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# A Metal Ion-binding Site in the Kringle Region of Bovine Prothrombin Fragment 1*

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**$^{45}$Ca(II) binding studies (equilibrium dialysis) on the kringle domain of bovine prothrombin fragment 1 were conducted using a mixture of peptides (residues 43–156 and 46–156) resulting from limited α-chymotryptic hydrolysis of fragment 1. Analysis of the Scatchard plot of these data indicates a single, low affinity Ca(II)-binding site to be present. Similar results were obtained from studies on the decarboxylated fragment 1 derivative, 10-γ-MGlu-fragment 1. Acetylation of bovine fragment 1 in the absence of Ca(II) or Mg(II) ions results in the loss of the metal ion-promoted quenching of the intrinsic Trp fluorescence of the protein and the Ca(II)-mediated binding to phosphatidylserine/phosphatidylcholine (PS/PC) vesicles. The acetylation of the $NH_2^\alpha$-group of Ala-1 has been shown (Welsch, D. J., and Nelsestuen, G. L. (1988) *Biochemistry* 27, 4946–4952) to abolish the PS/PC binding property of fragment 1. The present study demonstrates that acetylation of a second site possibly Ser-79 or Thr-81 using the conditions described in the preceding paper results in loss of both the fluorescence transition and the Ca(II)-mediated PS/PC binding of the resulting protein derivative. Removal of the *O*-acetyl group at the Ser-79/Thr-81 site is accomplished by aminolysis with 0.2 M hydroxylamine, pH 10, 50 °C; the fluorescence transition is partially restored. PS/PC binding is partially restored if the $NH_2^\alpha$-group of Ala-1 is trinitrophenylated but is not restored if the $NH_2^\alpha$-group of Ala-1 is acetylated. We conclude that the Ser-79/Thr-81 site may represent a portion of the metal ion-binding site within the kringle domain of fragment 1. Occupancy of this site by a Ca(II) ion appears to be important in the binding of the protein to PS/PC vesicles.**

In the preceding report (1) we described the Ca(II) binding stoichiometry and Ca(II)-mediated phospholipid binding (PS/PC)[1] of several bovine prothrombin fragment 1 (b-F-1) derivatives. These proteins were prepared using the acylation conditions developed by Welsch *et al.* (2–6). Acetylation of b-F-1 in the presence of Ca(II) ions provided $H_2N$-Ala-1-($N^\epsilon$-Ac-Lys)$_5$-F-1. This protein exhibited the characteristic metal ion-promoted quenching of the intrinsic tryptophan fluorescence and the Ca(II)-mediated binding to acidic phospholipid vesicles (PS/PC, 25:75). Acylation of b-F-1 in the presence of Mg(II) provided $N^\alpha$-Ac-NH-Ala-1-($N^\epsilon$Ac-Lys)$_5$-F-1. This derivative exhibited the same fluorescence quenching behavior as the native protein but did not bind to PS/PC vesicles in the presence of Ca(II) when measured by the light scattering assay (7). Welsch and Nelsestuen (6) also reported that acetylation of b-F-1 with acetic anhydride in the presence or absence of Ca(II) ions led to the irreversible modification of His-96. Although modification of His-96 did not affect the metal ion-mediated fluorescence transition or PS/PC binding, the unusual reactivity of this residue was noted.

Acetylation of b-F-1 in the absence of metal ions provided Ac-NH-F-1 which was tentatively assigned (5) as $N^\alpha$-Ac-Ala-1-($N^\epsilon$-Ac-Lys)$_5$-*N*-Ac-β-amido-Asn-101-F-1. Evidence for acetylation of the side chain at the amide nitrogen atom of Asn-101 also containing the *N*-linked carbohydrate of the glycoprotein has been presented (5). This derivative exhibited almost no quenching of the intrinsic fluorescence and no measurable binding to PS/PC vesicles.

