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Chemical Modification of Prothrombin Fragment 1: Documentation of Sequential, Two-Stage Loss of Protein Function†

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ABSTRACT: The amino groups of prothrombin fragment 1 (amino acids 1–156 of prothrombin) were derivatized by acetylation, amidination, and reductive methylation. Conditions that caused complete acetylation of protein amino groups produced a fragment 1 derivative which no longer displayed a metal ion dependent intrinsic fluorescence change and had lost its membrane binding capability as well. However, when derivatized in the presence of calcium ions, extensive acetylation yielded a product that underwent protein fluorescence quenching at metal ion concentrations similar to those observed for the native protein. This derivative bound to membranes in a calcium-dependent manner with only a small reduction in affinity. Several results showed the existence of a partially functional protein that was characterized by a high degree of calcium-dependent protein fluorescence quenching but which had a requirement for 10-fold higher calcium concentration. This derivative was produced by partial acetylation (>3 equiv) of metal-free protein. This partially acetylated protein had greatly diminished membrane binding. The calcium-protected amino group, therefore, was among the most reactive acetylation sites in the metal-free protein. The second site, responsible for abolishing all metal ion induced fluorescence change, was resistant to acetylation and became derivatized at the last stages of amino group acetylation. The second site did not function as a substitute for the first site. That is, both sites were shown to be essential for full protein function so that calcium actually protected both sites from acetylation. The second site, but not the first site, could undergo deacetylation under alkaline conditions and mild heat (pH 10, 50 °C) or with hydroxylamine and mild heat (0.2 M, 50 °C). Thus, the fully acetylated protein could be returned to its intermediate state of function (high calcium requirement, low membrane binding) by these treatments. Amidination and reductive methylation of fragment 1 produced derivatives which were similar to the partially acetylated protein. These derivatives underwent protein fluorescence quenching which required 10-fold more calcium than native protein and had greatly reduced membrane affinity. This indicated that these subtle changes abolished the function of the first site but did not alter the function of the second site.

Prothrombin, a plasma glycoprotein, is one of a group of proteins which requires vitamin K for the posttranslational carboxylation of glutamyl residues. The resulting calcium binding protein, which contains 10 γ-carboxyglutamyl (Gla)[1] residues in its amino-terminal domain, is capable of calcium-dependent membrane binding [reviewed in Nelsestuen (1984)]. Prothrombin fragment 1 (amino acids 1–156 of prothrombin) contains all of the Gla residues of prothrombin and has been used as a model peptide for calcium and membrane binding studies of vitamin K dependent proteins [Gitel et al., 1973; reviewed in Nelsestuen (1984)]. Recent X-ray crystallographic analyses of fragment 1 provide the potential for a detailed understanding of calcium ion binding to a Gla-containing protein (Tulinsky & Park, 1986). Relatively little is currently known about the precise metal ion binding sites in fragment 1. Loss of as few as two Gla residues results in considerable loss of protein function (Malhotra et al., 1985; Borowski et al., 1985; Wright et al., 1986). This has greatly restricted the ability for modification of Gla residues as an approach to understanding the metal binding functions of fragment 1.

The present investigations were initiated to expand our understanding of the fragment 1 structure and to identify specific groups important to the various functions of fragment 1. These approaches may also offer methods of producing chemically modified proteins which allow insertion of spectroscopic probes at precise sites in the protein. The results provided direct chemical evidence for involvement of amino groups in the calcium and membrane binding activities of prothrombin fragment 1. Two essential sites were found that were sensitive to acetylation. Both were protected from ace-

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[1] Abbreviations: Gla, γ-carboxyglutamic acid; fragment 1, amino acids 1–156 of the amino terminus of bovine prothrombin; EDTA, ethylenediaminetetraacetic acid; TNBS, trinitrobenzenesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; TSP, (trimethylsilyl)propionate sodium salt.

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tylation by calcium. However, the two sites had very different chemical reactivities which allowed sequential derivatization and production of partially active protein. The most reactive group lost function with even the modest structural changes associated with reductive methylation.

