Calcium and Phospholipid Binding Properties of Synthetic γ-Carboxyglutamic Acid-Containing Peptides with Sequence Counterparts in Human Protein C†

Tracey L. Colpitts and Francis J. Castellino*

Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, Indiana 46556

Received September 22, 1993; Revised Manuscript Received December 27, 1993®

ABSTRACT: Two peptides with counterpart sequences in the γ-carboxyglutamic acid (Gla) domain of human protein C (PC) have been synthesized and characterized. One peptide contained 38 amino acids (38-mer) and spanned the region from the amino terminus of the protein to the DNA splice junction between the Gla domain and the following short helical stretch, and the second peptide (48-mer) included a 10 amino acid extension that has been designed to incorporate the exon for the helical segment that is thought to play a role in stabilizing the Ca^{2+}-dependent conformation of the Gla domain of proteins of this class. The peptides were synthesized by solid-phase methodology, then oxidized to allow disulfide pairing, and finally purified by FPLC methodology. Chemical characterization showed that each peptide contained its full complement of Gla residues. Two types of Ca^{2+}-binding sites were found in these peptides, tighter sights (2–3) with K_d values of 60–370 μM and a weaker set of sites (7–10) with a range of K_d values from 0.8 to 3.1 mM. In general, the 48-mer interacted with Ca^{2+} more tightly than the 38-mer. As revealed by circular dichroism analysis, and by reactivity with monoclonal antibodies that recognize both the unfolded form of the Gla domain as well as the Ca^{2+}-dependent conformation of this same domain, the 38-mer and 48-mer underwent the Ca^{2+}-induced conformational changes characteristic of the intact protein. Both peptides displayed Ca^{2+}-dependent binding to negatively charged phospholipid vesicles (PL). The Ca^{2+} concentration required for 50% binding of the 48-mer to PL was approximately 2-fold lower than that for the 38-mer, while the dissociation constant (K_d) for binding of the 48-mer to the PL vesicles at 2 mM Ca^{2+} was approximately 3-fold lower than for the 38-mer. The K_d for binding of human plasma PC to PL at this Ca^{2+} concentration was approximately the same as the 48-mer, suggesting that these peptides contain essential PL-binding determinants found in the intact protein. The results of this investigation suggest that the short helical stretch immediately downstream of the Gla domain, which is a common feature of vitamin K-dependent coagulation proteins, does serve to enhance the Ca^{2+}-binding properties of the Gla domain. The peptide that contains both of the domains (48-mer) possesses most of the Gla-dependent Ca^{2+}- and PL-binding characteristics of the intact protein, suggesting that this is the minimal structure for nearly normal Ca^{2+}- and PL-binding properties of the Gla domain in intact PC. These findings allow simpler model systems to be employed to investigate the role of the Gla domain in the functioning of proteins of this type.

Several proteins involved in blood coagulation and anticoagulation reactions undergo a vitamin K-dependent posttranslational γ-carboxylation of specific precursor glutamic acid residues that results in a highly negatively charged region containing 9–13 Gla[1] residues, the first 10 of which are present in highly homologous positions within 30 amino acids of the amino terminus of these proteins. This modification generates Ca^{2+} and membrane binding sites in these proteins, thereby allowing elaboration of their functional properties (Gitel et al., 1973; Nelsestuen et al., 1974).

A considerable number of investigations have taken place that have resulted in elucidation of the Ca^{2+}-binding properties of several vitamin K-dependent proteins. Multiple classes of sites associated with large numbers of Ca^{2+} ions have been identified for all of these proteins (Nelsestuen & Suttie, 1972; Bajaj et al., 1975; Henriksen & Jackson, 1975; Amphlett et al., 1979, 1981a,b; Strickland & Castellino, 1980). In general, the Gla domains contain a relatively tight class of Ca^{2+}-binding sites that are necessary for conformational alterations required for PL binding (Nelsestuen, 1976; Keyt et al., 1982). Other divalent and trivalent cations can substitute for Ca^{2+} in this case (Prendergast & Mann, 1977). Additionally, a weaker class of Ca^{2+}-binding sites that interact with anionic sites on the acidic PL is present on this Ca^{2+}-dependent conformation of the Gla domain (Nelsestuen et al., 1976). Other domains exist in these proteins that may interact with Ca^{2+}, such as EGF-like regions, kringle domains, and the serine protease motif. These domains, particularly some of the EGF-like and protease regions, contain divalent and trivalent cation binding sites. The proteins factor VII (Wildgoose et al., 1993), factor IX (Morita & Kisiel, 1985; Astermark et al., 1991; Bajaj et al., 1992), factor X (Sugo et al., 1984; Persson et al., 1989; Selander-Sunnerhagen et al., 1992; Rezaie et al., 1993), PC (Johnson et al., 1983; Hill & Castellino, 1987; Ohlin et al., 1988; Rezaie et al., 1992), and protein S (Dahlback et al., 1990) contain cation binding sites outside of the Gla domain. These latter sites appear to be mainly responsible for interaction of these proteins with their protein cofactors, substrates, and cellular receptors.

† Supported by Grant HL-19982 from the National Institutes of Health, a graduate fellowship from the American Heart Association, Indiana Affiliate (to T.L.C.), and the Kleiderer/Pezold family endowed professorship (to F.J.C.).

* To whom to address correspondence.

® Abstract published in *Advance ACS Abstracts*, March 1, 1994.

[1] Abbreviations: Gla, γ-carboxyglutamic acid; Fmoc, 9-fluorenylmethyloxycarbonyl; PC, human protein C; PL, phospholipid; MAb, monoclonal antibody; *O-t*-Bu, *tert*-butyl ester; *O*-Bzl, benzyl ester; *t*-Boc, *tert*-butyloxycarbonyl; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; Bum, *tert*-butyloxymethyl; Z, benzyloxycarbonyl; *O*-Pfp, pentafluorophenyl ester; PAL, 5-[4-[[(9-fluorenylmethyloxycarbonyl)amino]-methyl]-3,5-dimethoxyphenoxy]valeric acid; CD, circular dichroism; NaB, 10 mM sodium borate/100 mM NaCl, pH 8.0.

0006-2960/94/0433-3501$04.50/0

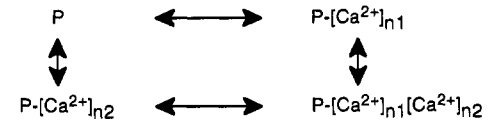
Due to the presence of several types of Gla domain-related and -nonrelated cation binding sites in vitamin K-dependent proteins, it would be of extreme importance to assess whether discrete regions of such proteins were able to interact with Ca^{2+} and PL in a manner similar to that of their counterpart regions in the intact proteins. If this were so, simplified systems would be available for design of experiments to better understand the functional roles of these multiple divalent cation sites that are contained in the different protein domains. In order to assess this possibility for the Gla domain, we have chemically synthesized two polypeptides encompassing the entire Gla domain of human PC, as well as this Gla domain and its trailing short helical stack that is believed to be important in stabilizing the Ca^{2+}-bound structure of the Gla domain (Soriano-Garcia et al., 1992). These polypeptides were then employed to examine a number of their Ca^{2+}-dependent functions. This paper presents a summary of these investigations.