These data suggested that a metal ion-binding site was located in the vicinity of Asn-101. Occupancy of this site by either Ca(II) or Mg(II) ions apparently blocked acylation of the amide side chain at Asn-101. Evidence in support of a metal ion-binding site in the kringle domain of b-F-1 was also available from the preliminary analysis of the x-ray crystallographic data obtained from Ca(II) crystals of b-F-1 (8). Crystals grown in the presence of Sr(II) ions yielded Sr(II)/b-F-1 crystals that were fairly isomorphous with the Ca(II)/b-F-1 crystals. A preliminary examination of the vectors of the difference Patterson map between the Sr(II) and Ca(II) crystals suggested the presence of Sr(II) ions at or near Gla-7, Gla-20, and Leu-100.[2] The present study was undertaken to evaluate the nature of the metal ion-binding site that appeared to be located in the kringle domain of the protein.

## EXPERIMENTAL PROCEDURES

### *Chemicals*

*N*-Glycanase (peptide:*N*-glycosidase F) was obtained from Genzyme. Trinitrobenzene sulfonic acid, Schiff's reagent, α-chymotryp-

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[1] The abbreviations used are: PS/PC, phosphatidylserine/phosphatidylcholine vesicles (25:75); Gla, γ-carboxyglutamic acid; γ-MGlu, γ-methyleneglutamic acid; b-F-1, bovine fragment 1 (residues 1–156 of bovine prothrombin); ($N^\epsilon$-Ac-Lys)$_5$, $N^\epsilon$-acetyl-Lys-3,11,44,57,97 residues in bovine fragment 1; Ac-NH-F-1, $N^\alpha$-acetyl-Ala-1-$N^\epsilon$-acetyl-Lys-3,11,44,57,97, *N*-(β-amido)-Asn-101,*O*-Ser-79 or Thr-81-fragment 1; TNP-Ala-1-($N^\epsilon$-Ac-Lys)$_5$-F-1, $N^\alpha$-2,4,6-trinitrophenyl-Ala-1-$N^\epsilon$-acetyl-Lys-3,11,44,57,97-fragment 1; b-F-1(HF), fragment 1 treated with anhydrous hydrogen fluoride; $Ac_2O$, acetic anhydride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.

[2] A. Tulinsky, unpublished observations.



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sin, and trypsin (from bovine pancreas) were obtained from Sigma. $^{14}$C-Acetic anhydride was obtained from Du Pont-New England Nuclear.

*Proteins*

Bovine prothrombin was isolated as described by Mann (10). Bovine prothrombin fragment 1 (b-F-1) was prepared by incubation of prothrombin with ecarin venom as reported by Pollock *et al.* (11). The modified fragment 1 derivatives, TNP-NH-Ala-1-($N^\epsilon$-Ac-Lys)$_5$-F-1 and Ac-NH-F-1 were prepared as described by Weber *et al.* (1). Modification of bovine fragment 1 with the formaldehyde-morpholine reagent according to the procedure of Zapata *et al.* (12) afforded 10-$\gamma$-MGlu-fragment 1. Incubation of fragment 1 with $\alpha$-chymotrypsin at an enzyme/protein ratio of 1:3000 according to the procedure of Pollock *et al.* (12) provided the mixture of peptides 1–42 and 1–45 and the corresponding kringle peptides 43–156 and 46–156. The kringle peptides were separated from the Gla domain peptides using a DEAE-Fractogel column as previously described. Sequence analysis of the kringle peptides indicated that Ala-43 and Thr-46 were the only $NH_2$-terminal residues released.

*Deglycosylated Proteins*

*b-F-1(HF)*—Treatment of bovine fragment 1 with anhydrous hydrogen fluoride as described by Pletcher *et al.* (13) provided a partially deglycosylated fragment 1 preparation, b-F-1(HF). The protein characterized by Pletcher *et al.* contains 2 residues each of *N*-acetylglucosamine (chitobiose) at Asn-77 and Asn-101, respectively. Protein concentrations were determined using $E^{1\%}_{280} = 10.1$ for bovine fragment 1 (14). The concentration of the modified and fully or partially deglycosylated proteins were determined using the BCA protein assay reagent (Pierce Chemical Co.).

