Amino-Terminal Alanine Functions in a Calcium-Specific Process Essential for Membrane Binding by Prothrombin Fragment 1†

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Received January 11, 1988; Revised Manuscript Received March 3, 1988

ABSTRACT: Two acetylation sites on prothrombin fragment 1 (amino-terminal 156 amino acid residues of bovine prothrombin) are essential for the tight calcium and membrane binding functions of the protein; calcium protects both of these sites from acetylation [Welsch, D. J., Pletcher, C. H., & Nelsestuen, G. L. (1988) *Biochemistry* (first of three papers in this issue)]. The ε-amino groups of the lysine residues (positions 3, 11, 44, 57, and 97) were not critical to protein function and were acetylated in the calcium-protected protein. The most reactive of the two essential acetylation sites was identified as amino-terminal alanine. To identify this site, fragment 1 was first acetylated in the presence of calcium to derivatize the nonessential sites. Removal of calcium and partial acetylation with radioactive reagent produced a single major radioactive peptide. Isolation and characterization of this peptide showed that the radioactivity was associated with amino-terminal alanine. In addition, sequence analysis of calcium-protected protein showed the presence of underivatized amino-terminal alanine. Surprisingly, covalent modification with a trinitrophenyl group did not alter membrane binding activity. Thus, the positive charge on the amino terminus did not appear critical to its function. Acetylation of amino-terminal alanine without acetylation of the second essential site produced a fragment 1 derivative which had a high requirement for calcium and which had lost most membrane binding function. However, this protein had only slightly altered affinity for magnesium ion. In agreement with this metal ion selectivity, protection of amino-terminal alanine was calcium specific, and magnesium ion did not protect this site from acetylation. Magnesium did protect the second essential acetylation site on the protein. Removal of the three amino-terminal amino acids from fragment 1 by limited plasmin digestion produced a protein with a new amino terminus (Gly-4). This derivative behaved as the protein with an acetylated amino terminus and displayed low calcium binding affinity and loss of membrane binding. These results showed that amino-terminal alanine functioned in a calcium-specific event that was essential to membrane binding.

Previous studies have shown that metal ions function in at least two roles in prothrombin–membrane binding (Nelsestuen, 1976; Nelsestuen et al., 1976). Although the details of how calcium mediates membrane binding are not well understood, some general proposals and properties have been identified. The two metal ion dependent processes can be distinguished by ion specificity. One process is very nonspecific for metals ions and causes major protein fluorescence quenching (Nelsestuen, 1976; Prendergast & Mann, 1977) and minor changes in protein CD spectra (Bloom & Mann, 1978). The second process is metal ion specific and is required for protein–membrane binding (Nelsestuen et al., 1976). The exact nature of this second process has been the basis of much interest.

An early proposal was that the calcium-specific event was related to sites at the protein–membrane interface (Nelsestuen et al., 1976). However, more recent observations are consistent with a calcium-specific protein process that is not detected by the spectroscopic methods. While many metal ions have been studied, the contrast between magnesium and calcium typifies the observations. Magnesium ion, which induces the fluorescence quenching event but does not allow membrane binding, actually enhances calcium binding to a subpopulation of sites (Bajaj et al., 1975; Deerfield et al., 1986). While this may suggest calcium-specific sites, other studies show an approximately similar number of total metal ion binding sites on fragment 1[1] regardless of the metal used (Nelsestuen et al., 1981). However, Deerfield et al. (1987) have provided evidence that there are one or two more calcium binding sites than magnesium binding sites on fragment 1.

Protein cross-linking reagents show calcium-specific cross-linking (Tarvers et al., 1982, 1986; Silversmith et al., 1983). While the latter has been attributed to calcium-specific protein dimerization (Tarvers et al., 1984), the same selectivity was observed for prothrombin (Tarvers et al., 1984) which does not undergo calcium-induced dimerization (Nelsestuen et al., 1981; Osterberg et al., 1980; Torbet & Freyssinet, 1987; Jackson et al., 1987). In addition, manganese causes dimerization of fragment 1 (Jackson et al., 1979; Nelsestuen et al., 1981) but does not enhance this cross-linking. Consequently, the cross-linking appears to be monitoring a calcium-specific event other than dimerization (Silversmith et al., 1983). Subpopulations of fragment 1 antibodies have been shown to have specificity for metal ion induced structures in fragment 1 (Tai et al., 1980; Madar et al., 1982). Furthermore, these antibodies can be separated into calcium conformation-specific antibodies (Borowski et al., 1986). This latter population does not recognize the product of the magnesium-induced conformational change. While these obser-

† This work was supported in part by Grant HL-15728 from the National Institutes of Health. Portions of this work have been presented in abbreviated form (Welsch & Nelsestuen, 1987).