MATERIALS AND METHODS

Synthesis of N^{α}-Fmoc-γ,γ'-di-O-t-Bu-L-Gla-OH. Gla was synthesized by previously described procedures, beginning with 1,3-di-*O*-*t*-Bu malonate. This latter compound was prepared by an acid-catalyzed transesterification of malonate (Colpitts & Castellino, 1993).

Peptide Synthesis. The following N^{α}-Fmoc-L-amino acids were employed for peptide synthesis: Ala-OH, Asp(*O*-*t*-Bu)-OH, Phe-OH, Ile-OH, Leu-OH, Asn-*O*-Pfp, Gln-*O*-Pfp, Ser-(*O*-*t*-Bu)-OH, and Thr(*O*-*t*-Bu)-OH (Sigma Chemical Co., St. Louis, MO); Lys(ϵ-*N*-*t*-Boc)-OH, His(Bum)-OH, and Arg-(Pmc)-OH (Calbiochem, La Jolla, CA); Cys(*S*-thio-*t*-Bu)-OH (Bachem, Torrance, CA). The peptides were synthesized using standard Fmoc protocols on a Milligen (Burlington, MA) Excell peptide synthesizer. Deprotection and cleavage from the solid support (PAL) resin were accomplished at room temperature over a 6–8-h period by employing 50% trifluoroacetic acid/CH_2Cl_2 with 5% (v/v) anisole/10% (v/v) thiophenol/10% (w/v) *p*-cresol/10% (w/v) indole as a scavenger solution. Disulfide bond pairing was accomplished in a N_2 atmosphere at basic pH, after removal of the *S*-*t*-Bu protecting groups with dithiothreitol. The resulting peptides were purified by ion-exchange chromatography using a MonoQ 10/10 column (Pharmacia, Piscataway, NJ). Full details of all of this methodology have been previously provided (Colpitts & Castellino, 1993).

Binding of Ca^{2+} to the Peptides. The exact methodology employed for determination of the thermodynamic parameters that characterize the binding of Ca^{2+} to the synthetic peptides, employing Ca^{2+}-selective electrode titrations, has been described previously (Colpitts & Castellino, 1993). The Ca^{2+} bound was derived from the potentiometric reading with use of a standard curve. The resulting data were treated by Scatchard analysis using the following model of two independent classes of ligand binding sites, with cooperativity within each class:



where P is the protein or polypeptide and n_1 and n_2 are the number of Ca^{2+} bound to sites 1 and 2.

The relationship $[P_{free}] = n[P^o] - [Ca^{2+}{}_{bound}]$, where $[P^o]$ is the total protein or polypeptide concentration, and the usual equilibrium constant expression provide

$$[Ca^{2+}{}_{bound}] = \left(\frac{K_d[Ca^{2+}{}_{free}]}{K_d + [Ca^{2+}{}_{free}]}\right)\left(\frac{[P^o]n}{K_d}\right)$$

Accounting for both sets of sites and cooperativity within each set:

$$[Ca^{2+}{}_{bound}] = \left(\frac{K_{d_1}[Ca^{2+}{}_{free}]^{H_1}}{K_{d_1} + [Ca^{2+}{}_{free}]^{H_1}}\right)\left(\frac{[P^o]n_1}{K_{d_1}}\right) + \left(\frac{K_{d_2}[Ca^{2+}{}_{free}]^{H_2}}{K_{d_2} + [Ca^{2+}{}_{free}]^{H_2}}\right)\left(\frac{[P^o]n_2}{K_{d_2}}\right)$$

where H_1 and H_2 are Hill coefficients for each set of sites. This is rearranged to the final form:

$$\frac{[Ca^{2+}{}_{bound}]}{[P^o][Ca^{2+}{}_{free}]} = \left(\frac{n_1}{K_{d_1}}[Ca^{2+}{}_{free}]^{H_1-1}\right)\left(1 - \frac{[Ca^{2+}{}_{free}]^{H_1}}{K_{d_1} + [Ca^{2+}{}_{free}]^{H_1}}\right) + \left(\frac{n_2}{K_{d_2}}[Ca^{2+}{}_{free}]^{H_2-1}\right)\left(1 - \frac{[Ca^{2+}{}_{free}]^{H_2}}{K_{d_2} + [Ca^{2+}{}_{free}]^{H_2}}\right)$$

which is solved by nonlinear least squares iterative best-fit analysis of the data with knowledge of $[Ca^{2+}{}_{free}]$, $[Ca^{2+}{}_{bound}]$, and $[P^o]$. The parameters minimized were n_1, n_2, K_{d_1}, K_{d_2}, H_1, and H_2.

Dependence on Ca^{2+} of the Binding of the Synthetic Peptides to the Monoclonal Antibody 7D7B10. This monoclonal antibody (donated by Dr. Dudley Strickland, Rockville, MD) recognizes a conformational epitope within the amino-terminal 15 residues of PC that is eliminated when the protein adopts its Ca^{2+}-dependent conformation (Orthner et al., 1989).

The peptides were bound to wells of microtiter plates with glutaraldehyde. A total of 100 μL of 2.5% glutaraldehyde in 100 mM $NaHCO_3$ was placed in each well and allowed to remain for 2 h, after which time the wells were washed three times with H_2O. A volume of 100 μL of 30 μM peptide in NaB/20 mM $CaCl_2$ was then added to each well and allowed to incubate overnight at 4 °C. The plate was then washed two times with a solution of NaB/2 mM EDTA, followed by two washes with H_2O. The remaining cross-linking sites in the wells were fully blocked by addition of 100 μL of a solution of 1% gelatin in NaB. After the plate was washed, 50 μL of solutions containing NaB, various concentrations of $CaCl_2$, and antibody 7D7B10 (0.44 mg/mL, final concentration) was added, and the solution was allowed to incubate overnight at 4 °C. After removal of the solutions, the wells were washed with NaB containing the same concentrations of $CaCl_2$ originally placed in the wells. Next, a secondary antibody, goat anti-mouse IgG conjugated to alkaline phosphatase (1:3000 dilution of affinity chromatography-purified antibody purchased from Bio-Rad, Richmond, CA), was added, and the solution was allowed to incubate for 1 h at room temperature. After being washed with NaB/$CaCl_2$ as above, the amount of secondary antibody present in each well was measured in a microtiter plate reader using the alkaline phosphatase substrate nitro blue tetrazolium (16.5 mg of nitro blue tetrazolium/0.5 mL of 70% (v/v) aqueous DMF/8.5 mg of bromochloroindolyl phosphate in 1 mL of H_2O, which was added to 50 mL of 0.1 M Tris-HCl/0.1 M NaCl/0.005 M $MgCl_2$, pH 9.5).