*Deglycosylation with N-Glycanase*—A 0.6-ml solution of b-F-1 (1.5 mg/ml) was incubated with 60 $\mu$l of *N*-glycanase solution (250 units/ml) at 37 °C in 0.02 M Tris buffer, pH 7.4 (0.1 M NaCl, 0.02% $NaN_3$) for 18 h (15). The solution was dialyzed overnight using a Pierce Chemical Co. microdialysis system against 0.02 M Tris buffer, pH 7.4 (0.1 M NaCl, 0.02% $NaN_3$). The composition of the product mixture was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) performed according to the procedure of Laemmli (16) using 12% acrylamide gel slabs, 0.75-mm thick. Glycoproteins were detected using Coomassie Blue (Bio-Rad), silver stain (Pierce Chemical Co.), and periodic acid-Schiff stain (Sigma) (17). Under these deglycosylation conditions (absence of $\beta$-mercaptoethanol), the deglycosylation product was a mixture consisting of approximately 10% unreacted b-F-1, 50% of b-F-1 containing one carbohydrate chain, and 40% of fully deglycosylated b-F-1 as estimated with Coomassie Blue-stained gels (data not shown).

When the reaction of b-F-1 with *N*-glycanase was conducted as described above except that 6 $\mu$l of 98% $\beta$-mercaptoethanol (1 $\mu$l of $\beta$-mercaptoethanol, 110 $\mu$l of solution) was added as suggested (Genzyme), complete deglycosylation of b-F-1 resulted (data not shown).

Complete deglycosylation of the acetylated TNP-NH-Ala-1($N^\epsilon$-Ac-Lys)$_5$-F-1 protein was accomplished without the addition of $\beta$-mercaptoethanol to the *N*-glycanase-protein solution. The acetylated TNP-NH-Ala-1-($N^\epsilon$-Ac-Lys)$_5$-F-1 was prepared with either $^{14}$C-acetic anhydride or cold acetic anhydride as described in the following section. A $4 \times 10^{-8}$ M (1.5 mg/ml) sample of the acetylated protein was incubated with 60 $\mu$l of *N*-glycanase (250 units/ml) in 0.02 M Tris buffer, pH 7.4 (0.1 M NaCl, 0.02% $NaN_3$) for 20 h at 37 °C. The reaction mixture was dialyzed overnight in a Pierce Chemical Co. microdialysis system against 0.02 M Tris buffer, pH 7.4 (0.1 M NaCl, 0.02% $NaN_3$).

Removal of the *N*-linked carbohydrate from acylated TNP-NH-Ala-1-($N^\epsilon$-Ac-Lys)$_5$-F-1 was evaluated by SDS-PAGE. The electrophoresis gel was cut in half. One part was stained with 0.1% Coomassie Brilliant Blue A-250. The other half was stained with periodic acid-Schiff reagent by the method of Dubray and Bezard (18).

*$^{14}$C-Acetic Anhydride Labeling of TNP-NH-Ala-1-($N^\epsilon$-Ac-Lys)$_5$-F-1*

A $1.28 \times 10^{-7}$ M sample of protein (2.1 mg/ml) was allowed to react with a 200 M excess of $^{14}$C-acetic anhydride ($2.5 \times 10^{-5}$ mol, 0.25 mCi). A 5-$\mu$l aliquot of the reaction mixture contained $20 \times 10^5$ cpm; the total cpm was $5.6 \times 10^8$ ($2.24 \times 10^{13}$ cpm/M). After addition of the acetic anhydride the pH was maintained at 7.4 by continuous addition of 0.5 N NaOH. After 30 min the reaction mixture was applied to a DEAE-Sepharose column, and the protein was eluted with 0.5 M NaCl. The protein peak was pooled and dialyzed twice at 4 °C against 0.02 M Tris buffer, pH 7.4, containing 0.1 M NaCl and 0.02% $NaN_3$. After dialysis 2.5 mg of protein ($1.07 \times 10^{-7}$ mol) remained containing $3.57 \times 10^6$ cpm. This $^{14}$C content is equivalent to 1.57 mol of $^{14}$C introduced/mol of modified fragment 1.