[1] Abbreviations: Gla, γ-carboxyglutamic acid; fragment 1, amino-terminal 156 amino acid residues of bovine prothrombin; Tris, tris(hydroxymethyl)aminomethane; HPLC, high-performance liquid chromatography; TNBS, trinitrobenzenesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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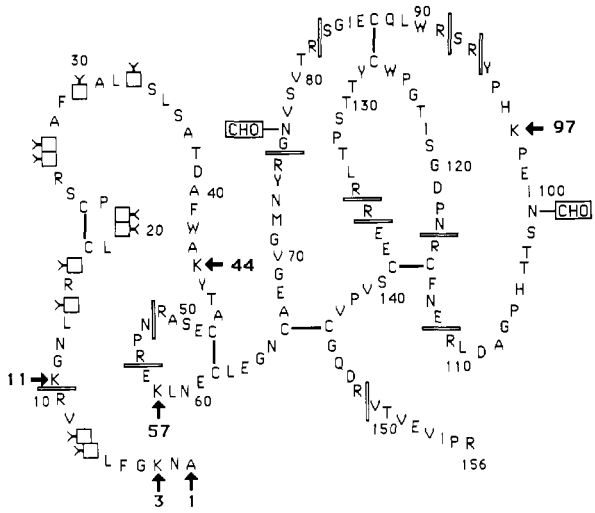
Scheme I: Amino Acid Sequence of Bovine Prothrombin Fragment 1[a]



[a] Adapted from Magnusson et al. (1975). Each letter denotes a single amino acid residue. The two carbohydrate groups which make up abbout 20% of the molecular weight are presented as "CHO". The γ-carboxyglutamic acid residues are shown as squares with branched extensions. The five disulfide bridges are shown as solid bars. The trypsin cleavage sites of fully acetylated protein are shown as open bars. Numbered arrows indicate the sites of the amino groups in fragment 1.

vations indicate a calcium-specific protein conformation, they do not suggest the nature of the calcium-specific process nor the region of the structure involved in the process.

Acetylation of prothrombin fragment 1 in the presence of calcium or magnesium ions revealed that amino groups were important to protein function and showed that calcium protected two acetylation sites on the prothrombin fragment 1 molecule (Welsch et al., 1988). Acetylation of the first site produced a protein that required high calcium concentrations to cause the fluorescence quenching event and had greatly reduced membrane binding. Acetylation of the second site abolished the calcium-induced fluorescence quenching event altogether.

For reference, Scheme I shows a schematic representation of the fragment 1 molecule, highlighting the amino groups as well as tryptic cleavage sites. The study reported here was initiated to identify the most reactive of the essential acetylation sites in prothrombin fragment 1. This site was identified as amino-terminal alanine.

Experimental Procedures

Materials. Isolation of prothrombin fragment 1 as well as the sources and experimental procedures used for much of the work presented here is described in the preceding paper (Welsch et al., 1988). Plasmin from bovine plasma, protease from *Streptomyces griseus* (type XIV, also known as Pronase E), and trypsin (type XIII) were purchased from Sigma.

Preparation of ^{14}C-Containing Derivatives of Fragment 1. Details of buffers and procedures used to obtain partially and fully acetylated fragment 1 are given in Welsch et al. (1988). To obtain fragment 1 containing radioactive acetate at the calcium-protected site, fragment 1 was first acetylated with saturating levels of acetic anhydride in the presence 25 mM $CaCl_2$. After dialysis to remove metal ions, 30 equiv (reagent to protein) of [^{14}C]acetic anhydride was added followed 0.5 h later by 300 equiv of unlabeled acetic anhydride. The protein was all dialyzed against 25 mM ammonium bicarbonate (pH 7.5), lyophilized, and stored at −70 °C. The specific activity of the [^{14}C]acetic anhydride used in these experiments (Amersham) was 25 mCi/mmol.

This radiolabeled protein was digested with trypsin [trypsin to fragment 1 ratio equal to 1:30 (w/w)] in 25 mM ammonium bicarbonate, pH 7.5, for 4 h at 37 °C. The digest was fractionated by gel filtration on a Sephadex G-50 column (2.0 × 100 cm) eluted with ammonium bicarbonate buffer (pH 7.5). The radioactive fractions were pooled and lyophilized. The lyophilized material was then fractionated on a Sephadex G-15 column (2.2 × 28.0 cm) eluted with 10% acetic acid. Radioactive fractions were pooled and lyophilized. This peptide (about 2.5 mg/mL) was treated with protease from *Streptomyces griseus* [5.6 units/mg; protease to fragment 1 ratio equal to 1:10 (w/w)] for 5 h at 37 °C in 50 mM Tris–0.10 M NaCl, pH 7.5. The digest was fractionated on the same Sephadex G-15 column (2.2 × 28.0 cm) eluted with 10% acetic acid. The radioactive fractions were pooled and lyophilized.