Experimental Procedures

Materials. The hexahydrates of $CaCl_2$, $MgCl_2$, and $MnCl_2$ were obtained from Aldrich in the highest purity available and were used without further purification. Tris base, Na_2EDTA, bovine brain phosphatidylserine, and egg phosphatidylcholine were purchased from Sigma. The phospholipids were reported to be quite pure (≥98%) and were used without further purification. Sephadex G-100 SF was from Pharmacia. All other reagents were reagent grade or better. All solutions were prepared with distilled and deionized water.

Prothrombin was isolated from citrated bovine plasma by barium citrate adsorption followed by ammonium sulfate fractionation and ion-exchange chromatography using DEAE-Sepharose (Nelsestuen & Suttie, 1972). Fragment 1 was prepared from pure prothrombin by digestion (6.5 h at 37 °C) with 1/1000 weight of thrombin in 0.1 M phosphate buffer at pH 7.5 containing approximately 0.25 M NaCl. The fragment 1 generated was purified by DEAE-Sepharose chromatography (Heldebrant & Mann, 1973) followed by gel filtration on a Sephadex G-100 SF column (3.2 × 70.0 cm). The purified fragment 1 intrinsic fluorescence decrease induced by 10 mM $CaCl_2$ was at least 55%. After dialysis into 25 mM ammonium bicarbonate buffer (pH 7.5), the protein was lyophilized and stored at −70 °C. Protein concentrations were determined by ultraviolet absorbance using an $E^{1\%}_{280\ nm}$ of 10.1 (Heldebrant & Mann, 1973). A molecular weight of 23 400 was used for calculations of molar concentrations of fragment 1.

TNBS Assay for Amino Group Determination. Free amino groups were determined by using trinitrobenzenesulfonic acid (TNBS) as previously described (Haynes et al., 1967) except that volumes were scaled down to one-tenth of those reported. The extinction coefficient ($1.09 \times 10^4\ M^{-1}\ cm^{-1}$ at 344 nm) for the (trinitrophenyl)amino group was used for estimation of the number of amino groups. In all cases, appropriate controls were determined and subtracted.

Acetylation of Fragment 1. Fragment 1 (0.3 mM) was acetylated at ambient temperature for 30 min with acetic anhydride in 1.0 M sodium acetate–0.25 M borate buffer, pH 8.5. Partially acetylated derivatives were prepared by varying the molar ratio of acetic anhydride to amino groups (see below). Divalent metal ion protected derivatives were prepared by preincubation of the protein with 25 mM metal ions in the acetate–borate buffer. The intrinsic fluorescence change under these conditions was the same as that observed in dilute buffer. To remove the metal ions from proteins, samples were exhaustively dialyzed against 25 mM ammonium bicarbonate (pH 7.5). Routinely, samples were either used directly from the ammonium bicarbonate buffer or lyophilized. The lyophilized samples were stored at −70 °C.

Reductive Methylation of Fragment 1. Fragment 1 was derivatized by reductive methylation using $NaCNBH_3$ according to the procedure described by Jentoft and Dearborn (1979). This relatively mild reducing agent reduces Schiff bases but does not reduce disulfide linkages. After thorough dialysis against 0.1 M borate buffer (pH 8.5), the modified protein was assayed for free amino groups as described above. The [^{14}C]formaldehyde (New England Nuclear, Boston, MA) was diluted with [^{13}C]formaldehyde (Merck, St. Louis, MO) to a specific activity of 2.2×10^8 cpm/mmol.

Amidination of Fragment 1. Ethyl acetimidate was used to amidinate fragment 1 in 0.1 M borate buffer (pH 8.5) as described (Hunter & Ludwig, 1972). The modified protein was dialyzed against 0.1 M borate buffer (pH 8.5) and assayed for free amino groups as was decribed above.

All of the modified proteins eluted as single symmetrical peaks from TSK 2000 SW steric exclusion chromatography. There was no evidence of intermolecular cross-linking. The steric exclusion chromatography was performed on an Altex (Berkeley, CA) Model 153 liquid chromatograph with a Varian (Palo Alto, CA) TSK 2000 SW steric exclusion column. A constant flow rate of 0.5 mL/min of 50 mM sodium phoshate buffer (pH 7.0) was used for elution.