The preparation of the acetylated TNP-NH-Ala-1-($N^\epsilon$-Ac-Lys)$_5$-F-1 was accomplished using the same conditions as described except that unlabeled acetic anhydride was employed.

*Stability Studies (pH 7.4 and 8.5) on $^{14}$C-Acylated TNP-NH-Ala-1-($N^\epsilon$-Ac-Lys)$_5$-F-1*

A convenient method for the assay of the stability of $^{14}$C-acetylated TNP-NH-Ala-1-($N^\epsilon$-Ac-Lys)$_5$-F-1 at pH 7.4 and 8.5 was gel filtration on a 1.2 × 50-cm P-6 (Bio-Rad) column. Elution of the radiolabeled protein and any $^{14}$C-acetate resulting from hydrolysis of the acetylated protein was accomplished using 0.2 M Tris buffer, pH 7.4 (0.1 M NaCl, 0.02% $NaN_3$). Gel filtration of a 20-$\mu$l aliquot of the purified $^{14}$C-acetylated protein in pH 7.4 Tris buffer and collection of the $^{14}$C counts eluting with the protein in the void volume and the salt in the total included volume allow estimation of the amount of radioactivity lost by hydrolysis.

*Phospholipid Binding and Fluorescence Quenching Studies*

Ca(II)-mediated binding of the proteins to PS/PC vesicles was conducted as previously described (1) using the light scattering assay (7).

*Peptide Mapping*—A limited $\alpha$-chymotrypsin and a trypsin digest of $^{14}$C-acetylated TNP NH-Ala-1-($N^\epsilon$-Ac-Lys)$_5$-F-1 was performed using a 1:100 (M/M) ratio of enzyme to protein. The digest was conducted for 16 h in the presence of $\beta$-mercaptoethanol. The released peptides were separated using a Waters liquid chromatograph equipped with a 150 × 4.5-mm Phenomex (18-W-porex) column. The peptides were separated using a 45 min gradient from 0% mobile phase A to 70% mobile phase B at a flow of 1.0 ml/min. Solvent A was 0.1% trifluoroacetic acid in water; solvent B was 0.1% trifluoroacetic acid in acetonitrile. The peptide fractions were collected using an ISCO V4 variable wavelength detector with scanning at 230 nm. A portion of each peak was transferred to a scintillation vial and examined for $^{14}$C radioactivity.

*Sequence Analysis*—Automatic Edman degradation of the released peptide fractions were performed using an Applied Biosystems Sequenator model 475 SA. The phenylthiohydantoin derivatives were identified by reverse-phase HPLC by comparison with standards at each cycle.

## RESULTS

The previous study of Welsch and Nelsestuen indicated that b-F-1 was acetylated at the $\alpha$-$NH_2$ of Ala-1, the $\epsilon$-$NH_2$ of the 5 lysine residues, *and* the $\beta$-amido nitrogen atom of Asn-101 when the acylation with acetic anhydride was conducted in the absence of Ca(II) or Mg(II) ions. This observation and the preliminary x-ray studies using Sr(II) crystals of b-F-1 suggested a metal ion-binding site within the kringle domain of b-F-1. We conducted equilibrium dialysis experiments with $^{45}$Ca(II) using the kringle peptide mixture (43–156/45–156) and the protein containing fully decarboxylated Gla residues, 10-$\gamma$-MGlu-F-1. Plots of $\bar{v}$ *versus* log of Ca(II) concentration are given in Fig. 7, *A* and *B* (*inset*), for the kringle peptides and 10-$\gamma$-MGlu-F-1, respectively. Scatchard plots are given in Fig. 7, *A* and *B*. These plots support the presumption that a single, weak Ca(II)-binding site ($k^{site}$ = 330 ± 30 $M^{-1}$ and 730 ± 40 $M^{-1}$, respectively) is indeed located within the kringle domain of b-F-1.