Isolation of Barium Citrate Adsorbing Peptides. Fragment 1 was acetylated to varying degrees by the procedures outlined above. The number of remaining amino groups was quantitated by the trinitrobenzenesulfonate reaction (Welsch et al., 1988). The protein was dialyzed, and 1-mL samples were subjected to trypsin digestion (1:100 w/w ratio at a fragment 1 concentration of 6 mg/mL) for 3 h at 37 °C in 0.05 M Tris buffer, pH 7.5. Dithiothreitol was added (2 mM final concentration), and the mixture was incubated for 1 h at 37 °C. Iodoacetamide (10 mM final concentration) was added and the reaction incubated for 1 h longer. Twenty milliliters of 0.05 M sodium citrate was added followed by 5 mL of 1 M $BaCl_2$. After thorough mixing, the precipitate was collected, washed 3 times by suspension in 20 mL of 0.1 M NaCl, and collected by centrifugation. The pellet was triturated in 2.0 mL of 1.0 M sodium sulfate. After centrifugation, the peptides in the supernatant were chromatographed on a Sephadex G-50 column (see below), and the barium citrate adsorbed peptides were pooled, lyophilized, and used for amino acid analysis.

^{1}H NMR Spectra. The ^{1}H NMR spectra were recorded on an NT 300WB spectrometer operating in the Fourier-transform mode at a frequency of 300 MHz. Spectra were recorded at 25 °C. The protein sample was dissolved in D_2O (99.96 atom % deuterium, Sigma Chemical Co.). The field was locked on the deuterium signal, and the chemical shifts are reported relative to external tetramethylsilane. Spectra were obtained by using an 8-μs pulse, a 4800-Hz spectral width, and an acquisition time of 0.85 s.

Preparation of Plasmin-Digested Fragment 1. Fragment 1 (3.2 mg in 1 mL) was incubated with 30 μg of plasmin at ambient temperature for 1 h in 50 mM Tris–0.10 M NaCl, pH 7.5. The protein was then dialyzed against 25 mM ammonium bicarbonate (pH 7.5) and subsequently lyophilized. The lyophilized protein was stored at −70 °C. The specific activity of the plasmin (Sigma Chemical Co.) was 3.6 units/mg (manufacturer's units).

Composition and Sequence Analysis. Samples for amino acid analysis were hydrolyzed in 6 N HCl at 110 °C for 16 h in evacuated, sealed tubes. Samples used for tyrosine analysis contained 1% phenol. Amino acid compositions were determined with a Beckman System 6300 high-performance analyzer. The compositions are expressed in mole ratios. Automatic Edman degradation was carried out in an ABI Model 470 A sequenator as previously described by Hewick et al. (1981). Phenylthiohydantoins were identified by reversed-phase HPLC.

Other Procedures. The peptide corresponding to residues 94–111 of fragment 1 was isolated by procedures presented