The [$C_{50,MAb}$–Ca^{2+}] (concentration of total Ca^{2+} at which a 50% decrease in binding occurs) was calculated from the titration curve using the following equation (Michaelis–Menten based) that in this case describes a saturable binding event: absorbance$_{405nm}$ = $x[Ca^{2+}]/([C_{50,MAb}–Ca^{2+}] + [Ca^{2+}]) + w$, where x is the rate of inhibition of binding and w is the initial binding value (at $[Ca^{2+}] = 0$). The data obtained were best fit to this equation using nonlinear least squares iterative curve fitting, as described in detail earlier (Menhart et al., 1991).

Dependence on Ca^{2+} of the Binding of the Synthetic Peptides to a Monoclonal Antibody JTC-1. This monoclonal antibody (provided by Drs. N. Aoki and K. Wakabayashi, Tokyo, Japan) recognizes a Ca^{2+}-dependent epitope within the Gla domain of PC (Wakabayashi et al., 1986). The antibody was labeled with ^{125}I as previously described (Zhang & Castellino, 1992). The final specific radioactivity of the antibody was 630 000 cpm/μg. The [$C_{50,MAb}$–Pep] (the concentration of Ca^{2+}-bound 48-mer required for 50% displacement of PC from the antibody) was calculated from the graph as the total concentration of PC or polypeptide required to reduce binding of antibody to the plate-bound PC by 50%.

Binding studies of the 38-mer and 48-mer to this antibody were performed by competition assays with PC. To individual wells of a microtiter plate was added a solution containing 10 μL of PC (0.9 μM) and 290 μL of NaB/3 mM $CaCl_2$. The solutions were allowed to incubate in the wells overnight at 4 °C. After being washed with NaB, 300 μL of a solution of 1% ovalbumin in NaB was used to block remaining protein adsorption sites in the wells. Various concentrations of peptides were dissolved in NaB/3 mM $CaCl_2$, and a total of 150 μL of this solution was added to each well. After addition of 5 μL of [^{125}I]-JTC-1 (0.014 μM, final concentration), the plate was allowed to stand for 9 h at room temperature. The solutions were then discarded, and the plate was washed three times with NaB/3 mM Ca^{2+}. Each well was then cut from the plate, and the amount of ^{125}I present was determined by γ counting.

Quasi-Elastic Light Scattering. PL vesicles containing 60/40 (w/w) bovine brain phosphatidylcholine/chicken egg phosphatidylserine (Sigma Chemical Co.) were prepared as described earlier (Beals & Castellino, 1986). The PL concentration was determined as total phosphate (Lowry & Lopez, 1946) using a conversion factor of 25 (w/w) to obtain the weight concentrations of the PL vesicles.

Our methods for determining binding parameters of proteins to PL by 90° relative light scattering and for calculating [$C_{50,PL}$–Ca^{2+}] and [$C_{50,PL}$–Pep] values at 2 mM Ca^{2+} (the concentration of polypeptide required for 50% binding to PL at 2 mM Ca^{2+}) have been described in detail earlier (Zhang & Castellino, 1993).

Circular Dichroism. CD spectra were recorded between the wavelengths 190–300 nm on an AVIV Model 62DS spectrometer. The polypeptide samples (600 or 6 μM) were dissolved in NaB or NaB containing the desired concentration of $CaCl_2$. The solutions were then purged with N_2 and spectra obtained at 27 °C. Cells of 1- and 0.01-cm path lengths were employed to optimize the signal. Each spectrum is the average of five separate scans and was deconvoluted using the computer program of Chang et al. (1978).

Titrations of changes in ellipiticity values as a function of [Ca^{2+}] were performed at polypeptide concentrations of 6 μM. The [$C_{50,CD}$–Ca^{2+}] values that characterize these titrations were calculated as in the case of the antibody 7D7B10 experiments, except that x now represents the change in ellipticities at the different concentrations of Ca^{2+}.

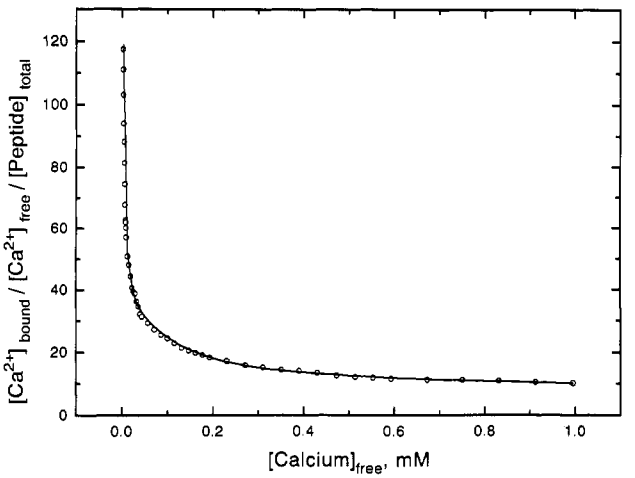


FIGURE 1: Scatchard plot of the binding of Ca^{2+} to the synthetic 48-mer. Ca^{2+}-selective electrode titrations were employed to determine the concentration of free Ca^{2+} in various Ca^{2+}/48-mer equilibrium mixtures at 25 °C, pH 7.5. Plotted in the graph is the $([Ca^{2+}_{bound}]/[Ca^{2+}_{free}])/[peptide_{total}]$ against the $[Ca^{2+}_{free}]$. After each addition of Ca^{2+}, the potentiometric response of the free [Ca^{2+}] was measured and its concentration determined from a standard curve under the same conditions, in the absence of peptide. The values for K_d (dissociation constant) and n (the concentration of Ca^{2+} bound per mole of polypeptide at saturation) were calculated from Scatchard-type plots and used to fit the data points shown in the graph. The concentration of polypeptide used in the titrations was 60 μM.

Analytical Methods. Our exact procedures for performance of amino acid analysis and amino acid sequencing of peptides (Chibber et al., 1990), as well as for the determination of their Gla contents (Zhang & Castellino, 1990), were as described.

RESULTS

The protected peptides prepared for this study were obtained in approximately 23–33% final yield, and no particular problems were encountered in their synthesis. The amino acid sequences of both peptides were determined through 30 residues. The results showed that the proper sequence was present and that <5% Glu was obtained at all positions wherein Gla was incorporated. This indicates that γ-decarboxylation did not occur to any significant extent during synthesis of the peptides or in any of the manipulations following synthesis. As confirmation of this point, Gla analysis of both peptides demonstrated that 8.7–9.0 mol of Gla/mol of peptide (theoretical, 9.0 mol/mol) was found in all cases.