In order to probe the molecular consequences of acylation of b-F-1 in the absence of Ca(II) or Mg(II) ions, we studied the acetylation of TNP-NH-Ala-1-($N^\epsilon$-Ac-Lys)$_5$-F-1. This derivative was selected for study since: 1) the $NH_2^\alpha$ and $NH_2^\epsilon$ groups are protected against further acetylation; yet, 2) the protein exhibits the characteristic metal ion-promoted fluorescence quench (34%, Table I) and Ca(II)-mediated binding to PS/PC vesicles ($K_d$ = 1.2 ± 0.61 $\mu$M) (1).

Acetylation of TNP-NH-Ala-1-($N^\epsilon$-Ac-Lys)$_5$-F-1 in the absence of metal ions at pH 7.4 provides a protein which does

TABLE I

*Summary of fluorescence quenching and PS/PC binding properties of modified bovine fragment 1 derivatives*

| Entry | Protein | Fluorescence quench[a] | Ca(II)-mediated PS/PC binding ($K_d$, μM) |
|---|---|---|---|
| | | % | |
| 1 | b-F-1 | 46 | + (0.4) |
| 2 | $H_2N$-Ala-1-($N^\epsilon$-Ac-Lys)$_5$-F-1 | 40 | + (1.1 ± 0.18) |
| 3 | TNP-NH-Ala-1-($N^\epsilon$-Ac-Lys)$_5$-F-1 | 34 | + (1.1 ± 0.61) |
| 4 | Entry 3 + acetic anhydride | 12 | ND[b] |
| 5 | Entry 4 + 0.2 M $NH_2OH$ (pH 10, 50 °C, 1 h) | 24 | +[c] |
| 6 | Entry 4 + *N*-glycanase (37 °C, 18 h) | 9 | ND[b] |
| 7 | Entry 6 + 0.2 M $NH_2OH$ (pH 10, 50 °C, 1 h) | 24 | +[c] |
| 8 | Entry 1 + 0.2 M $NH_2OH$ (pH 10, 50 °C, 1 h) | 46 | +[c] |
| 9 | AcNH-F-1 (entry 1 + acetic anhydride) | 8 | ND[b] |
| 10 | Entry 9 + 0.2 M $NH_2OH$ (pH 10, 50 °C, 1 h) | 24 | ND[b] |

[a] Experiments employed Ca(II) concentrations of 10 mM.
[b] ND = none detected (detection limits for this assay are ≥25 μM under the conditions used).
[c] $K_d$ value not determined.

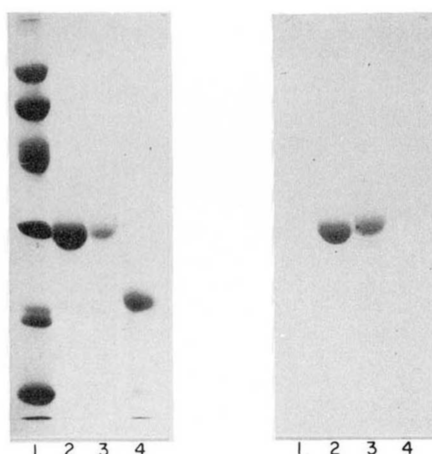



FIG. 1. **PAGE of fragment 1 derivatives using 12% SDS-PAGE gels; thickness, 0.75 mm.** *A*, gel stained with 1% Coomassie Brilliant Blue A-250. *B*, gel stained with periodic acid-Schiff reagent. *Lane 1*, low molecular weight standards; *lane 2*, b-F-1; *lane 3*, acetylation of TNP-NH$^\alpha$-Ala-1-($N^\epsilon$-Ac-Lys)$_5$-F-1 in the absence of metal ions; *lane 4*, protein in *lane 3* after digestion with *N*-glycanase.