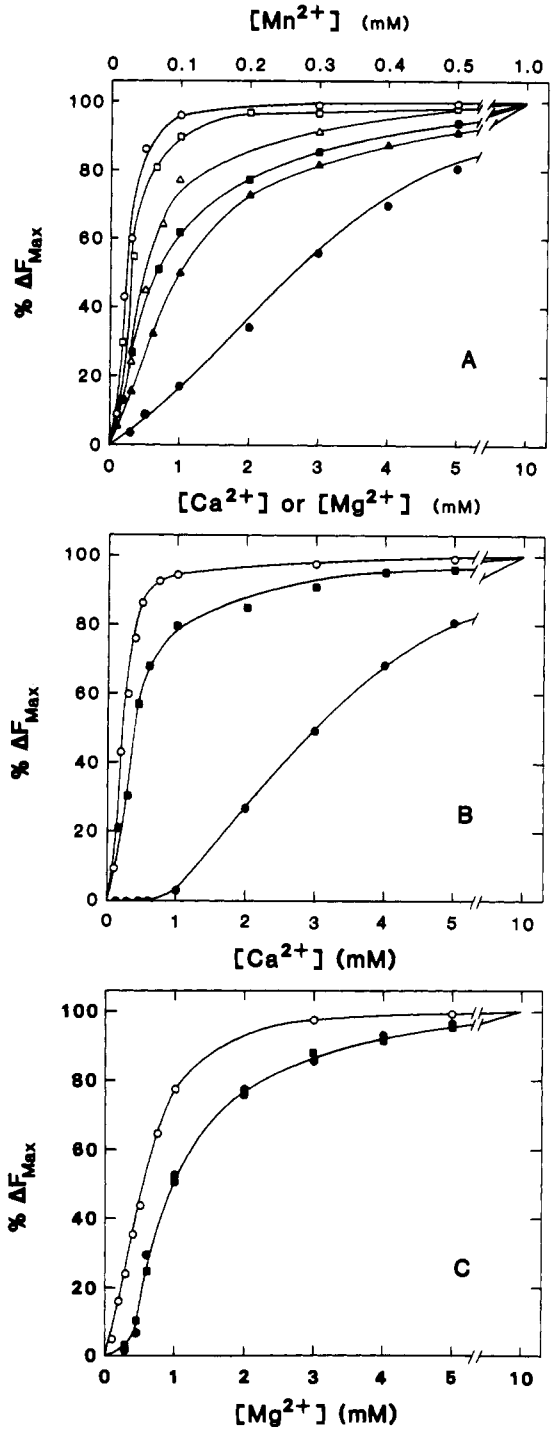


FIGURE 1: Metal ion titration of protein fluorescence. Part A shows the calcium (○, ●), magnesium (△, ▲), and manganese (□, ■) titrations of intrinsic protein fluorescence of native fragment 1 (open symbols) and fragment 1 which had 5 amino equiv acetylated (solid symbols). Part B shows calcium titration of intrinsic protein fluorescence of native fragment 1 (○) and fragment 1 exhaustively acetylated in the presence of magnesium (●) or calcium (■). Part C shows magnesium titration of native fragment (○) and fragment 1 exhaustively acetylated in the presence of magnesium (●) or calcium (■).

in Welsch and Nelsestuen (1988). Assay of free amino groups and formation of the trinitrobenzene derivative of fragment 1 are described in Welsch et al. (1988). Light-scattering and fluorescence methods are also described in the preceding paper (Welsch et al., 1988).

RESULTS

Calcium-Specific Nature of an Essential Amino Group in Fragment 1. Metal-free prothrombin fragment 1 was subjected to partial acetylation to produce the partially functional fragment 1 protein described by Welsch et al. (1988). Metal ion titrations showed that the major shift in metal ion dependence of the protein fluorescence quenching was unique to calcium; titration with magnesium and manganese showed only small changes compared to native protein (Figure 1A).

To examine this apparent metal ion specificity further, fragment 1 was subjected to thorough acetylation in the presence of either 25 mM calcium or 25 mM magnesium. The metal-free derivatives were isolated, and protein fluorescence quenching was examined (Figure 1B,C). The results showed that the calcium-protected protein had calcium and magnesium titration curves similar to those of the native protein. In contrast, magnesium-protected protein had dramatically lowered affinity for calcium. These results showed that magnesium failed to protect the first of the two essential calcium-protected acetylation sites in prothrombin fragment 1. This site apparently played little role in magnesium binding.

Identification of the Calcium-Protected Amino Group. Fragment 1 was thoroughly acetylated with unlabeled acetic anhydride in the presence of 25 mM $CaCl_2$. Reagents were removed by dialysis, and the protein was acetylated with [^{14}C]acetic anhydride for 30 min followed by addition of saturating amounts of unlabeled acetic anhydride. After dialysis against 25 mM NH_4HCO_3 (pH 7.5), the peptide was digested with trypsin and fractionated on a Sephadex G-50 column. The radioactivity eluted as one major peak (Figure 2A). The amino acid composition of this peak did not correspond to any single trypic peptide of fragment 1 (peptide A, Table I), and this material was chromatographed on a Sephadex G-15 column eluted with 10% acetic acid (Figure 2B) to give a pure peptide corresponding to the amino-terminal 10 residues of prothrombin fragment 1 (peptide B, Table I). Protein NMR analysis of the decapeptide showed three uncoupled peaks resulting from the hydrogens of acetate methyl groups (not shown). Comparison to known standards showed that a peak at 1.863 ppm was due to the amino-terminal acetyl group, a peak at 1.925 ppm was due to the acetyl group attached to the ε-amino group of lysine (position 3), and a peak at 1.811 ppm was from free acetate. The intensity ratio of

Table I: Amino Acid Composition Analysis

amino acid	peptide A[a]	peptide B[b]	peptide C[c]
D	1.96 (2.0)[e]	1.09 (1.0)	1.15 (1.0)
T	1.49 (2.0)	0.04 (0.0)	0.21 (0.0)
S	1.15 (1.5)	0.04 (0.0)	0.19 (0.0)
E	2.80 (3.0)	2.32 (2.0)	0.31 (0.0)
P	1.19 (1.5)	0.01 (0.0)	0.13 (0.0)
G	2.28 (2.5)	1.17 (1.0)	0.25 (0.0)
A	1.00[d] (1.0)	1.00 (1.0)	1.00 (1.0)
V	1.24 (1.0)	0.96 (1.0)	0.16 (0.0)
I	0.77 (1.0)	0.00 (0.0)	0.01 (0.0)
L	1.68 (2.0)	0.97 (1.0)	0.01 (0.0)
F	0.91 (1.0)	0.92 (1.0)	0.02 (0.0)
H	0.21 (0.0)	0.02 (0.0)	0.00 (0.0)
K	0.90 (1.0)	1.23 (1.0)	0.29 (0.0)
W	0.97 (1.0)	0.00 (0.0)	0.07 (0.0)
R	1.51 (2.0)	0.97 (1.0)	0.03 (0.0)

