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THE DETERMINATION OF A CALCIUM-DEPENDENT BINDING CONSTANT OF THE BOVINE PROTHROMBIN GLA DOMAIN (RESIDUES 1-45) TO PHOSPHOLIPID VESICLES

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Summary: Calcium-mediated binding of the radioiodinated peptide representing residues 1-45 of bovine prothrombin to single bilayer phospholipid vesicles composed of phosphatidylserine from bovine brain and synthetic 1-palmitoyl-2-oleoyl-phosphatidylcholine (25:75 PS/PC) has been studied over peptide concentrations from 0.33 μM to 3.75 μM and at a calcium concentration of 1.0 mM. The binding isotherm for the interaction between the radioiodinated peptide and PS/PC vesicles fits a model in which there is noncooperative binding of the peptide to non-interacting sites on the phospholipid bilayer. A dissociation constant determined at these conditions is 11.8 μM compared to 1.0 μM for prothrombin fragment 1.

The vitamin K-dependent carboxylation of specific glutamic acid residues on the N-terminal region of several of the coagulation and anticoagulation proteins forms a negatively charged amino acid sequence that contains the essential γ-carboxyglutamyl (Gla) residues [1-6]. The vitamin K proteins require Ca(II) for physiological function, and several essential coagulation steps are accelerated considerably with the presence of negatively charged phospholipid vesicles or platelets [6-10]. Previously, prothrombin fragment 1 was the smallest peptide shown to bind to phospholipid vesicles in the presence of Ca(II) [11]. In fact, it has been generally assumed that fragment 1 was the smallest portion of the prothrombin molecule that would exhibit Ca(II) mediated binding to PS/PC vesicles. Recently, the radioiodinated 1-45 peptide of prothrombin has been shown by qualitative experiments to bind to PS/PC vesicles [12,13]. The purpose of the present

ABBREVIATIONS: Gla, γ-carboxyglutamic acid; bovine fragment 1, residues 1-155 of bovine prothrombin, BSA, bovine serum albumin, SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

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study was to measure the binding constant for the radioiodinated 1-45 peptide and compare the binding of the bovine peptide with bovine fragment 1 under similar experimental conditions.

MATERIALS AND METHODS

Protein Preparations - Bovine prothrombin and fragment 1 were isolated as described by Mann [14]. Only fragment 1 preparations exhibiting 45% - 50% quenching in the presence of 10 mM Ca(II) were used to prepare the Gla domain peptide 1-45. The 1-45 peptide was prepared by a limited chymotrypsin digest followed by purification on ion exchange chromatography as described by Pollock et al. [12]. All peptide preparations used in the binding assays were determined to be homogeneous immediately before use by employing 13.5% non-SDS PAGE gels.

Radioiodinated 1-45 Peptide - Iodination of the 1-45 peptide was achieved via Iodo-beads, (Pierce Chemical Company)1 using conditions modified from those employed by Pollock et al. [12]. Fifteen beads were added to 200 µl buffer (0.01 M tris-HCl buffer, pH = 7.4, I = 0.1 M) which contained 500 µCi of 125-NaI. The suspension was allowed to stand for 5 min. at room temperature; 400-500 µg (3 mls) of the 1-45 peptide was added to the Iodo-bead suspension and allowed to stand for 30 minutes with occasional mixing. The iodination was terminated by pipeting the solution from the beads and adding this supernatent to 400-500 µg (3 mls) of 1-45 peptide that served as carrier protein. The iodination reaction mixture was loaded onto a plug of DEAE-Sephacyl (Pharmacia Chemical Co.) and washed overnight with the above buffer. Auto-radiographs were performed for each iodination to determine the homogeniety of the iodinated protein and to establish that all free iodine was separated. The specific activity of the [125-I] 1-45 preparations was 2×10^8 dpm/µmole.

25/75 Phosphotidylserine/phosphotidylcholine Vesicles - Phospholipid vesicles were prepared by the procedure of Barenholz et al. [15]. Individual phospholipids (Avanti Polar Lipids Inc.) were mixed in the desired ratios in chloroform and blown dry under nitrogen. The dry phospholipids were diluted to 1 mg/ml with buffer (0.02 M tris-HCl, pH = 7.4, I = 0.1 M). The cloudy solution was sonicated to clarity in a cool water bath sonicator for 30-60 minutes. The solution was ultracentrifuged at 100 x g for 1 hr and the solution in the top one-third of the centrifuge tube was used for the binding assay. Phospholipid concentrations were determined by an inorganic phosphate assay [16]. The vesicles were examined for size distribution on a Sepharose CL-4B column before and after centrifugation to establish that the size distribution was appropriate [17].