Metal Ion Dependent Intrinsic Fluorescence Quenching. Relative fluorescence intensities were determined on protein samples containing 0.075 ± 0.005 mg/mL protein in 0.05 M Tris base–0.10 M NaCl, pH 7.5, on a Perkin Elmer MPF-44A fluorescence spectrophotometer at 25 °C. The temperature was maintained at 25 °C using a Lauda RM-3 circulating water bath. Intrinsic protein fluorescence was measured with excitation at 280 nm and emission at 344 nm (8-nm slit widths).

Phospholipid Binding of Peptides. Single-bilayer vesicles (20% phosphatidylserine and 80% phosphatidylcholine) were prepared by a modification of the method of Huang (1969) as has been previously described (Nelsestuen & Lim, 1977). Phospholipid concentrations were determined by phosphorus analysis (Chen et al., 1956) using a phospholipid/phosphorus weight ratio of 25.

Relative light-scattering measurements were used to measure protein–phospholipid binding as described in detail elsewhere (Nelsestuen & Lim, 1977). Briefly, light-scattering intensity was used to estimate molecular weight ratios on the basis of the relationship

$$I_{s2}/I_{s1} = (M_2/M_1)^2[(\partial n_2/\partial c_2)/(\partial n_1/\partial c_1)]^2$$

where I_S is the light-scattering intensity, M is the molecular weight, and $\partial n/\partial c$ is the refractive index increment for the vesicles alone (subscript 1) and the protein–vesicle complex (subscript 2). In this study, the amounts of bound protein were used for relative comparisons of different proteins, and equilibrium constants were not accurately determined.

^{13}C NMR of Derivatized Protein. The ^{13}C NMR spectra were recorded on a Varian XL-100 spectrometer operating in the Fourier-transform mode at a frequency of 25.2 MHz. Spectra were recorded at 5 °C. The protein sample was dissolved in D_2O containing 0.1 M NaCl at pH 7.3. The field was locked on the deuterium signal, and the chemical shifts are reported in parts per million from external sodium (trimethylsilyl)propionate (TSP). Spectra were obtained with a 15-μs pulse, a 5646-Hz spectra width, and an acquisition time of 0.73 s.

Unless otherwise indicated, the buffer was 0.05 M Tris base containing 0.1 M NaCl at pH 7.5.

Results

Fragment 1 Acetylation. Acetic anhydride was used to derivative fragment 1 in the presence or absence of calcium (Figure 1). There are six amino groups in fragment 1 (five lysines and the amino-terminal alanine), and absorbance changes indicated formation of the TNBS derivative with about six amino groups. As detected by the TNBS assay of free amino groups, the addition of 25 mM $CaCl_2$ (Figure 1) to the derivitization reaction appeared to afford no protection from acetylation.

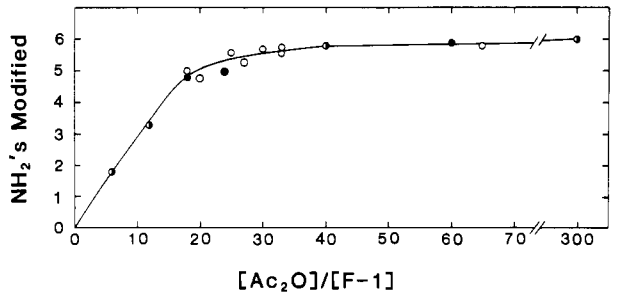

FIGURE 1: Acetylation of the amino groups of prothrombin fragment 1. Fragment 1 (7.5 mg/mL in borate–acetate buffer, pH 8.5) was acetylated with acetic anhydride in the absence (○) or presence (●) of 25 mM calcium ions. The number of amino groups modified was determined by the TNBS assay as described under Experimental Procedures.