[a] Radiolabeled tryptic peptide of Ca^{2+}-protected protein after Sephadex G-50 chromatography. [b] Radiolabeled tryptic peptide of Ca^{2+}-protected protein after Sephadex G-15 chromatography. [c] Radiolabeled tryptic peptide of Ca^{2+}-protected protein after protease digestion. [d] All amino acid mole fractions were calculated relative to alanine. [e] Predicted amino acid mole fractions are given in parentheses (predicted mole fractions for the radiolabeled tryptic peptide of Ca^{2+}-protected protein were calculated by assuming a 2 to 1 ratio of amino-terminal decapeptide to disulfide-linked amino acid residues 83–91 and 117–135).

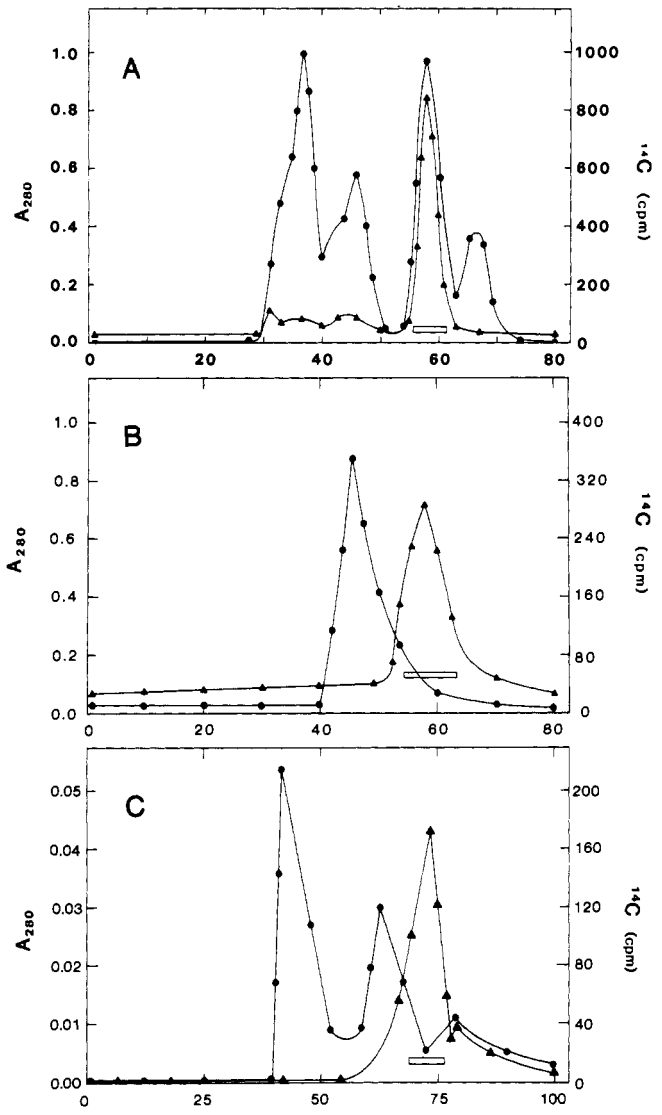


FIGURE 2: Isolation of the radiolabeled calcium-protected amino group. The tryptic digestion products from acetylated fragment 1 containing radiolabel at the calcium-protected amino group were fractionated on Sephadex G-50 (A). The fractions from part A indicated by the bar were pooled, lyophilized, and chromatographed on a Sephadex G-15 column eluted with 10% acetic acid (B). The fractions indicated in part B were pooled, lyophilized, and digested with protease to give the Sephadex G-15 elution profile shown in part C. The absorbance at 280 nm (●) and radioactivity (▲) of selected fractions are given.

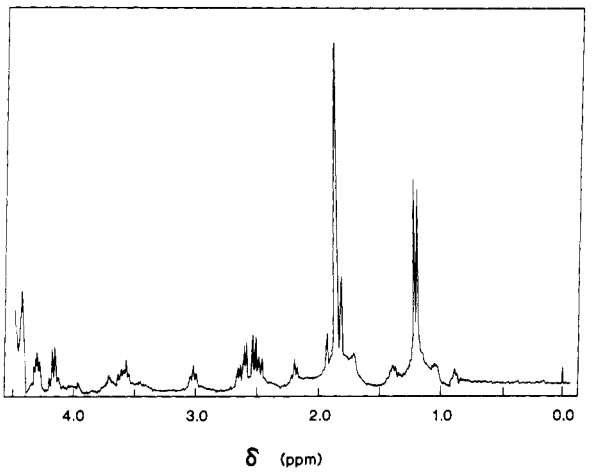


FIGURE 3: 300-MHz ^1H NMR spectrum of the peptide containing the calcium-protected amino group. The peptide isolated from part C of Figure 2 was used at a concentration of approximately 0.75 mM. The spectrum represents data accumulated from 1000 scans.