Peptide-Phospholipid Binding Determination - The binding of the [125-I] 1-45 peptide to phospholipid vesicles was determined using the gel filtration technique of Hummel and Dreyer [18]. All buffers contained 0.01 M tris-HCl pH = 7.4, 0.02% NaN3, 1.0 mM Ca(II), and enough NaCl to yield an ionic strength of 0.05 M. Stock solutions of Ca(II) (1.0 M) were prepared from ultrapure reagents; the concentrations were determined by atomic adsorption spectroscopy. Peptide samples were dialyzed against buffer (without calcium) overnight using a 1000 molecular weight cutoff dialysis tubing (Amicon Inc.). At least two column volumes of the peptide containing buffer (with the calcium added) were passed through the column prior to injection of the radiolabeled peptide-lipid vesicle sample. The peptide concentrations were determined using extinction coefficient E1% = 8.3 and the molecular weight = 5600g/mole [12]. The peptide concentrations were corrected to account for any precipitation of peptide: Ca(II) complex that may have occurred during the addition of Ca(II). The corrected peptide concentration was determined as follows:

$$[1\text{-}45]_{corrected} = [1\text{-}45]_{t=o} * \frac{(cpm\ supernatant)_t}{(cpm\ supernatant)_{t=o}} \quad t = experiment$$

where [1-45] at t=o is the peptide concentration without the addition of Ca(II); counts per minute in the supernatant at t = experiment and t=o are measured after a 15 minute centrifugation (Eppendorf Microfuge) of 250µl solutions of peptide (t=o) immediately prior to injection on the column. Only data with less that 5% precipitation was used to calculate a binding constant. Bio-Gel Agarose A - 0.5 m 100 to 200 mesh (Bio-Rad Laboratories) was found to be the most satisfactory column packing for the determinations. The columns were 0.6 x 55 cm and water jacketed. Constant flow was maintained with peristaltic pumps. Fractions were collected in plastic test tubes containing 1 ml of BSA; 500 µl aliquots of the fractions were transferred to a scintillation cocktail to be counted in a standard liquid scintillation counter. The counter was determined to be 98% efficient for counting the [125-I] samples and the peak data was analyzed to yield a binding isotherm. Estimates for error were obtained from estimates in the inherent error in each measured point (e.g. counting, pipeting, etc.) and then corrected by an experimental internal standard to give an accuracy correctional factor. The data were treated by nonlinear least square fits to a model with one class of non-interacting binding sites utilizing SAS (Cary, N.C.) on an IBM 3081 computer.

RESULTS

The binding isotherm of the [125-I] peptide interaction with PS/PC unilamellar vesicles at 25° C is given in Figure 1. The peptide does not cause fusion of the vesicles under the conditions of the binding experiments [12]. At these Ca(II) and peptide concentrations less than 5% precipitation of the radiolabeled peptide was observed. In a separate control experiment (data not shown), it was shown that high concentrations of Ca(II) (50 mM) induced soluble aggregates that do not elute in the void volume of the Hummel-Dreyer profile; therefore the profile peak is exclusively the 1-45:Ca(II):PS/PC complex. The presence of a trough which has an area equal to the area of the peak demonstrates that the formation of this complex is an equilibrium process. The model which best fit the data is the relationship:

$$n(1\text{-}45) + PS/PC = n(1\text{-}45):PS/PC$$

where n = 102 and Kd =11.8 µM (Figure 2). Use of the value n = 102 in the Hill equation yields a Hill coefficient of h = 1.13 (Figure 2 inset); hence cooperativity in this system is small or non-existent.

These studies coupled with the earlier qualitative studies [12] indicate that the [125-I] 1-45 peptide retains the Ca(II)-specific PS/PC binding properties associated with the vitamin K-dependent coagulation proteins.