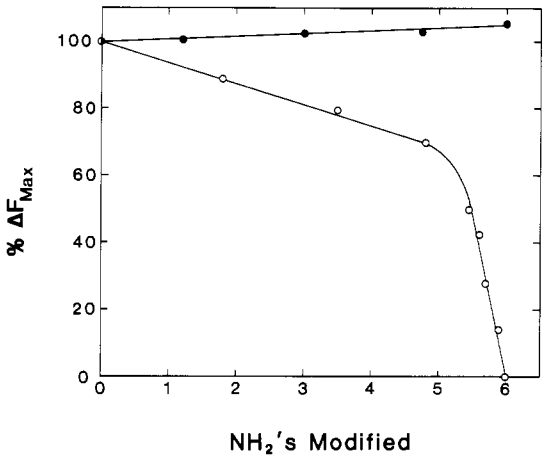

FIGURE 2: Maximum intrinsic fluorescence change as a function of the number of acetylated amino groups. The fluorescence change of fragment 1 differentially labeled in the absence (○) or presence (●) of 25 mM calcium ions was measured as a function of the number of amino groups derivatized. The number of amino groups modified was determined by using the TNBS assay. The percentage of maximum intrinsic fluorescence change, % ΔF_{Max}, was defined as the observed fluorescence quenching for the derivatized protein divided by the fluorescence quenching for native fragment 1. The fluorescence quenching was measured in 10 mM calcium. The assay was performed using 100 μg of protein in 1.5 mL of standard Tris buffer.

Addition of divalent cations to prothrombin fragment 1 quenches the intrinsic fluorescence of the protein (Nelsestuen, 1976; Prendergast & Mann, 1977). This quenching has been shown to be the result of a conformational change that is necessary but not sufficient for membrane binding (Nelsestuen et al., 1976; Marsh et al., 1979; Wright et al., 1986). Exhaustive acetylation in the absence of divalent metal ions completely eliminated this conformational change (Figure 2). In the absence of divalent cations, loss of the metal ion dependent protein fluorescence change was not linear with respect to the degree of acetylation; the very last stages of amino group derivatization accounted for 70% of the loss. This suggested that a relatively resistant acetylation site was critical to the fluorescence quenching event.

In contrast to the unprotected protein, calcium-protected samples retained the maximum fluorescence change at all levels of acetylation (Figure 2). This produced an apparent contradiction since the TNBS assay suggested that calcium afforded no protection to the amino groups in the molecule (Figure 1). However, subsequent studies (Welsch & Nelsestuen, 1988a,b) clearly showed the protection of one amino group.

Calcium titration of partially acetylated, unprotected fragment 1 revealed that the calcium concentration required

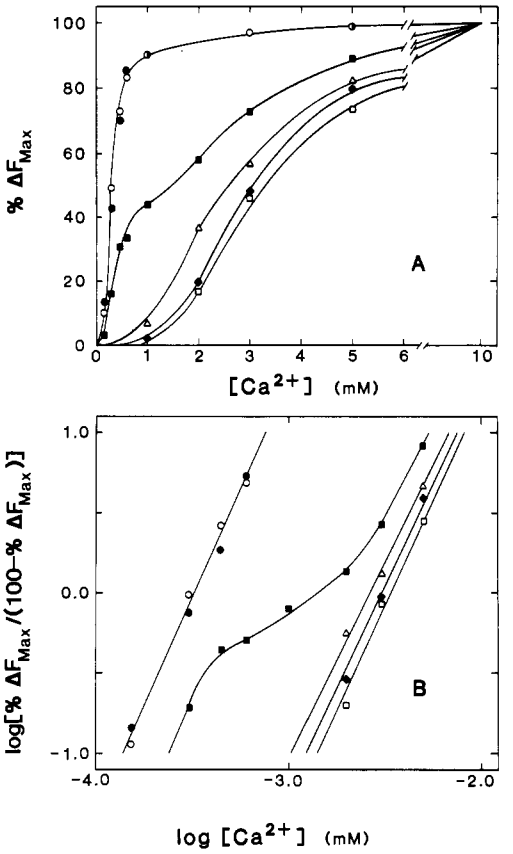

FIGURE 3: Calcium titration of the intrinsic fluorescence change of acetylated derivatives. Protein samples exhaustively acetylated in the presence of 25 mM calcium (●) or partially acetylated in the absence of metal ions [(○) 0.0, (■) 1.7, (△) 3.1, (♦) 4.5, and (□) 5.3 incorporated acetyl groups] were titrated with calcium. The percentage of maximum intrinsic fluorescence change for each derivative at the calcium concentration given, % ΔF_{Max}, is shown. Maximum fluorescence quenching at saturating calcium levels ranged from 30% for the protein with 5.3 acetyl groups to 55% for the native protein. Panel B is a Hill plot of the data given in panel A where % ΔF_{Max} is the percentage of the maximum fluorescence change at the calcium concentration shown.

for half-maximum fluorescence change gradually became 10-fold higher (Figure 3A). This shift to higher calcium requirement was completed when about three amino groups had been derivatized. The calcium requirement for half-maximum quenching remained at this level (3 mM) until the fluorescence change became too small to be accurately measured. For proteins with less than 3 equiv of derivatization, both native and elevated calcium-requiring species were present (Figure 3B). For calcium ion protected samples, the degree of acetylation had no effect on the calcium titration curve, so that the calcium required for half-maximum fluorescence change was always similar to that of the underivatized protein (0.3 mM).