The binding of Ca^{2+} to each of the peptides was determined by Ca^{2+}-selective electrode titrations. An example of such a titration is illustrated in Figure 1. The model used for deconvoluting these binding data, i.e., two classes of sites with cooperativity within each class, is likely not a unique model but is the simplest model which best fits the data. This model was chosen after having assessed the data with four of the simplest models, specifically (a) one set of sites without cooperativity, (b) one set of sites with cooperativity, (c) two sets of sites without cooperativity, and (d) two sets of sites with cooperativity. Relevant curve-fitting data for each of these cases are provided in Table 1. It is clear from this analysis that single-site models are not good fits of the data. The X^2 values, as well as the residual plots (not shown), are unsatisfactory. Each of the two-site models fits the data much better than the one-site models, and the simplest two-site model, *i.e.*, not involving cooperativity, provides a X^2 value nearly

Table 1: Deconvolution of the Ca^{2+}-Binding Isotherm of the Synthetic 48-mer to Various Models

model	binding parameters			
	n^a	$K_d{}^b$	H^c	$X^{2\,d}$
one set of noncooperative sites	1.5	0.011		93.4
one set of cooperative sites	15.2	1.71	0.56	20.4
two sets of noncooperative sites	0.4	0.001		2.5
	11.4	0.45		
two sets of cooperative sites	0.9	0.22	0.36	3.1
	14.8	0.80	0.95	

[a] Mole of Ca^{2+} bound per mole of sample. [b] Dissociation constant. [c] Hill coefficient. [d] Chi squared.

Table 2: Binding of Ca^{2+} to PC and Gla-Containing Synthetic Peptides As Revealed by Titrations with a Ca^{2+}-Selective Electrode

sample	binding parameters[a]		
	n^b	K_d (mM)[c]	H^d
PC	3–4	0.08–0.12	0.88–0.92
	>10[e]	0.5–1.5	1.5–1.8
48-mer	0.7–1.6	0.06–0.22	0.3–1.0
	>7[e]	0.8–1.1	1.0–1.7
38-mer	0.8–2.2	0.14–0.37	0.7–1.2
	>10[e]	2.0–3.1	1.2–1.5

[a] The ranges represent the extremes of three to four replicate experiments. [b] Mole of Ca^{2+} bound per mole of sample. [c] Dissociation constant. [d] Hill coefficient. [e] The values for the weak sites are not precise because of the lack of sensitivity of the models for weak binding sites. Thus, only a minimum number of such sites is provided.

equal to the more complex two-site cooperative model, both of which are much more reasonable choices than models with a single class of Ca^{2+}-binding sites. However, we rejected a two-class site noncooperative binding isotherm, since the value of n for the first set of sites was not an integer. An additional factor which played a role in our choice of the two-class of sites model with cooperativity was that the K_d for this first set of sites agrees far more suitably with the K_d values of the tightest class of sites in model peptides (Colpitts & Castellino, 1993) and with studies of several intact proteins of these types. In these latter cases, no previous indications of a K_d for Ca^{2+} of the order of 1 μM were obtained, the value that is provided by the noncooperative two-site model (Table 1). Thus, our basis for using this two-site cooperative model is based on best-fit minimization analysis of four of the simplest models, with a knowledge-based prejudice incorporated at the final stage. It is very likely that more complex models would also fit the data, but no basis for these selections could be made. Thus, in order to provide some quantitative analysis of our results, we selected a model based on the above rationale.

Using this model in the case of intact human plasma PC, deconvolution of the binding data revealed that three to four tight Ca^{2+} sites were present, with K_d values of 0.08–0.12 mM (class 1), as well as a weaker set of sites (class 2) characterized by K_d values of 0.5–1.5 mM. However, one to two of these tight sites in PC are located outside of the Gla domain (Esmon et al., 1983; Hill & Castellino, 1987; Rezaie et al., 1992). Our polypeptide data are consistent with this model since the 48-mer was found to contain one to two Ca^{2+} sites with K_d values of 0.06–0.22 mM as well as a set of weaker sites with K_d values similar to those sites in PC. The 38-mer displayed results similar to those of the 48-mer, except that somewhat weaker K_d values were found. A summary of these results is presented in Table 2. It should be pointed out that Ca^{2+} concentrations above 2 mM caused precipitation of the peptides. Thus the data could not be extended further than that reported in Figure 1.

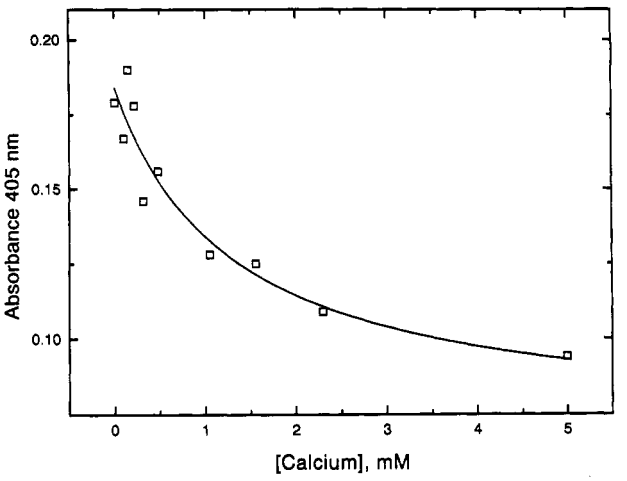


FIGURE 2: Effect of Ca^{2+} on the binding of the synthetic 48-mer to the Ca^{2+}-free Gla domain-directed monoclonal antibody 7D7B10. The polypeptide was bound *via* glutaraldehyde to wells of a microtiter plate, and solutions containing various amounts of Ca^{2+} (0–5 mM) and the antibody (0.44 mg/mL) were added to the wells. The amount of antibody 7D7B10 bound was determined after subsequent addition of a goat anti-mouse IgG/alkaline phosphatase conjugate using the alkaline phosphatase substrate nitro blue tetrazolium. Substrate hydrolysis was measured by absorbance at 405 nm at a time of 30 min after substrate addition. The [$C_{50,MAb}$–Ca^{2+}] (the Ca^{2+} concentration required for 50% decrease in antibody binding) was calculated from the graph by use of a Michaelis–Menten fit.

Two Ca^{2+}-specific monoclonal antibodies were employed to assess whether the synthetic peptides were able to adopt the Ca^{2+}-dependent conformation characteristic of PC. The antibody 7D7B10 recognizes an epitope in the amino-terminal region of the Gla domain that is eliminated upon binding of Ca^{2+} (Orthner et al., 1989). Titration of the Ca^{2+} dependency of the binding of the 48-mer to this antibody is illustrated in Figure 2 and shows that the epitope in this peptide is similarly lost concomitant with Ca^{2+} binding. From this titration, a [$C_{50,MAb}$–Ca^{2+}] value of 0.93 mM is obtained. Similar titration with PC and the 38-mer provided similar [$C_{50,MAb}$–Ca^{2+}] values of 1.0 and 0.83 mM, respectively.