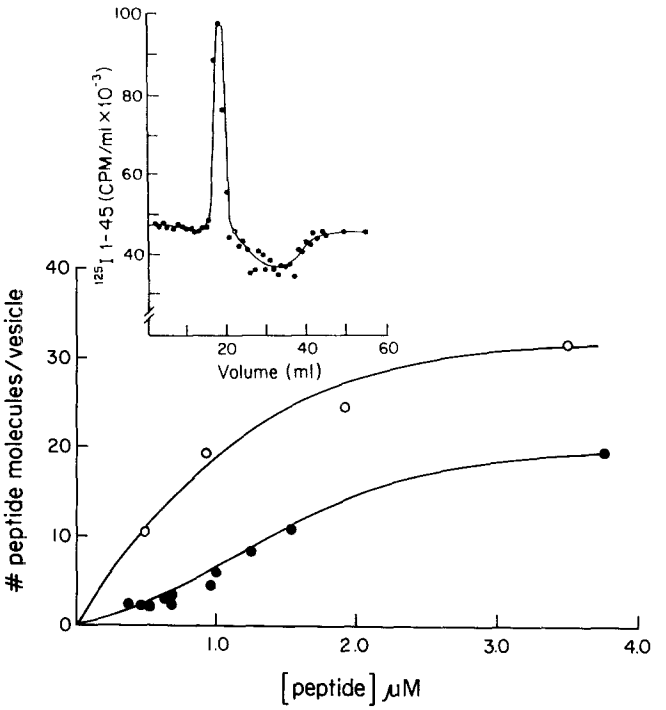

Figure 1: Binding isotherm of 3H-bovine prothrombin fragment 1 (O) and I-125 Bovine Prothrombin 1-45 (●) binding to 75/25 phosphatidylcholine/phosphatidylserine small unilamellar vesicles. Buffer, 0.010 M Tris-HCl, pH 7.5, ionic strength adjusted to 0.05 M with NaCl, 25 C. The inset shows typical Hummel-Dreyer column elution profile for the binding of I-125-prothrombin 1-45 to 25/75 PS/PC vesicles in the same buffer. The fragment 1 data is that of Dombrose et al. [11].

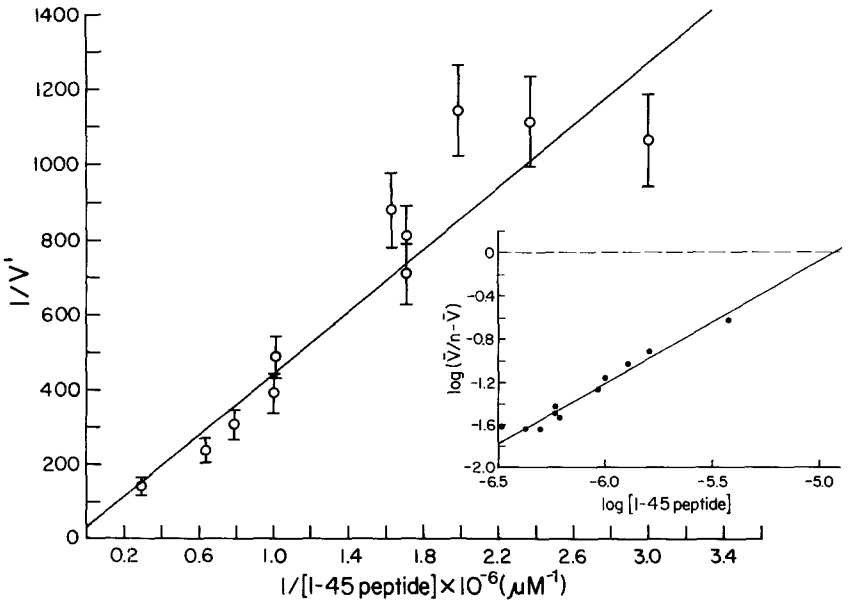

Figure 2: Plot of 1/v' vs. 1/[1-45] where v' is moles of protein bound/moles PL. The inset is a Hill plot of binding data with n = 102.

Neither the radioiodinated 1-45 peptide nor fragment 1 bind to PS/PC vesicles in the presence of Mg(II) ions. Fragment 1 competes with the [125-I] 1-45 peptide for PS/PC vesicles at 1.0 mM Ca(II) concentration [12]; the binding constant derived from Figure 2 reveals that the [125-I] 1-45 peptide binds with a Kd approximately one-tenth that of bovine fragment 1 (Kd = 1.0 μM) at this Ca(II) concentration using this methodology.

The [125-I] 1-45 peptide has been shown [12] to dimerize more readily than fragment 1 at similar concentrations of peptide or protein. The dimerization process for the peptide is enhanced by increasing NaCl concentrations and peptide concentration but is not affected by changes in Ca(II) concentration in the 0.5 - 1.5 mM Ca(II) range. The dimerization process may reduce the Ca(II)-promoted peptide:PS/PC interaction and thus NaCl concentrations (0.05 M) and iodination conditions were chosen to minimize dimerization. The [125-I] 1-45 peptide precipitates at Ca(II) concentrations greater than 2.0 mM Ca(II) [12], hence PS/PC binding measurement in the present study were conducted at non-precipitating Ca(II) concentrations.

These experiments establish that the 1-45 peptide derived from bovine fragment 1 contains sufficient structural organization to exhibit the Ca(II)-mediated binding to PS/PC vesicles inherent in the intact native proteins. Structure-function studies designed to establish the molecular nature of the peptide:Ca(II):PS/PC interaction will follow as a consequence of this finding.

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