Reductive Methylation and Amidination of Native Fragment 1. The amino groups of fragment 1 were completely derivatized by reductive methylation. As estimated by the TNBS assay, the product from this procedure had 5.6 derivatized amino groups. Derivatization of the protein (3.5 mg) with radiolabeled formaldehyde (2.2×10^8 cpm/mmol) showed that 12.8 ± 0.3 equiv of methyl groups had been incorporated into the protein (422 500 cpm/0.15 μmol of fragment 1). Assuming 2 methyl groups incorporated per amino group (Means & Feeney, 1971), 6.4 amino groups had been derivatized. Further evidence for complete amino group derivatization was provided by reductive methylation using [^{13}C]formaldehyde. The proton-decoupled ^{13}C NMR spec-

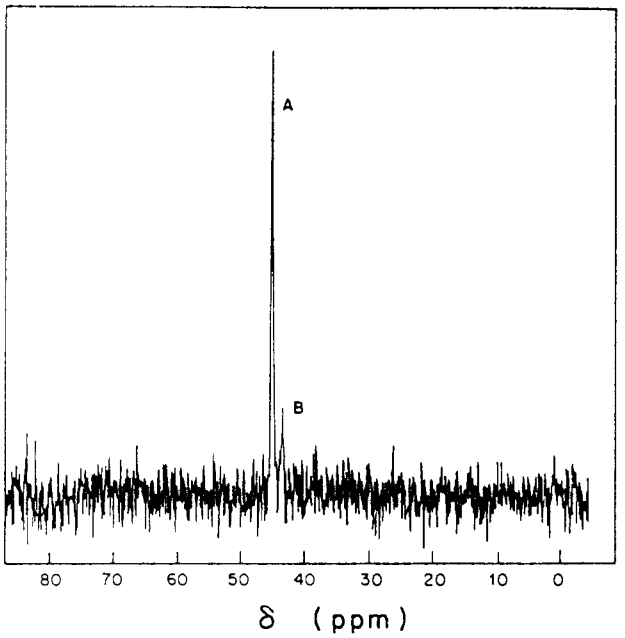

FIGURE 4: ^{13}C 25-MHz spectrum of ^{13}C-methylated prothrombin fragment 1. The protein concentration was 1 mM. The spectrum represents data accumulated from 10000 scans.

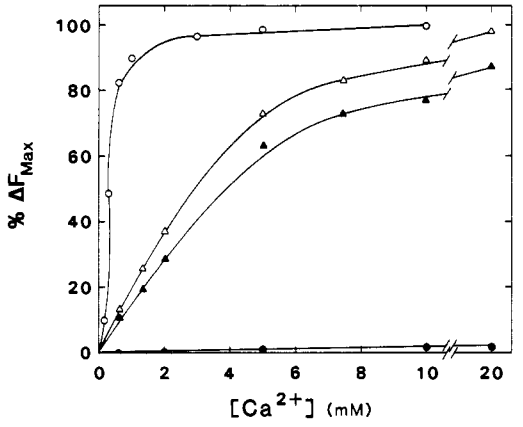

FIGURE 5: Intrinsic fluorescence quenching of derivatized fragment 1. Calcium titration of native (○), fully methylated (△), fully amidinated (▲), and fully acetylated (●) fragment 1. ΔF_{Max} is the fluorescence quenching observed for native protein at saturating calcium levels. The protein concentration for all titrations was 75 μg/mL.

trum observed for native fragment 1 which had been methylated with 90%-enriched [^{13}C]formaldehyde containing tracer levels of [^{14}C]formaldehyde (2.2×10^8 cpm/mmol) showed two peaks (Figure 4). The ratio of peak A to peak B areas was approximately 5 to 1, corresponding to the five N^{ϵ},N^{ϵ}-dimethyllysyl residues (peak A) and to the N^{α},N^{α}-dimethylamino terminus (peak B of Figure 4). No other NMR signals were detected between −3 and 200 ppm.

