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Modifications of Bovine Prothrombin Fragment 1 in the Presence and Absence of Ca(II) Ions

LOSS OF POSITIVE COOPERATIVITY IN Ca(II) ION BINDING FOR THE MODIFIED PROTEINS*

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Chemical modification of bovine prothrombin fragment 1 according to the procedure of D. J. Welsch and G. L. Nelsestuen (1988) [*Biochemistry* 27, 4946–4952 and earlier papers] provided a series of fragment 1 derivatives in which various nitrogen-containing side chains were *N*-acetylated and/or *N*-2,4,6-trinitrophenylated. In addition the des-[Ala-1,Asn-2]- and des-[Ala-1,Asn-2,Lys-3]-fragment 1 derivatives were prepared by limited enzymatic hydrolysis of fragment 1 using cathepsin C and plasmin, respectively. Quantitative studies on the Ca(II) binding of these proteins have been accomplished using ^{45}Ca(II) equilibrium dialysis. Binding of these fragment 1 derivatives to phosphatidylserine/phosphatidylcholine (PS/PC) vesicles (25:75) in the presence of Ca(II) ions has been studied using the light-scattering technique.

Acylation of the 5 lysine residues of fragment 1 by the action of acetic anhydride (500-fold molar excess) in the presence of 75 mM Ca(II), pH 8.0, results in loss of positive cooperativity in Ca(II) binding (Scatchard plot) and an increase in the number of Ca(II) ions bound. The Ca(II)-dependent PS/PC binding of the acylated protein is reduced. Removal of 2 and 3 residues from the amino terminus likewise leads to loss of positive cooperativity in Ca(II) binding and reduced binding affinity to PS/PC vesicles. The important role of the amino-terminal 1–10 sequence is discussed. We conclude that positive cooperativity in Ca(II) binding is not a prerequisite for the Ca(II)-dependent binding of bovine prothrombin fragment 1 to PS/PC vesicles.

The vitamin K-dependent carboxylation of specific glutamic acid residues in the amino-terminal region of several coagulation and anticoagulation proteins forms a negatively charged domain that contains the essential γ-carboxyglutamic acid (Gla)[1] residues (1–6). These proteins require Ca(II) for physiological function, and several essential coagulation steps are accelerated by the presence of negatively charged phospholipid vesicles or platelets (7–10). The amino-terminal 1–42 region (which contains all the Gla residues) of these coagulation proteins has a high degree of sequence homology. The 1–45 peptide derived from bovine prothrombin binds to phosphatidylserine/phosphatidylcholine (PS/PC) vesicles in the presence of Ca(II) ions with a reduced affinity compared to that of prothrombin fragment 1 (11–13). Selective chemical modification of Gla residues at positions 7, 8, and 33 has suggested that one or more of these residues are involved in the Ca(II)-phospholipid binding process (14).

Recently Welsch and Nelsestuen (15–19) reported a series of studies involving the action of acetic anhydride (pH 8.5) on bovine fragment 1. In the absence of metal ions the α-NH_2 group at Ala-1 and the ε-NH_2 groups of the 5 lysine residues at positions 3, 11, 44, 57, and 97 were acylated.

Under these reaction conditions asparagine 101 was converted to the imide derivative by acetylation of the nitrogen atom of the side-chain carboxamide group. This protein did not bind to PS/PC vesicles. When the acetylation was conducted in the presence of Ca(II) ions, the α-NH_2 of Ala-1 and Asn-101 were protected from acylation. This protein derivative displayed slightly diminished PS/PC binding. Acetylation in the presence of Mg(II) protected Asn-101 from acylation but did not protect the α-NH_2 of Ala-1. This protein did not bind to PS/PC vesicles. Welsch and Nelsestuen (19) report that His-96 is irreversibly modified by acylation in the presence or absence of Ca(II) ions. Thus this modification has no effect on Ca(II)-dependent binding of the protein to PS/PC vesicles. The reaction of bovine fragment 1 with TNBS was also evaluated in these studies. Surprisingly, the TNP-F-1 exhibited almost normal binding to PS/PC vesicles despite the attachment of a TNP group at the NH_2-group of Ala-1. In order to investigate the role of the amino-terminal region we have examined several modifications at this portion of the fragment 1 molecule.

EXPERIMENTAL PROCEDURES AND RESULTS[2]

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[1] The abbreviations used are: Gla, γ-carboxyglutamic acid; γ-MGlu, γ-methyleneglutamic acid; fragment 1, residues 1–156 of bovine prothrombin; des-[Ala-1,Asn-2]-F-1, residues 3–156 of bovine prothrombin; des-[Ala-1,Asn-2,Lys-3]-F-1, residues 4–156 of bovine prothrombin; *N*-Ac-F-1, N^{α}-acetyl-Ala-1-N^{ϵ}-acetyl-Lys-3,11,44,57,97-*N*(β-amido)-Asn-101-fragment 1; H_2N^{α}-Ala-1-$(N^{\epsilon}$-Ac-Lys$)_5$-F-1, N^{ϵ}-acetyl-Lys-3,11,44,57,97-fragment 1; TNP-Ala-1-$(N^{\epsilon}$-Ac-Lys$)_5$-F-1, N^{α}-trinitrophenyl-Ala-1-N^{ϵ}-acetyl-Lys-3,11,44,57,97-fragment 1; TNP-F-1, N^{α}-trinitrophenyl-Ala-1-N^{ϵ}-trinitrophenyl-Lys-3,11,44,57,97-fragment 1; *N*-Ac-Ala-1-(*N*-Ac-Lys$)_5$-F-1, N^{α}-acetyl-Ala-1-N^{ϵ}-acetyl-Lys-3,11,44,57,97-fragment 1; TNBS, 2,4,6-trinitrobenzenesulfonic acid sodium salt; N^{α}-DNB-Ala-1-$(N^{\epsilon}$-Ac-Lys$)_5$-F-1, *N*-2,4-dinitrobenzoyl-NH$^{\alpha}$-Ala-1-N^{ϵ}-acetyl-Lys-3,11,44,57,97-fragment 1; PTH, phenylthiohydantoin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PS/PC, phosphatidylserine/phosphatidylcholine; HPLC, high-performance liquid chromatography; PL, phospholipid.

[2] Portions of this paper (including "Experimental Procedures," part of "Results," Fig. 1, and Table I) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

$^{45}Ca(II)$ and PS/PC Binding Studies—Equilibrium dialysis studies employed the modified proteins (20 μM) and $^{45}Ca(II)$ at concentrations in the range of 0.056 to 20.0 mM. Scatchard plots of the binding data for the modified proteins are shown in Figs. 2, 3, and 5. The $^{45}Ca(II)$ binding data were analyzed using the SAS NLIN option as previously described (23). The data are tabulated in Table II. Bovine fragment 1 (entry 1, Table II) binds seven Ca(II) ions when measured under these conditions (31). The data are best fit to a model which involves three cooperative and four equivalent noninteracting Ca(II) sites (23).