Another monoclonal antibody, JTC-1, with an epitope located in the amino-terminal region of the Gla domain of PC that recognizes the Ca^{2+}-dependent conformation of this protein (Wakabayashi et al., 1986), has been similarly employed. An example of the displacement of PC from JTC-1 with the 48-mer is shown in Figure 3. The [$C_{50,MAb}$–Pep] in this case was found to be approximately 200 nM. This is compared with 250 nM for binding of the Ca^{2+}/38-mer and approximately 30 nM for binding of the Ca^{2+}/PC complex.

Further investigation of the conformational changes accompanying binding of Ca^{2+} to the 48-mer was accomplished by CD analysis. A plot of the mean residue ellipticities of this polypeptide as a function of wavelength in the presence and absence of Ca^{2+} is shown in Figure 4. A clear conformational alteration is observed which tends in the direction of increased percentage of α-helix. These spectra were deconvoluted using the program of Chang et al. (1978). In the absence of Ca^{2+}, no α-helix was observed, 65% is present as β-sheet, and 33% is in the random conformation. In the presence of 5 mM Ca^{2+} (free [Ca^{2+}] = 1.5 mM), these fractional conformations were 12% α-helix, 59% β-sheet, and 21% random structures. Titrations of the change in ellipticities in the wavelength range of 220–226 nm as a function of the concentration of Ca^{2+} have been performed, providing a [$C_{50,CD}$–Ca^{2+}] of approximately 1.5 mM.

The ability of the synthetic Gla domain polypeptides to interact with acidic PL has been assessed by 90° light scatter

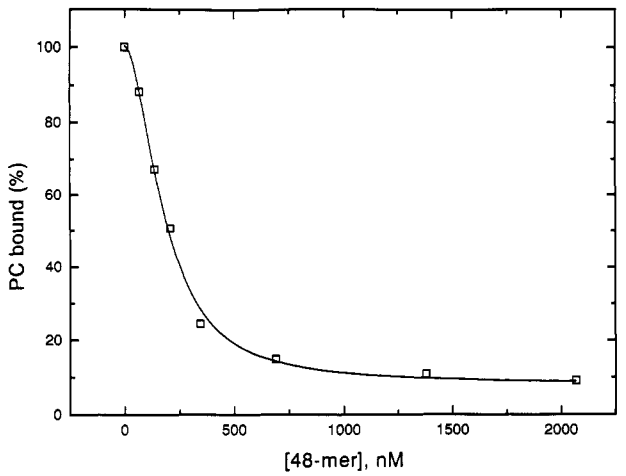


FIGURE 3: Displacement by the synthetic 48-mer of PC from the Ca^{2+}-dependent Gla domain-directed monoclonal antibody [^{125}I]-JTC-1. After individual wells of a microtiter plate were coated with PC, various concentrations of the synthetic 48-mer (0–2000 nM) were added, followed by [^{125}I]-JTC-1 (0.014 μM). The amount of antibody bound to the plate was then measured by γ counting. The [$C_{50,MAb}$–Pep] (the concentration of 48-mer required to displace 50% of the antibody from plate-bound PC at 2 mM Ca^{2+}) was determined directly from the graph. Ca^{2+} (2 mM) was present in all solutions.

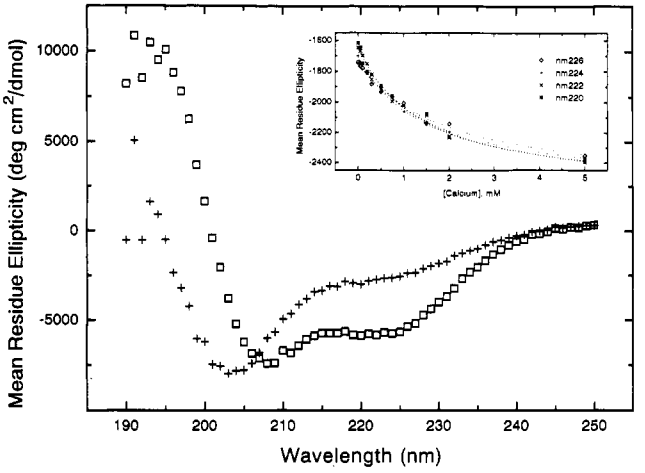


FIGURE 4: Circular dichroism analysis of the effect of Ca^{2+} on the secondary structure of the synthetic 48-mer. The mean residue ellipticities are plotted against the wavelengths for the polypeptide in the absence of Ca^{2+} (+) and in the presence of 5 mM (1.5 mM free) Ca^{2+} (□). The polypeptide concentration was 0.6 mM. Changes with Ca^{2+} in mean residue ellipticities at wavelengths of 220, 222, 224, and 226 nm are illustrated in the inset, at a polypeptide concentration of 0.006 mM. The [$C_{50,CD}$–Ca^{2+}] values (the [Ca^{2+}] required to change the ellipticity values by 50%) were calculated from the inset plot by use of a Michaelis–Menten data fit.

analysis. Titrations of the increase in scattering intensities as a function of the concentration of Ca^{2+} have been performed. These data were employed to calculate the fractional amounts of polypeptides bound to the PL vesicles, and the results are shown in Figure 5. From these plots, the [$C_{50,PL}$–Ca^{2+}] values were determined as 1.1 mM for the 48-mer and 2.0 mM for the 38-mer. Similar titrations with human plasma PC provided a [$C_{50,PL}$–Ca^{2+}] of approximately 0.5 mM. Additionally, titrations with the polypeptides at 2 mM Ca^{2+} have been accomplished, and the results are illustrated in Figure 6. The [$C_{50,PL}$–Pep] for the 48-mer was found to be 3.7 μM. This is compared to the [$C_{50,PL}$–Pep] of 1.9 μM for human plasma PC at 2 mM Ca^{2+}. The 38-mer showed an atypical sigmoidal titration curve under these conditions. A [$C_{50,PL}$–Pep] of 9.4 μM was calculated from this plot.

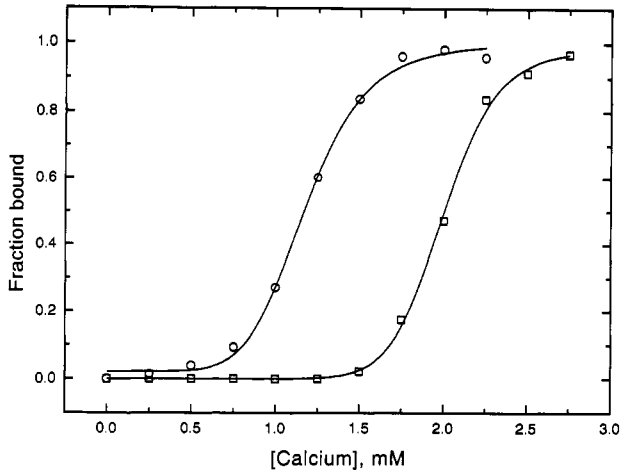


FIGURE 5: Effect of Ca^{2+} on the binding of the synthetic 38-mer (□) and 48-mer (○) to PL. The fractional amount of peptide bound was determined by comparison of the M_2/M_1 value to the final M_2/M_1 value at each [Ca^{2+}] and is plotted against the [Ca^{2+}].