Native fragment 1 was also amidinated to apparent saturation using ethyl acetimidate. This derivative contained 5.6 modified amino groups as determined by the TNBS assay.

When measured in excess calcium (20 mM), the magnitude of fluorescence quenching observed for the methylated protein was similar to that of the native protein while the extent of fluorscence change for the amidinated derivative was only slightly less (Figure 5). However, the calcium concentration required for the methylated and amidinated derivatives was shifted to about 10-fold above that for the native protein. This shift was similar to that observed for the partially acetylated derivative (see above). These relatively modest derivatizations

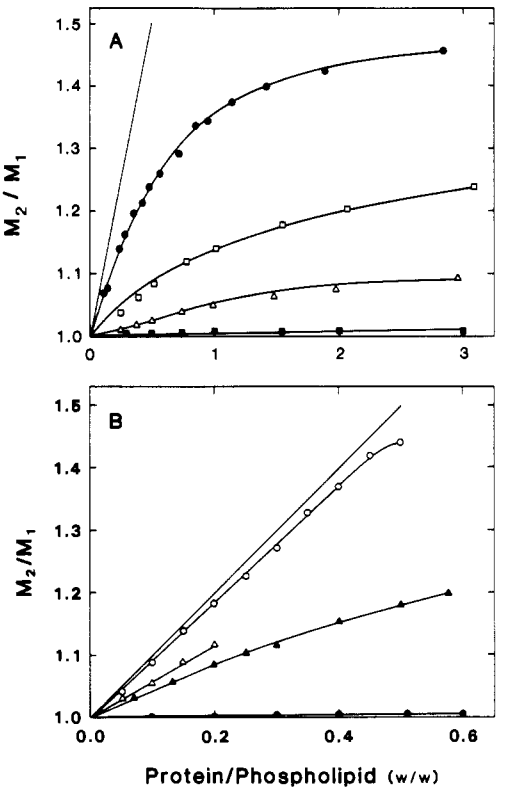

FIGURE 6: Protein–membrane binding. The molecular weight ratios of the protein–phospholipid complex to that of the phospholipid alone (M_2/M_1) are plotted as a function of the protein to phospholipid ratio. The straight line represents the expected M_2/M_1 ratio if all added proteins were bound. Part A shows results for native fragment 1 (●), fragment 1 which had been exhaustively acetylated in the absence (■) or presence of calcium ions (□), and fragment 1 which had 3 amino equiv acetylated in the absence of metal ions (△). Part B shows results for native (○), methylated (△), amidinated (▲), and acetylated (●) fragment 1. The experimental conditions in part A included 0.200 mg of phospholipid in 1.5 mL of buffer containing 10 mM calcium. In part B, a phospholipid concentration of 1.125 mg/mL was used. In all cases, protein binding was completely reversed with the addition of excess EDTA.

therefore appeared to eliminate the function of the first acetylation site. However, these derivatizations did not appear to influence the second essential acetylation site.

Membrane Binding by Derivatized Proteins. In the presence of calcium, prothrombin fragment 1 binds to phospholipid vesicles containing acidic phospholipids (Papahadjopoulos & Hanahan, 1964; Gitel et al., 1973). Binding of native and acetylated proteins to small unilamellar vesicles demonstrated that derivatives which required the elevated calcium concentration for the fluorescence change had greatly diminished affinity for membrane (Figure 6A). Binding of protein which had been acetylated in the presence of calcium showed less than a 2-fold reduction in membrane binding (Figure 6A). The basis for this small change was not determined. It is possible that protein molecules capable of fluorescence quenching but incapable of membrane binding accounted for the reduced membrane binding. In any event, the major finding was that this protein retained a high level of function.

In contrast, protein modified in the absence of calcium showed greatly diminished membrane binding. Estimations based on the plots shown indicated that the dissociation constant for protein which required higher calcium to induce maximum fluorescence change was at least 30-fold higher than that observed for native protein. This indicated that the conformational change monitored by fluorescence quenching was necessary but not sufficient for membrane binding. It also

suggested that the fluorescence quenching observed for proteins with the elevated calcium requirement did not provide the correct membrane binding element. As expected, derivatives that showed no fluorescence change were unable to bind membranes at all.