The Ca(II)-dependent binding of the modified fragment 1 derivatives to small unilamellar phospholipid vesicles (PS/PC, 25:75) was assayed by light-scattering measurements (28).

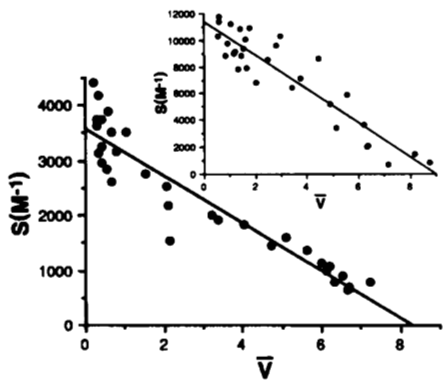

FIG. 2. **Ca(II) ion binding Scatchard plots of *N*-Ac-F-1 and (inset) H_2N-Ala-1-(N^{ϵ}-Ac-Lys)$_5$-F-1.**

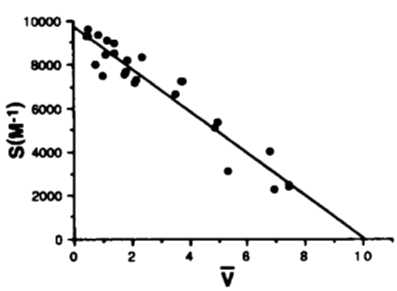

FIG. 3. **Ca(II) ion binding Scatchard plot of TNP-F-1.**

The calculated binding constants (Table II) were obtained from reciprocal plots of $1/\upsilon'$ *vs.* $1/[\text{protein}]_{free}$ (Appendix I). (υ' = moles of protein bound/moles of phospholipid). The phospholipid binding isotherms are shown in Figs. 4 and 6. Values for N (moles of protein bound/mole of phospholipid) are also listed for each modified protein (Table II). Error bars indicate the 95% confidence level as obtained by SAS NLIN option analysis of the reciprocal plots from separate determinations.

Acylation of fragment 1 in the presence of Ca(II) ions (entry 3, Table II, and Fig. 2, *inset*) results in the modification of the NH_2^{ϵ} of the 5 lysine residues. The positive charge of the protein at pH 7.4 is reduced, and the molecule becomes more negatively charged at this pH. The modified protein, H_2N^{α}-Ala-1-(N^{ϵ}Ac-Lys)$_5$-F-1, binds an increased number of Ca(II) ions relative to fragment 1. Positive cooperativity of Ca(II) binding is abolished; however the affinity of the nine equivalent, noninteracting sites is relatively high (k_{site} = 1270 M^{-1}). The modified protein retains the ability to bind to PS/PC vesicles although the K_d value is reduced relative to fragment 1. Acylation of the NH_2^{α} of Ala-1 (entry 4) and the lysine sidechains reduces Ca(II) affinity relative to entry 3 and abolishes positive cooperativity in Ca(II) binding. The modified protein does not bind to PS/PC vesicles. Acetylation of fragment 1 in the absence of metal ions at pH 8.0 (entry 5, Table II, and Fig. 2) possibly removed a Ca(II) site, (relative to entry 3 or 4), the positive cooperativity in Ca(II) binding, the PS/PC binding, and the metal ion promoted quenching of the intrinsic fluorescence. It is probably fair to say that the measured Ca(II) stoichiometry generally has an error of ±1; thus differences in stoichiometry of ±1 are not necessarily significant.

Removal of 2 (entry 6, Table II, and Fig. 5) or 3 (entry 7, Table II) residues from the amino terminus likewise results in a decrease in Ca(II) affinity as evidenced by the reduced k_{site} values and loss of positive cooperativity in Ca(II) binding. However the number of Ca(II) sites is unchanged in the des-[Ala-1,Asn-2,Lys-3] analog (entry 7, Table II) and Ca(II)-dependent PS/PC binding is still observed. The K_d of des-[Ala-1,Asn-2]-F-1 (entry 6) is lower than that of the des-[Ala-1,Asn-2,Lys-3]-F-1 derivative. Thus loss of 2 or 3 residues from the amino terminus decreases Ca(II) affinity but does

TABLE II

Ca(II) and PS/PC binding of bovine prothrombin fragment 1 derivatives

Entry	Protein	υ^a	Ca(II), k_{site}	PS/PC, K_d	n
			$(M^{-1})^b$	(μM)	
1	b-F-1[c]	7	Cooperative	0.4 ± 0.0.1	0.014
2	RCM-F-1[c]	9	7(463), 2(40)	ND[e]	
3	H_2N-Ala-1-(N^{ϵ}-Ac-Lys)$_5$-F-1 (b-F-1 + Ac_2O[d] + Ca)	9	1270	1.1 ± 0.0.2	0.009
4	N^{α}-Ac-Ala-1-(N^{ϵ}-Ac-Lys)$_5$-F-1 (b-F-1 + Ac_2O + Mg)	9	360	ND	
5	*N*-Ac-F-1 (b-F-1 + Ac_2O)	8	460	ND	
6	des-[Ala-1,Asn-2]-F-1	7	330	1.1 ± 0.5	0.011
7	des-[Ala-1,Asn-2,Lys-3]-F-1	9	430	2.2 ± 0.0.4	0.016
8	TNP-F-1 (b-F-1 + TNBS[f])	10	980	0.9 ± 0.0.6	0.017
9	TNP-des-[Ala-1,Asn-2]-F-1 (Entry 6 + TNBS)	9	750	0.5 ± 0.0.2	0.016
10	TNP-des-[Ala-1,Asn-2,Lys-3]-F-1 (Entry 7 + TNBS)	10	610	0.7 ± 0.0.1	0.010
11	TNP-Ala-1-(N^{ϵ}-Ac-Lys)$_5$-F-1 (Entry 3 + TNBS)	8	400	1.1 ± 0.0.6	0.011
12	N^{α}-DNB-Ala-1-(N^{ϵ}-Ac-Lys)$_5$-F-1 (Entry 3 + DNBAHSS)[f]	7	670	2.0 ± 0.0.4	0.010
13	TNP-F-1 + Ac_2O + Ca	8	740	2.1 ± 0.0.3	0.010
14	TNP-F-1 + Ac_2O	7	720	ND	

[a] Errors are ≤±1.0 site.
[b] Errors are ≤20%.
[c] Data reported in Ref. 11.
[d] Ac_2O, acetic anhydride.
[e] ND, none detected (detection limits for this assay are ≤25 μM under the conditions used).
[f] DNBAHSS, 2,4-dinitrobenzoic acid *N*-hydroxysulfosuccinimide ester.