Table II: Amino Acid Sequence Analysis

	cycle					
peptide	1	2	3	4	5	6
A[a]	A[d]	N	AcK[e]	G	F	L
B[b]	Y	P	H	AcK	P	E
C[c]	G	F	L	-	-	V

	cycle					
peptide	7	8	9	10	11	12
A	-	-	V	R	AcK	G

[a] Fragment 1 acetylated in the presence of Ca^{2+}. [b] Peptide corresponding to amino acids 94–111 isolated from calcium-protected acetylated fragment 1. [c] Amino-terminal sequence analysis of plasmin-digested fragment 1. [d] The single-letter abbreviation for the predominant amino acid released per cycle is given. A dash indicates that the amino acid released was not identified. The amount of the predominant amino acid identified was always at least 10-fold greater than the next most abundant amino acid. [e] Aminoacetyllysine is AcK.

these three peaks was 1.0:1.0:1.8, respectively.

Further digestion of the amino-terminal decapeptide with protease reduced its mass, and the radiolabel eluted as a smaller peptide from the Sephadex G-15 column (Figure 2C). The amino acid composition of this peptide showed the amino-terminal dipeptide (Ala-Asn) plus lesser amounts of lysine (peptide C, Table I). Since small quantities of amino-terminal tripeptide could account for the measured radioactivity, unequivocal determination of the location of the radiolabeled amino group (Ala-1 or Lys-3) was not possible by amino acid composition alone. However, ^1H NMR analysis (Figure 3) showed the presence of the peak at 1.863 ppm, corresponding to the acetyl group attached to the amino-terminal alanine, while the peak at 1.925 ppm corresponding to acetyllysine was nearly absent (<10% of the 1.863 ppm peak, Figure 3). The levels of lysine measured in the composition may result from contamination by peptides of the protease. Other easily identified peaks in the spectrum correspond to the methyl hydrogens of alanine (1.20 ppm), the methylene hydrogens of asparagine (eight peaks centered at about 2.56 ppm), the α-proton of asparagine (4.30 ppm), and the α-proton of alanine (4.17 ppm).

To further establish that the amino-terminal amino group was the calcium-protected site, calcium-protected acetylated fragment 1 was subjected to amino acid sequence analysis (sequence A, Table II). The amino-terminal alanine of calcium-protected, acetylated protein appeared to be fully available (yield was >80% of theoretical) and was therefore the site protected by calcium.

Calcium Did Not Protect Other Amino Groups in the Protein. Sequence analysis showed that the third and eleventh positions of calcium-protected protein had no detectable lysine and a high yield of a material corresponding to N^{ϵ}-acetyllysine (sequence A, Table II). These amino groups were therefore not protected by calcium. *N*-Acetyllysine-97 was also identified in direct sequence of the peptide isolated from calcium-protected protein (sequence B, Table II).

That Lys-44 was acetylated was determined by composition analysis of barium citrate adsorbing peptides. Acetylation of Lys-44 eliminates a trypsin cleavage site and results in extension of the Gla-containing tryptic peptide (normally residues 12–44; Nelsestuen & Suttie, 1973) to include residues 45–52 (Scheme I). The extended peptide now contains one tyrosine and two phenylalanines. The tyrosine:phenylalanine ratio of barium citrate adsorbed tryptic peptides at various states of

acetylation was determined. Before acetylation, the measured Tyr:Phe ratio was 0.09. In the presence of calcium, when 2.5 amino groups were acetylated, this ratio was to 0.39. When the protein was fully acetylated, this ratio approached 0.5. Thus, Lys-44 became acetylated in the presence of calcium.

The only amino group that was not directly identified was Lys-57. However, the results indicated either that this site was not protected by calcium and underwent acetylation or that it was equally unreactive under all conditions. First of all, no radiolabeled peptides corresponding to Lys-57 were present when calcium-protected protein was acetylated with [^{14}C]acetic anhydride (Figure 2A). Second, acetylation appeared to eliminate all sites for reaction with the trinitrobenzenesulfonate reagent (Welsch et al., 1988). This implied acetylation of all amino groups which would include Lys-57. This latter conclusion is tentative since this assay for free amino groups did not detect that the amino-terminal alanine was protected by calcium (Welsch et al., 1988). In any event, Lys-57 did not appear to be altered by the presence of calcium.

Plasmin-Digested Fragment 1. Previous investigations have shown that limited plasmin digestion is capable of selectively removing the amino-terminal three amino acids of fragment 1 (Nelsestuen et al., 1979). In order to investigate whether the newly generated glycine amino terminus could support protein function, fragment 1 was treated with plasmin, and the resulting protein was purified as outlined under Experimental Procedures. The amino-terminal amino acid sequence showed a highly pure protein with the expected amino-terminal sequence (sequence C, Table II).