The amidinated and methylated proteins both showed greatly reduced binding (Figure 6B). The concentration of phospholipid used to generate the data shown in Figure 6B was much higher than the dissociation constant for native fragment 1–membrane binding, and this results in a less sensitive demonstration of affinity. In any event, the data in Figure 6B indicated that the binding affinities for both the methylated and amidinated proteins were at least 15 times lower than those measured for native fragment 1. Again, the fully acetylated fragment 1 showed no binding to phospholipid vesicles.

Sequential Nature of the Reactions. The studies shown here indicated the function of two groups in the calcium and/or membrane binding functions of prothrombin fragment 1. At least two explanations for these properties are possible. First of all, there could be two sites that are both protected by calcium, and both are needed for full function. Alternatively, only one site may function at a time. In this case, when the first site is derivatized, the second site takes over the same function but in a diminished capacity.

To distinguish between these possibilities, fully acetylated, calcium-protected fragment 1 was reisolated in the metal-free state. This protein contains acetyl groups at all unprotected amino groups. Subsequent acetylation of this species in the absence of metal ions showed the same progress documented above. That is, mild acetylation conditions that normally derivatized three amino groups produced a partially functional protein that still underwent the protein fluorescence quenching event but required high calcium concentrations (data not shown). More rigorous acetylation then eliminated the calcium-induced fluorescence change all together. Thus, calcium had protected both groups from acetylation.

A further study that showed the sequential nature of the reactions consisted of deacetylation. Fragment 1 which had been completely acetylated in the absence of calcium and which had lost all spectroscopic reaction to calcium was treated with 0.2 M hydroxylamine. An intermediate state of the protein (fluorescence quenching by calcium required high calcium concentration) was generated after 30 min at 50 °C. The total fluorescence quenching of this protein was about 60% that of the native protein.

These conditions for deacetylation are unusual in that they are much more rigorous than anticipated for cleavage of an ester linkage but are much less rigorous than required for cleavage of an amide linkage. The latter was evident from the fact that the entire peptide backbone was maintained to provide the structure essential for the quenching events. In subsequent studies (Welsch & Nelsestuen, 1988b), we found that these conditions were sufficient to remove radioactive acetate from the second acetylation site.

Basis for Loss of Metal Ion Induced Fluorescence Change. Figure 7 shows additional properties of protein fluorescence quantum yield that appeared related to the second acetylation site. In all cases, fluorescence intensity was sensitive to alkaline pH and was quenched dramatically above pH 10. While this may result primarily from quenching by the phenolate anion of tyrosine (Cowgill, 1968), other properties show a correlation with the second acetylation site. The inset (Figure 7), shows that the quantum yield at neutral pH decreased dramatically in conjunction with acetylation of essential site 2. This suggested that derivatization of site 2 may cause the same fluorescence quenching event induced by metal ion binding. Addition of metal ions would then have no further effect on the protein fluorescence quenching.

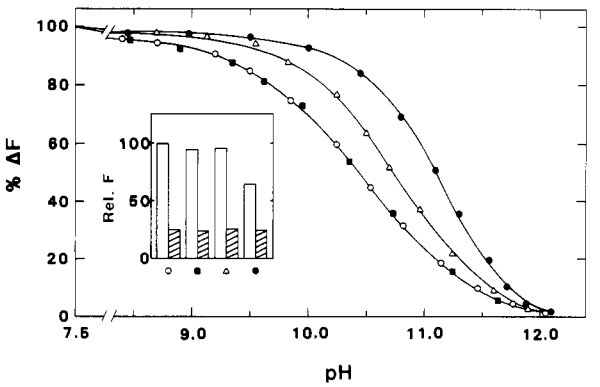

FIGURE 7: pH titration of intrinsic protein fluorescence. The intrinsic protein fluorescence for protein exhaustively acetylated in the presence of 25 mM calcium (■) or differentially acetylated [(○) 0.0, (△) 5.1, and (●) 6.0 incorporated acetyl groups] in the absence of metal ions was measured as a function of pH. For purposes of comparison, the titrations are presented as relative intensities with the intensity at pH 7.5 set at 100% and that at pH 12.2 set at 0%. Titrations were performed on 150 μg of protein in 1.5 mL of 0.02 M Tris buffer. The inset shows the fluorescence intensities for each of the four samples at pH 7.5 (open bars) and pH 12.2 (hatched bars).

