uncertainty of this kinetic data, the critical metal binding site(s) which must be occupied by Ca(II) for activation of Factor X is one (or both) of the high affinity sites on Factor X.

The similarities of certain structural features of the serine proteases make comparison of lanthanide interaction with bovine Factor X and bovine trypsinogen of interest. Trypsinogen and Factor X are zymogens of the serine proteases, trypsin and activated Factor X, respectively, whose active site and NH$_2$-terminal amino acid sequences demonstrate marked homology (33, 34). Lanthanide ions bind to two metal binding sites on trypsinogen (13), enhance the rate of trypsin-catalyzed trypsinogen activation (13), and are bound, albeit weakly, to a single metal binding site on porcine trypsin in close proximity of a tryptophan residue (20). It would appear that certain structural features of Factor X and trypsinogen, including metal binding properties, may have been preserved during evolution from a common ancestral protease. As is the case with transferrin (35) and trypsin (30) the 10,000-fold increase in the intensity of the Tb(III) emission is due to the energy transfer through a donor in the protein. Terbium(III) exhibits a characteristic fluorescence emission spectrum which is due to the $f-f$ electronic transition associated with irradiation by ultraviolet light (36). The magnitude of this emission is enhanced by energy transfer through contact or dipole-dipole interactions when Tb(III) is liganded in close proximity to a donor fluorophore which can participate as an energy donor. The excitation maximum for Tb(III) emission of 283 nm for the Factor X-Tb(III) complex and the association of the quenching of intrinsic tryptophan fluorescence with the binding of Tb(III) to Factor X suggests that a tryptophan residue may be the energy donor within the protein. Further studies of energy transfer in the Tb(III)-Factor X interaction should facilitate characterization of the metal binding sites of Factor X.

Successful applications of affinity chromatography to the purification of proteins have employed specific ligands covalently bound to an inert matrix and elution systems for the specific removal of proteins which interact with the derivatized matrix. For the purification of enzymes with protein substrates, affinity purification has been restricted to proteases for which specific low molecular weight substrate analog inhibitors are known (24). In the absence of specific inhibitors, the substitution of lanthanide ions for Ca(II) to inhibit enzymatic activity and facilitate protein complex formation offers a strategy for the affinity purification of proteins involved in Ca(II)-dependent protein-protein interactions using protein substrates. The purification of the venom protein using agarose-Factor X and lanthanide ions may serve as a model for the affinity purification of proteins involved in metal-dependent protein-protein interactions. Salient features necessary for the application of this approach to other protein complexes include requirements for the inhibition of metal-dependent enzyme catalysis by lanthanide ions, stable enzyme-substrate complex formation in the presence of lanthanide ions, the availability of one of the protein pair in purified or partially purified form for coupling to Sepharose, and an affinity constant, $K_d$, of $<10^{-4}$ to $10^{-5}$ M at pH 7.0 or lower for the two proteins. In addition to the use of lanthanide ions for nuclear magnetic resonance and optical spectroscopy, heavy metal ligands for x-ray crystallography, and kinetic studies of the metal specificity of metal-dependent enzyme reactions, we suggest that lanthanide ions may prove useful for the isolation and identification by affinity chromatography of metal-dependent protein complexes involved in blood coagulation, complement activation, muscle contraction, and other Ca(II)-dependent systems of biological import.

Acknowledgments—We wish to thank Dr. Alan N. Schechter for his helpful comments during the course of this work and his critical review of the manuscript. We also acknowledge the assistance of Dr. Peter Schiller in the interpretation of the fluorescence experiments and Drs. Ben Dunn and David Kosow for their helpful discussions concerning the kinetic experiments. We thank Ms. Patsy Waters for her excellent technical assistance.