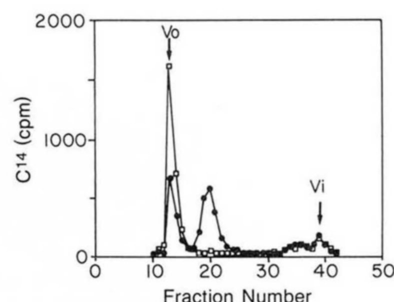



FIG. 2. **Bio-Gel P-6 chromatography of $^{14}$C-acetylated TNP-NH$^\alpha$-Ala-1-($N^\epsilon$-Ac-Lys)$_5$-F-1 prior to deglycosylation with *N*-glycanase, ■.** $^{14}$C-acetylated TNP-NH$^\alpha$-Ala-1($N^\epsilon$-Ac-Lys)$_5$-F-1 after deglycosylation with *N*-glycanase, $V_o$, void volume; $V_i$, total included volume of column.

not bind detectably to PS/PC vesicles and exhibits a fluorescence quench of 12% (Table I). Since the enzyme, *N*-glycanase, is known (15) to hydrolyze *N*-linked carbohydrate side chains of glycoproteins at the β-carbonyl group of the asparagine residue we examined the *N*-glycanase-catalyzed hydrolysis of acetylated TNP-NH-Ala-1-($N^\epsilon$-Ac-Lys)$_5$-F-1 (Fig. 1). Treatment of the acetylated protein with *N*-glycanase at pH 7.4 for 18 h provided the fully deglycosylated protein. Evidence for the complete removal of the acetylated carbohydrate at Asn-101 and the non-acetylated carbohydrate at Asn-77 from acylated TNP-NH-Ala-1-($N^\epsilon$-Ac-Lys)$_5$-F-1 was obtained as follows. After incubation with *N*-glycanase, the protein fraction was analyzed by PAGE. Half of the gel (Fig. 1) was stained with Coomassie Blue to identify protein components. The second half was stained with periodic acid-Schiff reagent to identify protein-bound carbohydrate. The presence of the deglycosylated protein was confirmed by the Coomassie Blue stain (*lane 4*, Fig. 1). The absence of a stained component with periodic acid-Schiff reagent (*lane 4*, Fig. 1) indicated the absence of carbohydrate side chains. Partially deglycosylated b-F-1(HF) containing 2 chitobiose residues at Asn-77 and -101 (13) stained with periodic acid-Schiff reagent and served as a positive control.

We then examined the *N*-glycanase hydrolysis of $^{14}$C-acylated TNP-NH-Ala-1-($N^\epsilon$-Ac-Lys)$_5$-F-1. Acylation with $^{14}$C-acetic anhydride at pH 7.4 followed by purification on a DEAE-Sepharose column provided the $^{14}$C-acetylated protein. Approximately 1.6 mol of $^{14}$C/mol of protein was incorporated under these conditions. Gel filtration of an aliquot of the $^{14}$C-labeled protein solution on a P-6 column (Fig. 2) indicated that 86% of the radioactivity eluted in the void volume ($V_o$) peak; 14% of the radioactivity appeared in the inclusion volume peak ($V_i$). Incubation of the $^{14}$C-acetylated protein with *N*-glycanase in pH 7.4 Tris buffer containing 1% β-mercaptoethanol to insure complete hydrolysis of the carbohydrate side chains followed by gel filtration on a P-6 (Bio-Rad) column provided the elution profile shown in Fig. 2. The peak eluting at the void volume contained 34% of the radioactivity. A second peak contained 53% of the radioactivity; the remaining $^{14}$C-label (10–12%) eluted in the salt peak. The identification of the void volume peak as the deglycosylated protein and the second peak as the [$^{14}$C]*N*-acetyl carbohydrate fraction was accomplished by conducting the *N*-glycanase hydrolysis on a larger scale. Gel filtration provided the elution profile shown in Fig. 3. Examination of the void volume fractions with the BCA protein assay indicated only protein was present in these fractions. Assay of peak two with the periodic acid-Schiff reagent at 555 nm indicated that only $^{14}$C-labeled carbohydrate was contained in this peak.