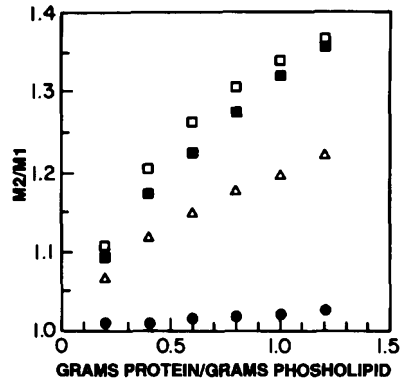

FIG. 4. **Phospholipid binding isotherms for native b-F-1 (□), TNP-F-1 (■), H_2N-Ala-1-(N^{ϵ}-Ac-Lys)$_5$-F-1 (△), *N*-Ac-F-1 (●).**

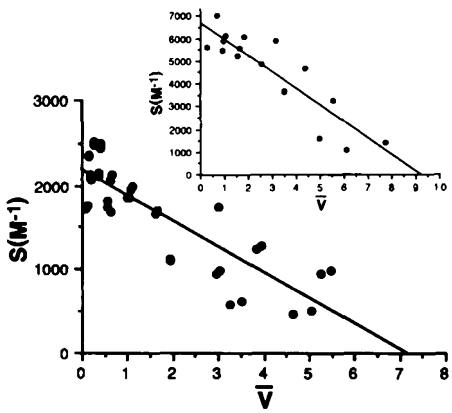

FIG. 5. **Ca(II) ion binding Scatchard plots of des-[Ala-1,Asn-2]-b-F-1 and (*inset*) TNBS-treated des-[Ala-1,Asn-2]-F-1.**

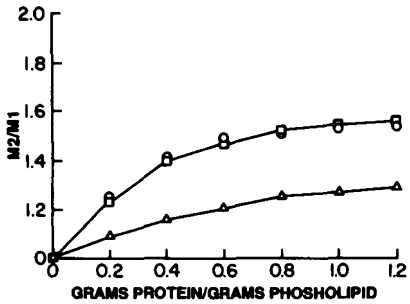

FIG. 6. **Phospholipid binding isotherms for native b-F-1 (□), des-[Ala-1,Asn-2]-F-1 (△), and TNBS-treated des-[Ala-1,Asn-2]-F-1 (○).**

not abolish Ca(II)-dependent PS/PC binding. Acylation of des-[Ala-1,Asn-2]-F-1 with acetic anhydride in the presence of Ca(II) yields a modified protein which in contrast to fragment 1, cannot be sequenced indicating that Lys-3 is fully acetylated.

Treatment of fragment 1 with excess TNBS (pH = 9.9) yields TNP-F-1 (entry 8, Table II, and Fig. 3). The protein exhibits decreased Ca(II) affinity relative to H_2N^{α}-Ala-1-(N^{ϵ}-Ac-Lys)$_5$-F-1 (entry 3) and contains 10 Ca(II) sites which are equivalent and noninteracting. Despite the loss of the electron donor properties of the NH_2^{ϵ}, the TNP-F-1 binds to PS/PC vesicles with approximately the same affinity as entry 3. Similar effects on Ca(II) affinity, Ca(II) stoichiometry, positive cooperativity in Ca(II) binding, and PS/PC affinity were noted when TNP-des-[Ala-1,Asn-2]-F-1 (entry 9, Table II, and Fig. 5, *inset*) and TNP-des-[Ala-1,Asn-2,Lys-3]-F-1 (entry 10) were examined. Both TNP-proteins displayed improved PS/PC binding over the corresponding NH_2^{ϵ}-free analogs. Treatment of entry 3 with TNBS yielded TNP-Ala-1-(N^{ϵ}-Ac-Lys-)$_5$-F-1 (entry 11, Table II). In contrast to entry 3 this protein apparently bound one less Ca(II) ion, at much lower affinity (k_{site} = 400 M^{-1}) although the affinity for PS/PC vesicles remained essentially the same.

In order to determine the effect of acetylation at H_2N^{α}-Ala-1 using a hydrophobic aryl acid we acetylated H_2N-Ala-1-(N^{ϵ}-Ac-Lys)$_5$-F-1 with 2,4-dinitrobenzoic acid *N*-hydroxysulfosuccinimide ester to obtain the *N*-2,4-dinitrobenzoyl derivative (entry 12, Table II). In contrast to N^{α}-Ac-Ala-1-(N^{ϵ}-Ac-Lys)$_5$-F-1 (entry 4), this modified protein, N^{α}-DNB-Ala-1-(N^{ϵ}-Ac-Lys)$_5$-F-1, bound to PS/PC vesicles with a low affinity and exhibited a Ca(II) binding stoichiometry and affinity that were not unlike TNP-Ala-1-(N^{ϵ}-Ac-Lys)$_5$-F-1 (entry 11).

Acylation of TNP-F-1 in the presence of Ca(II) ions was expected to lead to little change in the properties of the product. However, the resulting product (entry 13, Table II) exhibited an apparently reduced Ca(II) stoichiometry and a reduced PS/PC affinity relative to entry 3 or 8. As expected the reaction of TNP-F-1 with acetic anhydride in the absence of Ca(II) (entry 14, Table II) abolished PS/PC binding. The Ca(II) stoichiometry and k_{site} values of entry 5 and 14 were similar. Thus acylation of fragment 1 (entry 5) or the TNP-F-1 derivative (entry 14, Table II) in the absence of Ca(II) ions yields proteins that are quite similar in Ca(II) binding properties to reduced and carboxymethylated fragment (compare entries 5 and 14 to entry 2, Table II).

DISCUSSION

The principal focus of this study was to quantitatively evaluate the effects of chemical modification at the amino groups of bovine fragment 1 on Ca(II) and PS/PC binding. Several conclusions regarding the "Ca conformation" required for Ca(II)-dependent binding of fragment 1 to PS/PC vesicles emerge from this study.

We have previously suggested that the positive cooperativity observed in Ca(II) binding to bovine fragment 1 and other vitamin K-dependent proteins was a prerequisite for the Ca(II)-conformation required for Ca(II)-mediated binding to PS/PC vesicles. We based this argument on: 1) the absence of observed cooperativity in ^{28}Mg(II) binding to fragment 1 (23) and the lack of PS/PC binding by the fragment 1-Mg(II) complex; and 2) on the properties of 7,8,33-γ-MGlu-F-1 (14). The latter studies indicated that the ^{28}Mg(II) binding to the 7,8,33-γ-MGlu-F-1 was identical to ^{28}Mg(II) binding to fragment 1. The metal ion-promoted quenching of the intrinsic fluorescence was retained by the 7,8,33-Gla-modified protein; however this protein had lost positive cooperativity in Ca(II) binding, exhibited reduced Ca(II) affinity and, importantly, did not bind to PS/PC vesicles. The acylation studies of Welsch and Nelsestuen (15–19) that lead to H_2N^{α}-Ala-1-(N^{ϵ}-Ac-Lys)$_5$-F-1 upon acylation in the presence of Ca(II) ions and N^{α}-Ac-Ala-1-(N^{ϵ}-Ac-Lys)$_5$-F-1 in the presence of Mg(II) ions also support the idea of different conformational populations induced by the interaction of different ions (calcium and magnesium with different charge densities, coordination states, and ligand preferences) with fragment 1. Nevertheless, it is clear from the data reported in Table II that positive cooperativity in Ca(II) binding to the various fragment 1 derivatives is not a precondition reflective of, or required for, PS/PC binding. Furthermore, the Ca(II) affinity of the particular protein does not appear to be a good predictor of PS/PC binding. For example, des-[Ala-1,Asn-2]-F-1 (entry 6, Table II) exhibits a k_{site} value of 330 M^{-1} which is quite similar