REFERENCES

1. Furie, B. C., and Furie, B. (1974) Fed. Proc. 33, 1599
2. Jackson, C. M., and Hanahan, D. J. (1968) Biochemistry 7, 4900-4917
3. Esnouf, M. P., and Williams, W. J. (1962) Biochem. J. 84, 62-71
4. Fukiwaka, K., Legaz, M. E., and Davie, E. W. (1972) Biochemistry 11, 4882-4890
5. Furie, B. C., Furie, B., Gottlieb, A. J., and Williams, W. J. (1974) Biochim. Biophys. Acta 365, 121-132
6. Hoggie, C. (1959) Proc. Exp. Biol. Med. 110, 132-135
7. MacFarlane, R. G., Biggs, R., Ash, B. J., and Denson, K. W. E. (1964) Brit. J. Haematol. 10, 530-541
8. Williams, W. J., and Esnouf, M. P. (1962) Biochem. J. 84, 52-62
9. Kosov, D. P., Furie, B. C., and Forastieri, H. (1974) Thromb. Res. 4, 219-227
10. MacFarlane, R. G., and Barnett, B. (1934) Lancet 11, 985
11. Darnell, E. R., Gomez, J. E., and Darnell, D. W. (1970) J. Amer. Chem. Soc. 92, 3287-3288
12. Williams, R. J. P. (1970) Quart. Rev. Chem. Soc. London 24, 331-365
13. Darnell, D. W., and Darnell, E. R. (1973) J. Biol. Chem. 248, 6484-6486
14. Levitzki, A., and Reuben, J. (1973) Biochemistry 12, 41-44
15. Campbell, J. S., Dobson, C. M., Williams, R. J. P., and Xavier, A. B. (1973) Ann. N.Y. Acad. Sci. 222, 163-174
16. Nierboer, E., Kast, D., Cohen, J. S., Furie, B., and Schechter, A. N. (1973) Proceedings of Tenth Rare Earth Research Conference, pp. 763-769, May 1973, Carefree, Arizona, United States Department of Commerce, Springfield, Va.
17. Darnell, D. W., and Darnell, E. R. (1973) Biochemistry 12, 3489-3491
18. Furie, B., Eastlake, A., Schechter, A. N., and Anfinsen, C. B. (1973) J. Biol. Chem. 248, 5821–5825
19. Matthews, B. W., and Weaver, L. H. (1974) Biochemistry 13, 1719–1720
20. Epstein, M., Levitzki, A., and Reuben, J. (1974) Biochemistry 13, 1777–1782
21. Furie, B., Griffin, J. H., Feldmann, R. J., Sokoloski, E. A., and Schechter, A. N. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 2839–2847
22. Esnouf, M. P., Lloyd, P. H., and Jesty, J. (1975) Biochem. J. 131, 781–789
23. Weber, K., and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412
24. Cuatrecasas, P., Wilchek, M., and Anfinsen, C. B. (1968) Proc. Natl. Acad. Sci. U. S. A. 61, 636–643
25. Coldrick, S. P., and Womack, F. C. (1969) J. Biol. Chem. 244, 774–777
26. Bray, G. A. (1960) Anal. Biochem. 1, 279–285
27. Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51, 660
28. Yankeeov, J. A., Jr. (1963) Anal. Biochem. 6, 287–289
29. Dixon, M. (1953) Biochem. J. 50, 170–171
30. Furie, B., Gottlieb, A. J., and Williams, W. J. (1970) Fed. Proc. 29, 709
31. Weber, G., and Young, L. B. (1964) J. Biol. Chem. 239, 1415–1423
32. Yue, R. H., Starr, T., and Gertler, M. M. (1972) Fed. Proc. 31, 241
33. Leveson, J. E., and Esnouf, M. P. (1969) Brit. J. Haematol. 17, 173–178
34. Tifani, K., Hermodson, M. A., Fujikawa, K., Ericsson, L. H., Walsh, K. A. Neurath, H., and Davie, E. W. (1972) Biochemistry 11, 4899–4903
35. Luk, C. K. (1971) Biochemistry 10, 2838
36. Sinha, S. P. (1966) Complexes of the Rare Earths Pergamon, Oxford
Interaction of lanthanide ions with bovine factor X and their use in the affinity chromatography of the venom coagulant protein of Vipera russelli.

B C Furie and B Furie

J. Biol. Chem. 1975, 250:601-608.

Access the most updated version of this article at http://www.jbc.org/content/250/2/601

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/250/2/601.full.html#ref-list-1