A second property associated with acetylation of site 2 was a shift in the pH dependence of fluorescence quenching. The fully acetylated protein appeared to have lost a major quenching event at pH 10. The final fluorescence intensity at high pH was similar in all cases. Thus, acetylation of site 2 may cause quenching of the metal ion dependent fluorophore, and this same fluorophore may also be quenched by a group ionizing at about pH 10. This latter group may correspond to a specific tyrosine residue with somewhat lower than average p*K* or to ionization of acetylation site 2 itself. Further studies are needed to distinguish between these possibilities.

Discussion

This study clearly showed the existence of two acetylation sites that function in the metal ion binding and membrane binding functions of prothrombin fragment 1. The remaining amino groups in fragment 1 appeared to have little importance for either metal ion or membrane binding functions. With the minor qualifications outlined above, the various derivatives formed in this study can be summarized as follows:

fully active	→ partially active	→ completely inactive
(1) native protein	(1) acetylation at site 1	(1) acetylation at sites 1 and 2
(2) calcium-protected acetylated protein	(2) reductively methylated protein	
	(3) amidinated protein	

The fully active protein displayed normal fluorescence quenching in the presence of calcium and bound to membranes with high affinity. It was clear that calcium protected both sites from derivatization. Failure to detect calcium protection of any amino groups by the TNBS assay appeared to be an artifact. The partially active protein was produced in the absence of calcium and showed a nearly normal magnitude of fluorescence quenching but required about 10 times as much calcium to cause that quenching. This derivative also had lost most of its membrane binding function. The completely inactive protein lacked both calcium-induced fluorescence quenching and membrane binding characteristics.

The acetylation proceeded with very different chemical reactivities toward the first and second acetylation site. The

conditions for acetylation are generally considered mild and potentially specific for amino groups (Means & Feeney, 1971). However, the ability to remove the second acetyl group with hydroxylamine at 50 °C was unexpected for an acetamide derivative.

The first site appeared to be very sensitive to even modest changes in structure. On the basis of studies with other proteins (Osheroff et al., 1980; Einspahr & Bugg, 1977), it is reasonable that acetylation of the amino groups may disrupt polar interactions, thus causing an unfolding of the protein and a loss of activity. However, the more subtle modifications afforded by amidination or methylation, where the net positive charge on the protein was unaltered, also greatly affected calcium binding. Both the methylated and amidinated proteins showed a 10-fold shift in the calcium concentration required to give half-maximum protein fluorescence change. That this shift was similar in magnitude to that observed for partially acetylated protein suggested that these derivatives had virtually eliminated the function of site 1. This property suggests that site 1 participated in a very strict steric environment or that it functioned in a specific hydrogen-bonding arrangement which was disrupted by these derivatives.

Site 2 was primarily responsible for the fluorescence quenching events associated with metal ion binding. However, the results showed that this quenching monitors only a portion of the events needed for complete protein function. Site 2 was also anomalous due to its relatively low reactivity but ability to undergo reversal with hydroxylamine. Acetylation of this second site began before all amino groups were derivatized and required about 10-fold as much reagent for complete derivatization. An accompanying study reveals that this site corresponds to a new protein acetylation site that appears unique to fragment 1 (Welsch & Nelsestuen, 1988b).

From these investigations, it is clear that acetylation sites and other modifications of amino groups have major effects on fragment 1 function. Amino groups are seldom implicated as calcium ligands, and it is more likely that these groups are required for some other function such as maintaining proper protein folding. These studies add to the general observation that calcium binding requires substantially more structure than is provided by simple placement of Gla residues in a primary protein sequence (Nelsestuen et al., 1975). Accompanying papers (Welsch & Nelsestuen, 1988a,b) serve to identify the locations of the first and second acetylation sites. Future studies will hopefully demonstrate the precise functions of these groups in calcium and membrane binding.

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