to that of N^{α}-Ac-Ala-1-(N^{ϵ}-Ac-Lys)$_5$-F-1 (entry 4, Table II). However, the former protein binds to PS/PC vesicles, whereas the latter does not. Thus the ability of fragment 1 to exhibit Ca(II)-mediated phospholipid binding must depend on conformational features that are mediated by the Ca(II) binding process.

Positive cooperativity in Ca(II) binding to bovine fragment 1 involves three of the seven Ca(II) sites (23). The positive cooperativity is abolished when the amino-terminal region of the protein is modified. For example, positive cooperativity of Ca(II) binding is abolished by: removal of 2 or 3 residues from the amino terminus; modification of Gla residues 7 and 8; introduction of a N^{α}-TNP or N^{α}-acyl group at the amino terminus; or acetylation of the N^{ϵ}-amino groups of the 5 Lys residues. Since positive cooperativity is not observed in Mg(II) binding to the protein, the phenomena must involve some property of Ca(II) ions as well as some property of the ligand (fragment 1).

Hodgson *et al.* (32, 33) have suggested that the major reason for the presence of Gla rather than Glu in the family of Ca(II) binding coagulation proteins is the availability in the former of the additional carboxylate group. Hodgson *et al.* note that the additional carboxylate permits polymeric arrays of Ca(II) ions in the crystallographic structures of malonate complexes with Ca(II) or Ba(II). Malonate complexes with smaller metal ions such as Mg(II) or Be(II) are invariably monomeric. Polymeric arrays were also observed by Zell *et al.* (37) in their Ca(II) α-ethylmalonate crystal study.

One might expect to observe positive cooperativity in the formation of a polymeric system involving two or more Ca(II) ions that interact through a network of carboxylate ligands. The positive cooperativity observed with Ca(II) binding to fragment 1 might reflect the formation of such a polymeric matrix involving three Ca(II) ions. Each Ca(II) ion would be bound to two or more Gla carboxylate groups in hepta- or octacoordinate arrays as observed by Hodgson *et al.* (32, 33). Each Ca(II) ion would interact with the negative ligands (*i.e.* Gla carboxylates) positioned by the previous Ca(II) ion leading to the observed positive cooperativity associated with Ca(II) binding by the native protein.

The negatively charged Gla carboxylates in the protein can be stabilized by Ca(II) ions and/or by salt bridges with available amino groups. Chemical modification of the amino groups will affect salt bridge formation and might also affect interactions between the bound Ca(II) ions. Thus, acetylation of fragment 1 in the presence of Ca(II) ions might be expected to yield a modified protein (entry 3, Table II) which retained the essential Ca(II)-promoted interactions between Ca(II) ions, amino groups, and the Gla carboxylate groups. Viewed in this way, the α-NH_2 group of Ala-1 must be an integral feature of the polymeric Ca(II)-Gla domain matrix. The involvement of the α-NH_2 group of Ala-1 in the positive cooperativity of the Ca(II) binding process is evident from a comparison of fragment 1 (entry 1, Table II) with either des-[Ala-1,Asn-2]-F-1 (entry 6, Table II) or des-[Ala-1,Asn-2,Lys-3]-F-1 (entry 7, Table II). Removal of Ala-1 abolishes the cooperativity of Ca(II) binding. The involvement of the ϵ-NH_2 groups of Lys residues in the Ca(II) binding process is seen by comparison of the Ca(II) binding stoichiometry of the N^{ϵ}-acetyl- or N^{ϵ}-TNP fragment 1 derivatives. Modification of the ϵ-NH_2 groups leads to an increase in Ca(II) ions bound by Gla carboxylate groups as the Gla-ϵ-NH_2 salt bridges are abolished. Cooperativity in Ca(II) binding is lost in all cases suggesting that Gla-Lys salt bridges are involved in this event as well.

Church *et al.* (34, 35) have described a murine monoclonal antibody, H-11, which binds to a conserved epitope involving Phe-5 in the bovine prothrombin sequence. Binding of H-11 is inhibited by Ca(II), Mg(II), and Mn(II) ions. Church *et al.* (34, 35) conclude that Phe-5 is buried and thus inert to H-11 upon divalent metal ion binding to bovine fragment 1. Thus the amino-terminal 1–10 sequence must adopt an ordered conformation as a result of divalent ion binding. This proposal is supported by the present studies which indicate that subtle changes in the 1–10 region such as modification of Ala-1, acetylation of des-[Ala-1,Asn-2]-F-1 or des-[Ala-1,Asn-2,Lys-3]-F-1, and conversion of Gla-7,8 to γ-methyleneglutamyl residues can abolish or modify PS/PC binding by the protein.

We wished to demonstrate that the PS/PC binding exhibited by the TNP derivatives of the various fragment 1 derivatives was in fact a Ca(II)-dependent process. Two control experiments indicate the Ca(II) requirement. In the first experiment EDTA was added to the Ca(II)·TNP-F-1·PS/PC binding complex. The complex immediately dissociated. The second control utilized the modified protein, 10-γ-MGlu-F-1, (14) in which all Gla residues are modified. This protein does not bind to PS/PC vesicles in the presence of Ca(II) ions. Treatment of this protein with excess TNBS, isolation of the TNP-10-γ-MGlu-F-1, and incubation with Ca(II) and PS/PC vesicles gave no indication of protein-phospholipid binding. Thus the binding observed by incorporation of a TNP residue at Ala-1 or Lys-3 or Gly-4 in the appropriate fragment 1 derivative must reflect the importance of the Ca(II):Gla interactions in establishing the phospholipid binding conformation. The effect of a dinitro- or trinitrophenyl substituent at the amino terminus on PS/PC binding also suggests the presence of an ordered conformation in the 1–10 region of the protein. Recent studies by Schwalbe *et al.* (36) indicate that Ca(II) but not Mg(II) protected the amino terminus of the amino-terminal peptides from human II (residues 1–41 and 1–44, 60:40, v/v), bovine X (residues 1–44), and bovine IX (residues 1–42). The introduction of a TNP group at the amino-terminal residue of these peptides also promoted Ca(II)-dependent binding of the peptides to PS/PC vesicles.