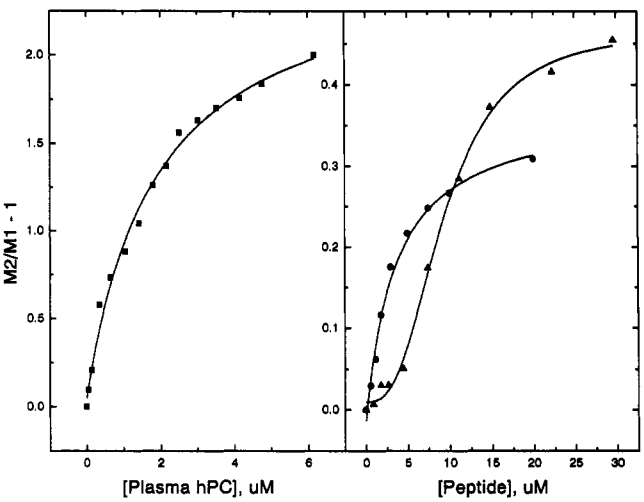


FIGURE 6: Dependence on protein and polypeptide concentrations on their interactions of PL (0.6–1.0 μg/mL) in the presence of 2 mM Ca^{2+}. The molecular weights of the human plasma PC/PL, 48-mer/PL (●), and 38-mer/PL (▲) complexes were determined by relative light scattering after subtraction of the light scattering of the nonbound protein or polypeptides.

DISCUSSION

The purpose of this investigation was to evaluate the extent to which the isolated Gla domain of PC mimicked the behavior attributed to this same Gla domain in the intact protein, with particular emphasis on its Ca^{2+}- and PL-binding properties. If a defined relationship between the isolated fragment and the fragment contained in the entire protein could be established, then it would be possible to investigate more thoroughly the properties of a much simpler system and relate these to the intact protein. We decided to undertake this investigation by chemically synthesizing the entire exon containing the Gla domain, as well as a second peptide also containing the next exon—a short (nine residue) stretch of amino acids with an α-helical conformation that has been proposed to stabilize the Ca^{2+}-dependent conformation of the Gla domain (Persson & Stenflo, 1992; Soriano-Garcia et al., 1992). We felt that chemical synthesis offered a great advantage over limited protein proteolysis methods or recombinant techniques to obtain the Gla domain, since large amounts of well-defined products could be obtained with the additional opportunity to selectively label amino acid residues for NMR investigations (Colpitts & Castellino, 1993). The

chemical syntheses were successfully accomplished by the methods described, and two peptides corresponding to residues 1–38 and 1–48 of PC were obtained. The former polypeptide contained only the Gla domain, and the latter polypeptide contained the Gla domain plus the entire exon of the immediately downstream helical stack. These polypeptides were characterized by amino acid sequence and Gla analysis and were found to contain their entire complements of Gla residues in the expected locations.

The binding characteristics of Ca^{2+} to the synthetic polypeptides have been directly measured and compared to this same property of intact human plasma PC (Table 2). The data show that the high-affinity Ca^{2+} binding sites of protein C are present in the 48-mer, except that one to two fewer sites exist in this polypeptide. This is consistent with the observation that a high-affinity Ca^{2+} binding site in PC is present outside of the Gla domain (Johnson et al., 1983; Hill & Castellino, 1987; Ohlin et al., 1988; Rezaie et al., 1992). These high-affinity sites are also present in the 38-mer, except that they are slightly weaker in nature. The weak class of binding sites of PC also exists in both polypeptides in similar numbers and binding strengths, suggesting that the weak class of binding sites mainly exists in the Gla domain of PC. Thus, it appears from these studies that Ca^{2+} binding to the 48-mer closely parallels that of the Gla domain of the intact protein whereas Ca^{2+} binding to the 38-mer shows slightly altered characteristics.

The ability of the synthetic polypeptides to adopt the proper Ca^{2+}-dependent conformation of the Gla domain of intact PC has been assessed by use of two conformationally directed monoclonal antibodies, *viz.*, 7D7B10, which recognizes an epitope in the Ca^{2+}-free form of the Gla domain (Orthner et al., 1989), and JTC-1, which possesses an epitope in the Ca^{2+}-dependent conformation of the Gla domain. With regard to the former antibody, the results demonstrated that both the 38-mer and the 48-mer interacted with this antibody in the absence of Ca^{2+} and showed Ca^{2+} dependencies similar to those of intact PC in loss of this reactivity upon Ca^{2+} binding. Similarly, neither synthetic polypeptide interacted with antibody JTC-1 in the absence of Ca^{2+} but did display such reactivity in the presence of Ca^{2+}. From competition binding experiments with PC in the presence of 2 mM Ca^{2+}, the [$C_{50,MAb}$–Pep] for binding of the Ca^{2+}-bound polypeptides was approximately 200–250 nM. This value was somewhat higher than that obtained from competition of PC by solution-phase PC, suggesting that some small differences in the epitopes were present in PC and in the peptides and/or that an extended epitope for this antibody is present in the intact protein. We conclude from these experiments that each of the synthetic polypeptides undergoes a Ca^{2+}-dependent conformational transition similar to that of the Gla domain of PC when this latter domain is present in the intact protein.

Another method of assessing the Ca^{2+}-induced transition of PC and similar proteins is through alterations in their intrinsic fluorescence properties. X-ray crystallography studies of the bovine prothrombin fragment 1/Ca^{2+} complex (Soriano-Garcia et al., 1992) suggest that this fluorescence change is due to the interaction of an aromatic cluster comprising Phe^{41}, Trp^{42}, and Tyr^{45} with the Cys^{18}–Cys^{23} disulfide bond in the Ca^{2+}-induced structure of the protein. This interaction is then likely reported by a Trp residue(s), which is believed to be Trp^{42} (Schwalbe et al., 1989). Since all of these residues are present in the 48-mer, its ability to undergo the fluorescence transition was determined. We did not observe such a change, a result similar to that obtained for the polypeptide containing residues 1–45 of bovine prothrombin (Pollock et al., 1988). This could be due to an altered conformation of the Ca^{2+}-bound polypeptides or the possibility that the reporter Trp for this event is not actually present within residues 1–45. We believe that the latter is the probable situation, based on the fact that bovine and human protein S do not possess a Trp residue at sequence positions homologous to 42 in bovine prothrombin (Tyr residues are present), but yet undergo the Ca^{2+}-induced fluorescence quenching characteristic of other proteins of this class,[2] and on the observation that mutation of Trp^{41} to Tyr in recombinant PC does not alter the property of this protein to undergo Ca^{2+}-dependent fluorescence quenching.[2] Thus, we contend that the fluorescence change(s) accompanying formation of the Ca^{2+}-dependent structure of the Gla domain depend(s) upon other Trp residues in the intact protein, a result consistent with other published studies (Welsch & Nelsestuen, 1988; Rezaie et al., 1992). Therefore, the lack of Ca^{2+}-dependent fluorescence quenching of the isolated Gla domain does not necessarily reflect its ability to adopt the proper Ca^{2+}-induced conformation at the normal levels of Ca^{2+}. Indeed, CD analysis of the polypeptides in the presence and absence of Ca^{2+} (Figure 4), along with the antibody binding results discussed above, demonstrates that a conformational alteration does occur. The abnormally high [Ca^{2+}] required for 50% fluorescence quenching found in proteolytically derived Gla domain-containing polypeptides (with variable amounts of the intact hydrophobic stack) from other proteins, *viz.*, 4 mM for the Gla domain of fIX containing residues 1–43 (Schwalbe et al., 1989; Astermark et al., 1991) and >2 mM for the Gla domain of fX containing residues 1–44 (Persson et al., 1991), may be associated with additional weak types of Ca^{2+}-binding events with lower binding affinities that are reported by the homologous Trp^{42} residue.