These results suggested that acylation of TNP-NH-Ala-1-($N^\epsilon$-Ac-Lys)$_5$-F-1 in the absence of metal ions occurred at the β-amido nitrogen of Asn-101 as reported (7) and in addition at another site in the protein. The radiolabeled carbohydrate fraction (*peak 2*, Figs. 2 and 3) presumably represented the 1-*N*-[$^{14}$C]acetylamino-2-deoxy-*N*-acetylamino carbohydrate released by deglycosylation with *N*-glycanase (53% of the total incorporated radioactivity, Fig. 2). The presence of a second acylation site, inert to the action of *N*-glycanase, was

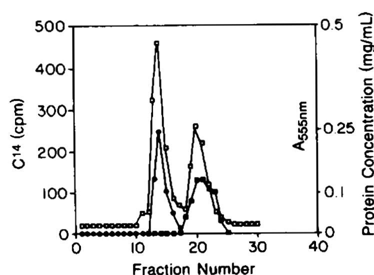



FIG. 3. **Bio-Gel P-6 chromatography of a mixture of TNP-NH$^{\alpha}$-Ala-1-($N^{\epsilon}$-Ac-Lys)$_5$-F-1 acetylated with $^{14}$C- and non-radioactive acetic anhydride.** ■, $^{14}$C cpm; ●, total protein, BCA assay; ■, carbohydrate determined using periodic acid-Schiff reagent.

indicated by the presence of the $^{14}$C-labeled protein peak that eluted in the void volume fractions (34% of the radioactivity, Fig. 2).

The appearance of approximately 14% of the total radioactivity in the salt peak of the elution profile prior to deglycosylation (Fig. 2) suggested some hydrolysis of the incorporated [$^{14}$C]acetyl groups occurred at pH 7.4. A control experiment in which an aliquot of $^{14}$C-acetylated TNP-NH-Ala-1-($N^{\epsilon}$-Ac-Lys)$_5$-F-1 was stored in pH 7.4 Tris buffer containing 1% $\beta$-mercaptoethanol for 12 h at 37 °C was conducted. Gel filtration of the protein solution indicated that 82% of the radioactivity eluted in the protein peak at the void volume; 18% of the radioactivity eluted in the salt peak. No radiolabeled carbohydrate peak was observed. Thus, even at pH 7.4 slow but observable hydrolysis of the incorporated [$^{14}$C]acetyl groups results.

In order to examine the effect of pH and temperature on the hydrolysis of the incorporated radioactivity, we stored $^{14}$C-acetylated TNP-NH-Ala-1-($N^{\epsilon}$-Ac-Lys)$_5$-F-1 in a pH 8.5 Tris buffer (0.1 M NaCl, 0.02% $NaN_3$) containing 1.6 $\mu$l of $\beta$-mercaptoethanol at 37 °C. Aliquots (30 $\mu$l) were withdrawn at various times and assayed by gel filtration on the P-6 column for the amount of radioactivity remaining in the protein peak. The time and percentage of radioactivity remaining in the protein are as follows: 30 min (54%); 60 min (43%); 240 min (40%); 360 min (38%). In these studies radioactivity appeared only in the protein peak eluting at the void volume and the salt peak at the inclusion volume. A radiolabeled carbohydrate peak was not observed. Incubation of the $^{14}$C-acetylated TNP-NH-Ala-1-($N^{\epsilon}$-Ac-Lys)$_5$-F-1 protein with $N$-glycanase for 20 h at 37 °C in pH 8.5 Tris buffer in the absence of $\beta$-mercaptoethanol followed by gel filtration provided a radiolabeled protein peak (13% of the total cpm), a carbohydrate peak (15% of the total cpm), and a salt peak (70% of the total cpm). Incubation of the $^{14}$C-acetylated protein in pH 8.5 Tris buffer containing 1% $\beta$-mercaptoethanol for 20 h at 37 °C followed by gel filtration provided a protein peak (24% of the total cpm), no carbohydrate peak, and a salt peak (76% of the total cpm).

These data are consistent with a pattern of radiolabeling involving [$^{14}$C]acetyl groups at Asn-101 and another site. The second site cannot involve His-96 since acetylation at this position occurs in either the presence or the absence of Ca(II) ions. Indeed the ultraviolet spectrum of $NH_2$-Ala-1-($N^{\epsilon}$-Ac-Lys)$_5$-F-1 indicated that the absorption at 250 nm of this protein (indication of His-96 modification) was enhanced compared to b-F-1. Thus, acetylation and irreversible decomposition of His-96 occurred when b-F-1 was originally treated with labeled acetic anhydride in the presence of Ca(II) ions as observed by Welsch and Nelsestuen (6).