Acylation of fragment 1 in the presence of Mg(II) or Ca(II) protected the β-amido nitrogen atom of Asn-101 from imide formation. Acylation in the absence of metal ions leads to the production of the Asn-101 imide and perhaps modification at other sites. Acylation under these conditions results in the loss of the intrinsic fluorescence transition and PS/PC binding. Evidence suggesting a role for a non-Gla domain metal ion binding site in this region of the kringle domain is presented in the accompanying manuscript (30).

APPENDIX

Derivation of a Model of a Single Class of Noninteracting Sites for Protein Binding to Phospholipid Vesicles

1. $$\upsilon' = \frac{\text{protein molecules bound}}{\text{phospholipid molecules}}.$$

2. Protein + PL = protein:PL.

$$K_a = \frac{[\text{protein:PL}]}{[\text{protein}][\text{PL}]}$$

3. The fraction of sites on the phospholipid bound = h.

a. $$h = \frac{[\text{protein:PL}]}{[\text{protein:PL}] + [\text{PL}]}$$

b. $$h = \frac{K_a[\text{PL}][\text{protein}]}{[\text{PL}] + K_a[\text{PL}][\text{protein}]}$$

c. $$h = \frac{K_a[\text{protein}]}{1 + K_a[\text{protein}]}$$

4. $hN = v'$, where N is the moles of protein bound/mol of PL at saturation.

5. $$h = \frac{v'}{N} = \frac{K_a \text{ protein}}{1 + K_a[\text{protein}]}$$

   or

   $$\frac{N}{v'} = \frac{1}{K_a[\text{protein}]} + 1.$$

   Therefore,

   $$\frac{1}{v'} = \frac{1}{NK_a[\text{protein}]} + \frac{1}{N}$$

6. Plot of $1/v'$ *versus* 1/[protein] gives N and K_a. For a typical strong binder $1/N = 71.42$; $K_a = 2.5 \times 10^6\ \text{M}^{-1}$; $K_a = 0.4\ \mu\text{M}$.

REFERENCES

1. Suttie, J. W. (1980) *Fed. Proc.* **39,** 2730–2735
2. Suttie, J. W. (1980) *CRC Crit. Rev. Biochem.* **8,** 191–223
3. Furie, B. C., Borowski, M., Keyt, M., and Furie, B. (1984) *Calcium and Cell Function* (Cheung, W. Y., ed) Vol. II, Academic Press, New York
4. Nelsestuen, G. L. (1984) *Metal Ions in Biological Systems* (Sigel, H., ed) Vol. 17, pp. 353–381, Marcel Dekker, New York
5. Suttie, J. W., and Jackson, C. M. (1977) *Physiol. Rev.* **57,** 1–70
6. Esmon, C. T. (1987) *Science* **235,** 1348–1352
7. Nesheim, M. E., Tracy, R. P., and Mann, K. G. (1984) *J. Biol. Chem.* **259,** 1447–1453
8. Papahadjopoulos, D., and Hanahan, D. J. (1964) *Biochim. Biophys. Acta* **63,** 436–439
9. Walker, F. (1986) *Biochemistry* **25,** 6305–6311
10. Walker, F. J. (1981) *J. Biol. Chem.* **256,** 11128–11131
11. Pollock, J. S., Zapata, G. A., Weber, D. J., Berkowitz, P., Deerfield, D. W., II, Olson, D. L., Koehler, K. A., Pedersen, L. G., and Hiskey, R. G. (1988) in *Current Advances in Vitamin K Research A Steenbock Symposium* (Suttie, J. W., ed) pp. 325–334, Elsevier Science Pub., New York
12. Pollock, J. S., Shepard, A. J., Weber, D. J., Olson, D. L., Klapper, D. G., Pedersen, L. G., and Hiskey, R. G. (1988) *J. Biol. Chem.* **263,** 14216–14223
13. Weber, D. J., Pollock, J. S., Pedersen, L. G., and Hiskey, R. G. (1988) *Biochem. Biophys. Res. Commun.* **155,** 230–235
14. Zapata, G. A., Berkowitz, P., Noyes, C. M., Pollock, J. S., Deerfield, D. W., II, Pedersen, L. G., and Hiskey, R. G. (1988) *J. Biol. Chem.* **263,** 8150–8156
15. Welsch, D. J., and Nelsestuen, G. L. (1988) in *Current Advances in Vitamin K Research* (Suttie, J. W., ed) pp. 371–378, Seventeenth Steenbock Symposium, Elsevier Press, New York
16. Welsch, D. J., Pletcher, C. H., and Nelsestuen, G. L. (1988) *Biochemistry* **27,** 4933–4938
17. Welsch, D. J., and Nelsestuen, G. L. (1988) *Biochemistry* **27,** 4939–4945
18. Welsch, D. J., and Nelsestuen, G. L. (1988) *Biochemistry* **27,** 4946–4952
19. Welsch, D. J., and Nelsestuen, G. L. (1988) *Biochemistry* **27,** 7513–7519
20. Mann, K. G. (1977) *Methods Enzyml.* **45,** 123–156
21. Staros, J. V. (1982) *Biochemistry* **21,** 3950–3955
22. Fields, R. (1972) *Methods Enzymol.* **38,** 464–469
23. Deerfield, D. W., II, Olson, D. L., Berkowitz, P., Byrd, P. A., Koehler, K. A., Pedersen, L. G., and Hiskey, R. G. (1987) *J. Biol. Chem.* **262,** 4017–4023
24. Scatchard, G. (1949) *Ann. N. Y. Acad. Sci.* **51,** 660–672
25. Barenholz, Y., Gibbes, D., Litman, B. J., Goll, J., Thompson, T. E., and Carlson, F. D. (1977) *Biochemistry* **16,** 2806–2810
26. Grant, R. T. (1959) *J. Biol. Chem.* **234,** 466–468
27. Huang, C. (1969) *Biochemistry* **8,** 344–352
28. Nelsestuen, G. L., and Lim T. K. (1977) *Biochemistry* **16,** 4164–4171
29. Mycek, M. K. (1970) *Methods Enzymol.* **28,** 285–315
30. Berkowitz, P., Huh, N.-W., Brostrom, K. E., Panek, M. G., Weber, D. J., Tulinsky, A., Pedersen, L. G., and Hiskey, R. G. (1992) *J. Biol. Chem.* **266,** 4570–4576
31. Deerfield, D. W., II, Berkowitz, P., Olson, D. L., Wells, S., Hoke, R. A., Koehler, K. A., Pedersen, L. G., and Hiskey, R. G. (1986) *J. Biol. Chem.* **261,** 4833–4839
32. Yokomori, Y., Flaherty, K. A., and Hodgson, D. J. (1988) *Inorg. Chem.* **27,** 2300–2306
33. Hodgson, D. J., and Asplund, R. O. (1990) *Inorg. Chem.* **29,** 3612–3615
34. Church, W. R., Messier, T., Howard, P. R., Amiral, J., Meyer, D., and Mann, K. G. (1988) *J. Biol. Chem.* **263,** 6259–6267
35. Church, W. R., Boulanger, L. L., Messier, T., and Mann, K. G. (1989) *J. Biol. Chem.* **264,** 17882–17887
36. Schwalbe, R. A., Ryan, J., Stern, D. M., Kisiel, W., Dahlback, B., and Nelsestuen, G. L. (1989) *J. Biol. Chem.* **264,** 20288–20296
37. Zell, A., Einspahr, H., and Bugg, C. E. (1985) *Biochemistry* **24,** 533–537