The PL-membrane binding properties of the two synthetic polypeptides are similar to those of intact plasma PC (Figures 5 and 6). The Ca^{2+} dependency for binding of the 48-mer to PL was only 2-fold greater than that obtained with plasma PC. Further, the [$C_{50,PL}$–Pep] for binding of the 48-mer to PL at 2 mM Ca^{2+} was 3.7 μM, a value quite close to that of 1.9 μM ([$C_{50,PL}$–PC]) that was obtained for plasma PC.[3] Thus, we believe that the 48-mer possesses the required Ca^{2+} binding sites required for PL binding and that the 48-mer represents the minimal structure in PC needed for such PL binding. This conclusion is in accord with a similar observation published earlier regarding the PL-binding properties of bovine prothrombin fragment 1–45 (Pollock et al., 1988). On the other hand, the 38-mer also appears to possess such a Ca^{2+}-dependent structure, but it is somewhat less effective in PL binding that that of the 48-mer. We conclude that the hydrophobic helical stack present in the 48-mer does indeed confer improved Ca^{2+}- and PL-binding properties on the Gla domain.

Other investigations have addressed the question as to minimal structures in proteins of this class needed for various functions. In recent studies with protein Z (Persson & Stenflo, 1992), factor IX (Astermark et al., 1991), factor X (Persson et al., 1991), and PC (Ohlin et al., 1990), Ca^{2+}-binding experiments with a fragment containing the Gla domain and

[2] L. Zhang, unpublished experiments.

[3] We have published previously PC/PL binding data obtained at 20 mM Ca^{2+} and therein showed that human plasma PC and wtr-PC from 293 cells displayed nearly identical [$C_{50,PL}$–PC] values of 0.30–0.38 μM (Zhang & Castellino, 1993). However, at 2 mM Ca^{2+}, while the [$C_{50,PL}$–PC] for wtr-PC remains at approximately 0.4 μM (Zhang & Castellino, 1994), that for human plasma PC is increased to approximately 1.9 μM.

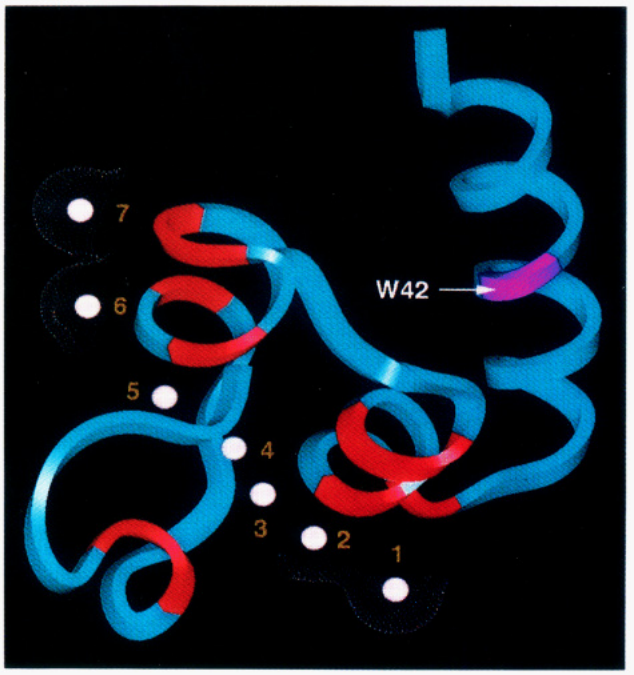


FIGURE 7: X-ray crystal structure of the prothrombin fragment 1/Ca^{2+} complex. The backbone conformation of residues 1–46 is illustrated (blue). The positions of each of the Gla residues are indicated in red. The Ca^{2+} ions associated with the Gla domain are present as white spheres with the extents of their solvent accessibilities as shaded areas. The position of Trp^{42} (Trp^{41} in PC) in the backbone structure of the helical stack is highlighted in purple.

the first EGF-like fragment were compared to those of the Gla domain alone. From these studies it was suggested that the EGF fragment was needed for normal Ca^{2+}-binding properties of the Gla domain and that the EGF domain provides a scaffold for folding of the Gla domain. However, these experiments were conducted using Gla domains liberated from the proteins by limited chymotryptic cleavage, which excises the Gla domains at a position corresponding to Trp^{41} in PC (Trp^{42} in bovine prothrombin), a residue conserved at homologous positions in most of these proteins, and/or cleavage by lysyl endopeptidase which cleaves at Lys^{43} of bovine factor IX (Astermark et al., 1991). Using the crystal structure of bovine prothrombin (Soriano-Garcia et al., 1992) as a model of the structure of the Gla domains of these proteins, it is seen from Figure 7 that these particular fragments are cleaved within the hydrophobic helical stack, likely preventing any helix formation in this region. We believe from studies herein that the entire helical stack is needed for provision of normal Ca^{2+}- and PL-binding properties of the Gla domain. A similar situation may exist regarding the necessity of the helical stack in conserving the tight Ca^{2+}-binding site present in the first EGF-like region of bovine factor X (Velcarce et al., 1993). Thus, our results do not necessarily disagree with previous work on this same topic, but we do feel that the first EGF-like region does not markedly influence the Ca^{2+}- and PL-binding properties of the Gla domain. A complete helical stack that resides in a domain between the Gla and EGF-like modules plays a more important role here. While the EGF-like domain may also play some role in defining the final Ca^{2+}-dependent structure of the Gla domain, it does not appear to be the dominant factor in this regard. Support for this conclusion is found in the study of the PL-binding properties of a fragment of bovine prothrombin containing residues 1–45, which includes the Gla domain and a more complete hydrophobic stack (Pollock et al., 1988). The studies described in that report were consistent with the conclusions of the current paper and showed that the relevant Ca^{2+}-binding sites important to the PL-binding properties of bovine prothrombin were retained in the polypeptide containing residues 1–45.