The calcium-induced intrinsic fluorescence quenching of this modified protein showed greater than 90% of the total quenching measured for native protein. However, titration revealed that 10-fold more calcium was required to achieve half-maximal fluorescence change (Figure 4A). Furthermore, the derivative no longer bound to membranes in a calcium-dependent manner (Figure 4B). Consequently, the new amino terminal, Gly-4, did not substitute for Ala-1 in forming the critical calcium structure of fragment 1.

Previous studies (Nelsestuen et al., 1979) on plasmin-treated fragment 1 examined total metal ion induced fluorescence quenching at saturating calcium levels and did not detect the more subtle but critical changes induced by this amino-terminal modification.

Trinitrophenyl Derivatives of Fragment 1. Metal ion free fragment 1 was allowed to react with saturating levels of TNBS reagent. The protein was isolated and analyzed for function. Amino acid sequence analysis showed no detectable level of any amino-terminal residue (<5% of the peptide present; data not shown). Due to quenching by the trinitrophenyl group, protein fluorescence could not be studied. However, membrane binding studies of the derivatized protein showed calcium-dependent interactions typical of native protein (Figure 4B).

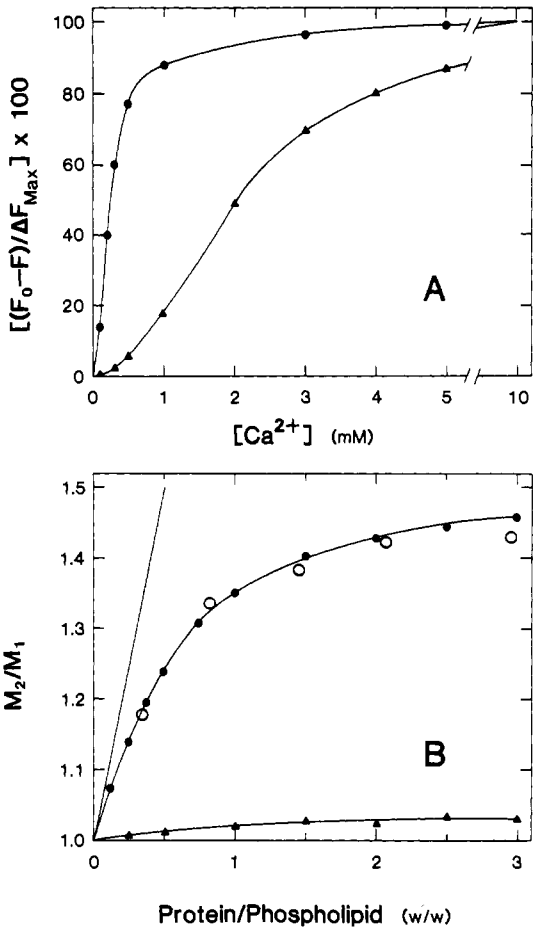


FIGURE 4: Properties of plasmin-digested fragment 1. Calcium titrations of the intrinsic fluorescence (F) for native (●) and plasmin-digested (▲) fragment 1 are given (A) where F_0 is the intensity of metal-free protein. Maximum fluorescence quenching (ΔF_{Max}) at saturating calcium was 55% for native protein and 51% for plasmin-digested fragment 1. Part B shows protein–membrane binding for native (●) and plasmin-digested (▲) fragment 1. The molecular weight ratio of the protein–phospholipid complex (M_2) to that of the phospholipid alone (M_1) is plotted as a function of the protein to phospholipid ratio. The straight line represents the expected M_2/M_1 ratio if all added proteins were bound. The experimental conditions included 0.200 mg of small unilamellar phospholipid vesicles (20% phosphatidylserine, 80% phosphatidylcholine) in 1.5 mL of buffer containing 10 mM calcium. Protein binding was completely reversed by the addition of excess EDTA. Also shown in part B is the binding of fragment 1 which had been extensively modified with TNBS (○).

Discussion

The amino group protected from acetylation by calcium ion corresponded to Ala-1. The amino terminal therefore appeared to be involved in a specific calcium binding function required for formation of the essential membrane binding structure of fragment 1. Magnesium did not offer adequate protection for this site. This could arise from complete lack of magnesium association with this site, from incorrect association with this site, or from a relatively low affinity such that 25 mM magnesium did not provide adequate protection. This latter possibility describes a situation where the various sites are filled in different sequence by different metal ions (Nelsestuen et al., 1981). This description appeared to fit the metal binding site(s) associated with the second acetylation site (Welsch & Nelsestuen, 1988).

That is, protein which was acetylated at the amino terminal actually preferred magnesium but still underwent fluorescence quenching at high calcium concentrations. This property may explain why magnesium ion promotes calcium binding to a population of sites on prothrombin fragment 1 (Bajaj et al., 1975; Deerfield et al., 1986).