Supplemental Material To:
Modifications of Bovine Prothrombin Fragment 1 in the Presence and Absence of Ca(II) Ions: Loss of Positive Cooperativity in Ca(II) Ion Binding for the Modified Proteins

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Experimental Procedures

H$_2$N$^{\alpha}$Ala-1(N$^{\varepsilon}$-Ac-Lys)$_4$-F-1. Fragment 1 preparations (20) that exhibited 45-50% fluorescence quenching in the presence of 10 mM Ca(II) were employed for the acetylation studies. Acylation of fragment 1 was conducted using modified conditions from those reported by Welsch et al. (15,16). Fragment 1 (2 mg/ml) was incubated in a buffer containing 0.02 M Tris-HCl, pH 8.0, 0.02% sodium azide, 0.1 M NaCl with 75 mM Ca(II) for 30 min. Acetic anhydride was added to the cold protein solution (0°) at two successive 15 min intervals to provide a final 500 molar excess of reagent. After each addition of acetic anhydride the pH was maintained at 8.0 (pH-stat) by continuous addition of 1N NaOH. After 30 min the protein was dialyzed against 0.02 M Tris-HCl, pH 7.4, 0.02% sodium azide, 0.1 M NaCl for 24 h at 4°C. The homogeneity of the modified protein was established using 11% SDS-PAGE gels and 6% native PAGE gels and by amino acid sequence analyses (Table I).

DNB-NH$^{\alpha}$Ala-1(N$^{\varepsilon}$-Ac-Lys)$_4$-F-1. The N-hydroxysulfosuccinimide ester of 2,4-dinitrobenzoic acid was prepared as described by Staros (21). The crude active ester was used in the protein modification reaction without further purification. A solution of NH$_2^{\alpha}$-Ala-1-(N$^{\varepsilon}$-Ac-Lys)$_4$-F-1 (1 mg/ml) in 0.02M HEPES-HCl (0.1 M NaCl, 0.02% sodium azide, pH = 7.8) was incubated with 75 mM Mg(II) and cooled to 0°C. The cold solution was treated with a 200 molar excess of the N-hydroxysulfosuccinimide ester of 2,4-dinitrobenzoic acid for 30 min. The active ester was added in two aliquots and the pH was adjusted to pH = 7.8 using 1M NaOH before the second addition. The protein was dialyzed several times against 0.02 M Tris-HCl (0.1 M NaCl, 0.02% sodium azide, pH = 7.4) containing 0.001 M EDTA in the first dialysis.

The protein could not be sequenced and exhibited an enhanced UV absorption maxima at 270 nm compared to the starting protein. The preparation was homogeneous in the 11% SDS PAGE gel system and on 6% PAGE gels.

Des-[Ala-1,Asn-2]-fragment 1. Des-[Ala-1,Asn-2]-fragment 1 was prepared by incubation of 4 mg of bovine fragment 1 (2mg/ml) with 0.46 units of dipeptidyl-peptidase 1 (Cathepsin C, Sigma) to give 2.7 units enzyme/μmole fragment 1. The digestion was performed at 25°C for 24 hrs in buffer (0.05 M sodium acecate pH 5.0).

The enzyme was separated from des-[Ala-1,Asn-2]-fragment 1 by gel permeation chromatography of the reaction mixture using a Sephadex G 75 column (35x1.5 cm) (data not shown). The bovine des-[Ala-1,Asn-2]-fragment 1 was determined to be homogeneous on an 11% SDS gel. Amino acid sequencing gave the expected phenylthiohydantoin (PTH) amino acid derivatives (Table I).

Des-[Ala-1,Asn-2,Lys-3]-fragment 1. Des-[Ala-1,Asn-2,Lys-3]-fragment 1 was prepared by incubation of 62 mg of bovine fragment 1 (2mg/ml) with 3.1 units of plasmin (Sigma) (1.2 units enzyme/mole fragment 1). The digestion was performed in 0.02 M Tris-HCl (0.1 M, NaCl, 0.02% NaN_3, pH = 7.4) buffer for 2 hr. The protein was dialyzed against the same buffer overnight at 4°C. The bovine des-[Ala-1,Asn-2,Lys-3]-fragment 1 was determined to be homogeneous by 11% SDS PAGE and by amino acid sequencing (Table I).

TNBS Modifications

Using conditions modified from those reported by Fields [22], solutions of bovine fragment 1 or the modified bovine fragment 1 proteins, (2-3 mg/ml) previously dialyzed vs distilled deionized water, were diluted with one volume of borate buffer (0.1 M $Na_2B_4O_7$, M NaOH, pH = 9.9). Immediately, an aqueous TNBS solution was added as either a 600 molar excess of a solution prepared by dissolving TNBS sodium salt in water (50% by weight) or as a 300 molar excess of 5% by weight TNBS aqueous solution (Sigma). The reddish orange solution was incubated at 25°C for 0.5 h. The excess reagent was removed by exhaustive dialysis against 0.02 M Tris HCl buffer (0.1 M NaCl, 0.02% sodium azide, pH = 7.4). The TNBS created protein was determined to be homogeneous on 11% SDS-PAGE.

Sequence Analysis

Automatic Edman degradation was performed using an Applied Biosystems Sequenator Model 475SA. The phenylthiohydantoins were identified by reverse-phase HPLC by comparison to standards at each cycle.

Equilibrium Dialysis Calcium Binding Studies

The procedure for the metal ion equilibrium dialysis experiment has been previously described [23]. All the experiments were performed at 25°C. The protein concentration was generally 20 μM in a Tris-HCl buffer, pH = 7.4, and 0.1 M NaCl. For N^{α}-DNB-Ala-1-(N^{ϵ}-Ac-Lys)$_4$-F-1 and N^{α}-Ac-Ala-1-(N^{ϵ}-Ac-Lys)$_4$-F-1 protein concentrations of 40 μM were used for the equilibrium dialysis measurements. The higher protein concentrations gave more accurate and reproducible results. Ca(II) concentrations in the range of 0.056-20 mM were employed. In each case saturation of protein with Ca(II) was confirmed by the leveling of the Ca(II) bound points of the Ca(II) binding isotherms (data not shown). In all cases the data were analyzed using a Scatchard analysis ($\bar{S}$ vs. $\bar{S}$; $\bar{S}$ = $\bar{\nu}$/[metal free], $\bar{\nu}$ = [metal bound]/[protein total] assuming a non-sequential loading of the ligands [24]. The error bars reported represent with 95% confidence the sum of standard deviations from several experiments with different protein preparations. The data were fit to a linear model with one class of non-interacting binding sites utilizing SAS NLIN option (Cary, N.C.) on an IBM 3090 computer.