In conclusion, we have shown that the short helical stretch comprising a complete exon in PC encompassing amino acid residues 38–46 serves to enhance the Ca^{2+}- and PL-binding properties of the Gla domain. The 48-mer underwent the important Ca^{2+}-dependent conformational transition that is prerequisite for PL binding and also possessed the Ca^{2+}-binding sites needed for the second stage of PL binding by the Gla domain. Thus, this polypeptide contains the minimal structures needed for nearly normal Ca^{2+} and PL binding that is characteristic of the Gla domain in intact PC. These findings allow simpler model systems to be employed to investigate the role of the Gla domain in the functioning of proteins of this type.

REFERENCES

Amphlett, G. W., Byrne, R., & Castellino, F. J. (1979) *J. Biol. Chem. 254*, 6333–6336.

Amphlett, G. W., Kisiel, W., & Castellino, F. J. (1981a) *Biochemistry 20*, 2156–2161.

Amphlett, G. W., Kisiel, W., & Castellino, F. J. (1981b) *Arch. Biochem. Biophys. 208*, 576–585.

Astermark, J., Bjork, I., Ohlin, A.-K., & Stenflo, J. (1991) *J. Biol. Chem. 266*, 2430–2437.

Bajaj, S. P., Butkowski, R. J., & Mann, K. G. (1975) *J. Biol. Chem. 250*, 2150–2156.

Bajaj, S. P., Sabharwal, A. K., Gorka, J., & Birktoft, J. J. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 152–156.

Beals, J. M., & Castellino, F. J. (1986) *Biochem. J. 236*, 861–869.

Chang, C. T., Wu, C. C., & Yang, J. T. (1978) *Anal. Biochem. 91*, 13–31.

Chibber, B. A. K., Urano, S., & Castellino, F. J. (1990) *Int. J. Pept. Protein Res. 35*, 73–80.

Colpits, T. L., & Castellino, F. J. (1993) *Int. J. Pept. Protein Res. 41*, 567–575.

Dahlback, B., Hildebrandt, B., & Linse, S. (1990) *J. Biol. Chem. 265*, 18481–18489.

Esmon, N. L., DeBault, L. E., & Esmon, C. T. (1983) *J. Biol. Chem. 258*, 5548–5553.

Gitel, S. N., Owen, W. G., Esmon, C. T., & Jackson, C. M. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1344–1348.

Henriksen, R. A., & Jackson, C. M. (1975) *Arch. Biochem. Biophys. 170*, 149–159.

Hill, K. A. W., & Castellino, F. J. (1987) *Arch. Biochem. Biophys. 254*, 196–202.

Johnson, A. E., Esmon, N. L., Laue, T. M., & Esmon, C. T. (1983) *J. Biol. Chem. 258*, 5554–5560.

Keyt, B., Furie, B. C., & Furie, B. (1982) *J. Biol. Chem. 257*, 8687–8695.

Lowry, D. H., & Lopez, J. A. (1946) *J. Biol. Chem. 162*, 421–428.

Morita, T., & Kisiel, W. (1985) *Biochem. Biophys. Res. Commun. 130*, 841–847.

Nelsestuen, G. L. (1976) *J. Biol. Chem. 251*, 5648–5656.

Nelsestuen, G. L., & Suttie, J. W. (1972) *Biochemistry 11*, 4961–4964.

Nelsestuen, G. L., Zytkovicz, T. H., & Howard, J. B. (1974) *J. Biol. Chem. 249*, 6347–6350.

Nelsestuen, G. L., Broderius, M., & Martin, G. (1976) *J. Biol. Chem. 251*, 6886–6993.

Ohlin, A.-K., Linse, S., & Stenflo, J. (1988) *J. Biol. Chem. 263*, 7411–7417.

Ohlin, A.-K., Bjork, I., & Stenflo, J. (1990) *Biochemistry 29*, 644–651.

Orthner, C. L., Madurawe, R. D., Velander, W. H., Drohan, W. N., Battey, F. D., & Strickland, D. K. (1989) *J. Biol. Chem. 264*, 18781–18788.

Persson, E., & Stenflo, J. (1992) *FEBS Lett. 314*, 5–9.
Persson, E., Selander, M., Linse, S., Drakenberg, T., Ohlin, A.-K., & Stenflo, J. (1989) *J. Biol. Chem. 264*, 16897–16904.
Persson, E., Bjork, I., & Stenflo, J. (1991) *J. Biol. Chem. 266*, 2444–2452.
Pollock, J. S., Shepard, A. J., Weber, D. J., Olson, D. L., Klapper, D. J., Pedersen, L. G., & Hiskey, R. G. (1988) *J. Biol. Chem. 263*, 14216–14223.
Prendergast, F. G., & Mann, K. G. (1977) *J. Biol. Chem. 252*, 840–850.
Rezaie, A. R., Esmon, N. L., & Esmon, C. T. (1992) *J. Biol. Chem. 267*, 11701–11704.
Rezaie, A. R., Neuenschwander, P. F., Morrissey, J. H., & Esmon, C. T. (1993) *J. Biol. Chem. 268*, 8176–8180.
Schwalbe, R. A., Ryan, J., Stern, D. M., Kisiel, W., Dahlback, B., & Nelsestuen, G. L. (1989) *J. Biol. Chem. 264*, 20288–20296.
Selander-Sunnerhagen, M., Ullner, M., Persson, E., Teleman, O., Stenflo, J., & Drakenberg, T. (1992) *J. Biol. Chem. 267*, 19642–19649.
Soriano-Garcia, M., Padmanabhan, K., de Vos, A. M., & Tulinsky, A. (1992) *Biochemistry 31*, 2554–2566.
Strickland, D., & Castellino, F. J. (1980) *Arch. Biochem. Biophys. 199*, 61–66.
Sugo, T., Bjork, I., Holmgren, A., & Stenflo, J. (1984) *J. Biol. Chem. 259*, 5705–5710.
Velcarce, C., Selander-Sunnerhagen, M., Talmitz, A.-M., Drakenberg, T., Bjork, I., & Stenflo, J. (1993) *J. Biol. Chem. 268*, 26673–26678.
Welsch, D. J., & Nelsestuen, G. L. (1988) *Biochemistry 27*, 4946–4952.
Wildgoose, P., Foster, D., Schiodt, J., Wiberg, F. C., Birktoft, J. J., & Petersen, L. C. (1993) *Biochemistry 32*, 114–119.
Zhang, L., & Castellino, F. J. (1992) *J. Biol. Chem. 267*, 26078–26084.
Zhang, L., & Castellino, F. J. (1993) *J. Biol. Chem. 268*, 12040–12045.
Zhang, L., & Castellino, F. J. (1994) *J. Biol. Chem.* (in press).