Derivatization of fragment 1 has also been accomplished by a chemical modification (Wright et al., 1986) which converts γ-carboxyglutamate residues to γ-methyleneglutamyl residues. Derivatization to the level of three Gla residues per fragment 1 molecule resulted in a protein with properties similar to those of fragment 1 with a derivatized amino terminal. The three sites of reaction were reported to be Gla-7, -8, and -33 (Zapata et al., 1987). This protein displayed full fluorescence quenching but had a severalfold increase in the calcium requirement and only small changes in the magnesium required for protein fluorescence quenching. This protein had

lost calcium-dependent membrane binding ability as well. The similarities with fragment 1 that contains a blocked amino terminal may suggest similar bases for the changes. That is, the amino terminal plus one or more of Gla residues 7, 8, and 33 may participate in a calcium-specific binding site. The amino terminal may also be involved in forming the antigenic determinant recognized by antibodies specific for the calcium–fragment 1 structure (Borowski et al., 1986).

A recent report has shown that a modified gene for blood coagulation factor VII produced a protein with an additional serine residue at the amino terminal (Busby et al., 1987). This protein had low function in coagulation assays, and it was postulated that the amino-terminal modification might result in altered carboxylation and reduced Gla formation. However, the modified factor VII produced was quantitatively precipitated by barium citrate which indicates the presence of Gla residues. All vitamin K dependent proteins of the plasma are closely related in their amino-terminal regions. Our results with prothrombin fragment 1 therefore provide the alternative explanation that the altered factor VII protein lacked function due to loss of membrane binding ability as a result of a modified amino terminal. Further studies are needed to differentiate these and other possible explanations for the low activity of the modified factor VII molecule.

It appeared that no other amino groups in fragment 1 were protected by calcium. Lysine residues 3, 11, 44, 57, and 97 therefore did not appear to serve critical roles in the calcium and membrane binding functions of fragment 1. This was unexpected since an accompanying study (Welsch et al., 1988) showed that two acetylation sites in fragment 1 were protected by calcium. Further studies have now shown that the second calcium-protected site, which corresponds to the magnesium-protected site, is an acetylation site not previously identified in other proteins (Welsch & Nelsestuen, 1988).

The amino terminal should ionize with a pK_a of about 7.5–7.9 while the lysine side chain should have a pK_a of about 10.5 (Metzler, 1977). Amino-terminal alanine of fragment 1 appeared to have normal reactivity since it was selectively acetylated in the apoprotein and was quantitatively derivatized by the time three of the six amino groups were derivatized (Welsch et al., 1988). In light of this, a surprising observation was that calcium binding to fragment 1, detected by fluorescence quenching, was altered only modestly between pH 7.0 and 9.0 (Resnick & Nelsestuen, 1980). These results were repeated with the same proteins used here, and less than a 50% increase in the calcium titration midpoint at pH 9 relative to that at pH 7 was obtained (data not shown). These properties suggested that protonation of amino-terminal alanine had only a small effect on calcium binding. While this is unexpected, it might be consistent if hydrogen bonding were the primary function of the amino terminal.

It is interesting to note that some previous metal binding investigations have implicated a group titrating near pH 7.5. Nelsestuen and Suttie (1972) reported that calcium binding to prothrombin increased at least 5-fold as the pH was raised from 7 to 9. This contrasted with subsequent studies (Nelsestuen & Suttie, 1973). However, a similar pH effect was reported by Scott et al. (1979), who used intrinsic fluorescence to examine metal binding. These workers observed approximately a 10-fold increase in the association constant for calcium binding to fragment 1 as the pH was raised from 7 to 8. Changes in ^{25}Mg NMR signals at about pH 7.5 have also been reported (Marsh et al., 1979). Involvement of the amino-terminal group would be consistent with all these pH effects. However, as stated above, we have been unable to demonstrate these effects in recent studies. While the bases for these inconsistencies are not known, it is possible that modified forms of the protein have been isolated in the various studies. Further investigations are needed to clarify these reports.

A further unexpected observation was that fragment 1 which was derivatized with a trinitrophenyl group at the amino terminal retained membrane binding function. In contrast, it appeared that this amino group was unable to function when subjected to reductive methylation (Welsch et al., 1988). The latter treatment would normally seem less disruptive since it does not alter the charge properties of the amino group. Overall, the function of the amino-terminal group did not appear to be described by simple charge interactions.

In the apoprotein, Lys-3 was available to proteolysis by plasmin. We have found that calcium caused loss of selective plasmin digestion at lysine-3.[2] Consequently, several residues at the amino terminal appeared to be unstructured and available in the apoprotein but highly structured and protected when calcium was bound.

Acknowledgments

We thank Tom Krick for performing the NMR analyses and Dr. Wohlheuter of the Microchemical Facility, The University of Minnesota, for performing amino acid and sequence analyses.

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