Phospholipid Binding Assay

Small unilamellar phospholipid vesicles were prepared by the method of Barenholz et al. [25]. Phosphatidylserine (bovine brain) and synthetic 1-palmitoyl-2-oleoyl lecithin (Avanti Polar Lipids) were used at 25/75 molar ratio, respectively. Phospholipid content of the vesicle preparation was determined by the inorganic phosphate assay of Grant [26]. The vesicle size was characterized by the method of Huang [27].

Relative light scattering measurements were performed as described by Nelsestuen and Lim (28) with excitation and emission wavelengths both set at 320 nM and with bandpasses of 2 nM. Ca(II) concentrations were generally 10 mM for all the assays; for TNP-NH^{α}-Ala-1-(N^{ϵ}Ac-Lys)$_4$-F-1, N^{α}-DNB-Ala-1-(N^{ϵ}-Ac-Lys)$_4$-F-1 and N^{α}-Ac-Ala-1-(N^{ϵ}-Ac-Lys)$_4$-F-1 Ca(II) concentrations of 20 mM were used. Small unilamellar phospholipid vesicle (PS/PC 25:75) concentrations were determined by the inorganic phosphate assay [26]. Binding isotherms plot M_2/M_1 (where M_1, the initial molecular weight of the vesicles is set equal to one, and M_2 is the relative molecular weight of the vesicle-protein complex) vs. the ratio of protein to phospholipid weight. The calculated binding constants were obtained utilizing reciprocal plots assuming a model in which the protein binds to non-interacting sites on the phospholipid bilayer (Appendix I). The data were best fit to this model utilizing SAS NLIN option (Cary, N.C.) on an IBM 3090 computer. The error bars reported (95% confidence) are derived from standard deviations from several experiments with different protein preparations.

Table I
Amino Acid Sequence of Modified Bovine Fragment 1 Derivatives

Protein	PTH Derivative Identified in Cycle 1	Cycle 2	Cycle 3
bovine Fragment (bF1) (A.N.K.G.F...)	A	N	K
bF1 + Ac_2O[a]	blocked		
bF1 + Ca(II) + Ac_2O	A	N	AcK[b]
bF1 + TNBS[c]	blocked		
Des[Ala-1,Asn-1]F1	K	G	F
Des[Ala-1,Asn-2,]F1 + Ca(II) + Ac_2O	blocked		
Des[Ala-1,Asn-2,Lys-3]F1	G	F	L

[a]Acetic Anhydride (500-fold molar excess, pH = 8.0)

[b]N^{ϵ}-Acetyllysine

[c]2,4,6-Trinitrobenzene sulfonate

High Performance Liquid Chromatography

A Phenomenex C18 W porex (250 x 4.6 mm) column was used to separate peptide mixtures. Chromatography was performed on a Waters/ISCO chromatograph equipped with a ISCO V4 variable wavelength detector and a peak separator. Peptides were suspended in 1 ml of 0.1% TFA in water (mobile phase A) and were eluted with a 30 min. gradient from 0% to 100% of mobile phase B (0.1% TFA in acetonitrile) at a flow rate of 1.0 ml/min. The detector wavelength was set at 215 nm.

Results

Characterization of Modified Bovine Fragment 1 Derivatives. Acylation of Fragment 1 in the Presence and Absence of Ca(II). The acylation of fragment 1 in the presence of 75 mM Ca(II) using a 500-fold molar excess of acetic anhydride at pH = 8.0 was examined in some detail. Welsch et al. reported the structure of the acylation product as N^{ϵ}-acetyl-Lys-3,11,44,57,97-fragment 1 although verification of the acylation of Lys-57 was presumptive. We verified the protection of NH_2^{α} of Ala-1 and the acylation of the NH_2^{ϵ} of Lys-3,11 and 44 by the amino acid sequencing profile of the first 44 residues of NH_2^{α}-Ala-1-(N^{ϵ}-Ac-Lys)$_4$-F-1. The PTH derivatives of N^{ϵ}-acetyl lysine appeared at the expected positions and no PTH derivatives of Lys were observed.

In our initial studies the possibility of incomplete acylation of the lysine residues was suggested when TNBS treatment of a H_2N^{α}-Ala-1-(N^{ϵ}-Ac-Lys)$_4$-F-1 preparation yielded a TNP derivative with an ultraviolet absorbance at 340 nm larger than that expected for the incorporation of a single TNP-group at Ala-1. A limited α-chymottrypsin digest of this preparation yielded four peptides (the 1-42 and 1-45 Gla peptides and the 43-156 and 46-156 kringle peptides). The UV spectra of the latter pair indicated a TNP-group had been incorporated in the kringle region. In order to locate the TNP group the 43-156/46-156 mixture was subjected to trypsin digestion; two peptides exhibiting 340 nm adsorption were collected (HPLC) and sequenced.

Both peptides corresponded to tryptic cleavage at Arg-55. The sequencing profile exhibited a PTH derivative derived from N^{ϵ}-acetyllysine-57 and N^{ϵ}-TNP-Lys-57. Based on the UV absorbance of the kringle peptides at 340 nm and using ε TNP for N^{ϵ}-TNP-Lys as 1.18×10^4 M^{-1} cm^{-1}, the amount of N^{ϵ}-TNP-Lys-57 was estimated as 30%. Based on the quantity of N^{ϵ}-Ac-Lys-57 produced in the sequence analysis of the 55-77 tryptic peptide the amount of N^{ϵ}-TNP-Lys-57 was 25%. In subsequent acylation experiments a somewhat longer reaction time was employed to minimize the amount of unreacted Lys-57. This residue reacts with acetic anhydride at a considerably slower rate than the other lysine residues in the fragment 1 molecule. With longer reaction times the amount of unreacted Lys-57 was less than 10%.

The absence of a TNP group at Lys-97 was confirmed by isolation and sequence analysis of a tryptic fragment corresponding to residues 93-111. In this peptide only the PTH derivative of N^{ϵ}-Ac-Lys-97 was obtained upon sequence analysis. As noted by Welsch and Nelsestuen [19] His-96 is acylated and the imidazole side chain is degraded even under these relatively mild conditions. The PTH derivative of His-96 was not obtained when the 93-111 peptide was sequenced.

Fragment 1 acetylated in the absence of Ca(II) provided a protein derivative, N-acetyl-F-1, with properties similar to those reported by Welsch et al. [15-17]. The TNBS assay of this derivative indicated no appreciable absorbance at either 340 or 420 nm suggesting that all primary amine groups were acetylated. The NH_2^{α} of Ala-1 was blocked to sequence analysis indicating that Ala-1 was completely acetylated.

Acetylation of Fragment 1 in the Presence of Mg(II). Fragment 1 acylated in the presence of 75 mM Mg(II) provided N^{α}-Ac-Ala-1-(N^{ϵ}-Ac-Lys)$_4$-F-1. Sequence analysis indicated that the NH_2^{α} of Ala-1 was completely acetylated. The TNBS assay suggested that all of the NH_2^{α}-amino groups were also acetylated.

Des-[Ala-1,Asn-2]-F-1. The conversion of fragment 1 to des-[Ala-1,Asn-2]-F-1 is based on the observation that dipeptidylaminopeptidase I (Cathepsin C) will not hydrolyze a peptide containing an N-terminal lysyl residue [29]. After hydrolysis of Ala-1 and Asn-2 the N-terminal lysyl residue of des-[Ala-1,Ala-2]-F-1 blocks further hydrolytic action of the enzyme. The modified fragment 1 preparation obtained from the Cathepsin C digest was determined to be homogeneous by SDS-PAGE gels and amino acid sequencing (Table I). On addition of Ca(II) ions, bovine fragment 1 is known to exhibit a biphasic fluorescence quenching curve (T_1^{Ca} = 0.34±0.09mM) [11]. The bovine des-[Ala-1, Asn-2]-F-1 exhibited similar behavior (T_1^{Ca} = 1.04±08 mM) upon making the solution 10 mM in Ca(II) (data not shown).

Treatment of des-[Ala-1,Asn-2]-F-1 with TNBS at pH 9.9 provided the corresponding TNP-derivative. The N^{α}-TNP-des-[Ala-1,Asn-2]-F-1 could not be sequenced indicating that the N-terminal residue Lys-3 was fully trinitrophenylated. Acylation of des[Ala-1,Asn-2]-F-1 in the presence of 75 mM Ca(II) provided an N-acetyl derivative that could not be sequenced. In contrast to N^{α}-Ac-Ala-1-(N^{ϵ}-Ac-Lys)$_4$-F-1 the N^{α}-Ac-des[Ala-1,Asn-2]-(N^{ϵ}-Ac-Lys)$_4$-F-1 exhibited a very low but discernible level of Ca(II)-dependent binding to PS/PC vesicles. Acylation of des-[Ala-1,Asn-2]-F-1 in the absence of Ca(II) ions provided a product that could not be sequenced and did not bind to PS/PC vesicles.

Des-[Ala-1,Asn-2,Lys-3]-F-1. Treatment of bovine fragment 1 with plasmin for limited periods of time (<2h) provided des-[Ala-1,Asn-2,Lys-3]-F-1. After removal of plasmin on a DEAE Sephacel column the protein migrated as a single band on SDS-PAGE gels and exhibited a single N-terminal Gly-4 residue upon sequence analysis (Table I). The des-[Ala-1,Asn-2,Lys-3]-F-1 exhibited biphasic intrinsic fluorescence quenching that was virtually identical to the des-[Ala-1,Asn-2]-F-1 (data not shown). Treatment of the des-[Ala-1,Asn-2,Lys-3]-F-1 with TNBS provided the corresponding TNP-derivative. Acylation of des-[Ala-1,Asn-2,Lys-3]-F-1 with acetic anhydride in the presence of 75 mM Ca(II) afforded a protein that could not be sequenced (Table I).

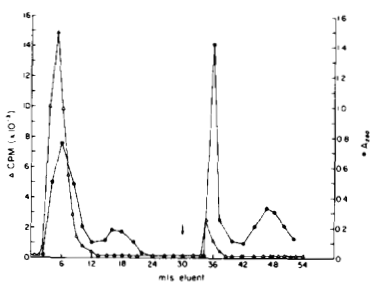

Fig. 1. DEAE - fractogel elution profile monitored by (Δ) cpm and (●) A_{280} of a (1:100) chymotrypsin digest of TNP-b-F1 created with 14-C labeled acetic anhydride in the absence of Ca(II) ions. At the arrow a 0.1 - 0.5 M salt gradient was applied.

14-C-Acetic Anhydride Labeling of TNP-F-1. In order to examine the question of whether sidechain functional groups other than those at Ala-1, Lys-3,11,44,57,97, His-96 and Asn-101 reacted with acetic anhydride in the absence of Ca(II) we allowed TNP-F-1 to react with 14-C labeled acetic anhydride (500-fold molar excess) in the absence of Ca(II). After dialysis the resulting protein was subjected to a 100:1 chymotrypsin digest and the cleavage products (the 1-42 and 1-45 peptides plus the corresponding kringle peptides) were separated on DEAE-fractogel (Fig. 1) as previously described (11,12). The fractions in the wash and gradient peaks were pooled separately.

The wash peak which contained peptides resulting from digestion of the 43-156 and 46-156 peptides accounted for 91% of the incorporated radioactivity (Fig. 1). Separation of the peptides by reverse phase HPLC indicated that one of the four major peaks identified by A_{280} absorbance contained the radioactivity present in the wash peak. Sequence analysis of this radioactive fragment indicated the peptide resulted from chymotrypsin cleavage of the Trp-90/Arg-91 and Phe-114/Cys-115 bonds. The expected sequence from residue 91 to residue 114 was obtained with the exception that PTH derivative for His-96 was lower than expected and Asn-101 was not present. No 14-C counts were present in any cycles. We conclude in agreement with Welsch et al. (18) that His-96 is irreversibly modified and Asn-101 contains the unusual imide formed by 14-C acetic anhydride acylation of the β-amido sidechain of that residue. The 14-C counts thus remain with the Asn-101 linked carbohydrate associated with this residue. More definitive data on this point is provided in the accompanying manuscript [30].

The gradient peak from the limited chymotryptic digest (9% of 14-C counts) eluted from DEAE-fractogel somewhat slower than native 1-42 because of the decrease positive charge resulting from the TNBS modification of the amino groups of the two lysine residues and the amino terminal alanine (Fig. 1). The peptide precipitated in the presence of 50 mM Ca(II) as previously described for the 1-42 peptide [11,12]. The peptide moved on a 13.5% native PAGE gel as one band. Thus we conclude that the gradient peak represented the TNP labeled 1-42 peptide. The TNP labeled peptide was further purified by HPLC; no detectable 14-C counts remained in the purified 1-42 peptide. We conclude that the small amount of radioactivity that eluted with the 1-42 fraction was not covalently incorporated in the 1-42 peptide. Further support of this conclusion was provided by a autoradiograph of the mixture of 14-C labeled peptides obtained from the chymotryptic digest. Using a autoradiograph enhanced with PPO/DMS (20:80) > 99% of the 14-C counts were localized in the kringle peptide. No radioactive counts could be detected in the 1-42/1-45 peptides after a 15 day exposure.