Experiments designed to evaluate the effect of acylation on the Ca(II)-mediated fluorescence transition and lipid binding were conducted as follows. Deglycosylation of $^{14}$C-acetylated TNP-NH-Ala-1-($N^{\epsilon}$-Ac-Lys)$_5$-F-1 with $N$-glycanase followed by gel filtration of an aliquot of the digest provided the elution profile shown in Fig. 4. Treatment of a second aliquot of the digest with 0.2 M hydroxylamine solution, pH 10.0, at 50 °C for 1 h followed by gel filtration removed most of the radioactivity from the protein and carbohydrate fractions (Fig. 4). The distribution of radioactivity was protein peak, 10%; carbohydrate peak, 8%; salt peak, 82%.

A preparative sample of TNP-NH-Ala-1-($N^{\epsilon}$-Ac-Lys)$_5$-F-1 was acetylated with labeled acetic anhydride and digested with $N$-glycanase in the absence of $\beta$-mercaptoethanol. Dialysis against pH 7.4 Tris buffer provided the deglycosylated protein containing an acetyl group in the second site. The Ca(II)-mediated PS/PC binding isotherms of the original protein (*curve 1*, Fig. 5), the acetylated protein (*curve 2*), the deglycosylated protein obtained from $N$-glycanase hydrolysis (*curve 4*), and the acetylated protein treated with 0.2 M hydroxylamine solution (*curve 3*) are given in Fig. 5. It is apparent from these results that the PS/PC binding properties of the protein are lost upon acetylation (*curve 2*) and are not restored by deglycosylation with $N$-glycanase (*curve 4*). However, removal of both of the acetyl groups at Asn-101 and the second site with hydroxylamine does lead to partial restoration of the protein-lipid binding interaction (*curve 3*).

Similar results were observed when the Ca(II)-mediated quenching of the intrinsic tryptophan fluorescence was monitored. The fluorescence quenching of TNP-NH-Ala-1-($N^{\epsilon}$-Ac-Lys)$_5$-F-1 was 34% (entry 3, Table I). Acetylation (entry 4, Table I) reduced the fluorescence of the protein to 12%. Removal of the carbohydrate by $N$-glycanase hydrolysis did not restore the fluorescence quench (entry 6, Table I). However, aminolysis of the incorporated acetyl groups using hy-

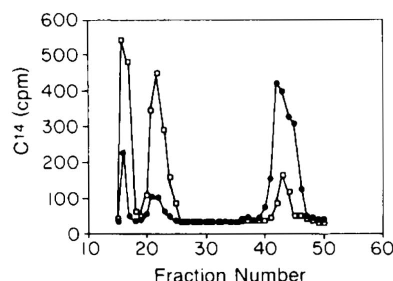



FIG. 4. **Bio-Gel P-6 chromatography of TNP-NH$^{\alpha}$-Ala-1-($N^{\epsilon}$-Ac-Lys)$_5$-F-1 acetylated with $^{14}$C-acetic anhydride in the absence of metal ions followed by deglycosylation with $N$-glycanase.** ■, before reaction with 0.2 M hydroxylamine; ●, after reaction with 0.2 M hydroxylamine, pH 10, 50 °C, 1 h.

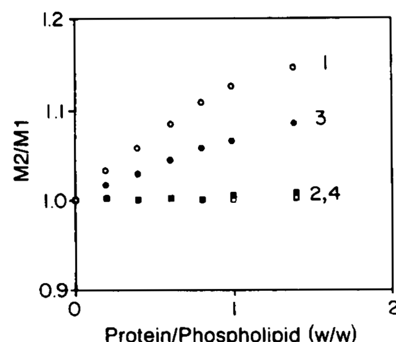



FIG. 5. **Phospholipid binding isotherms using light scattering assay (7).** *1*, TNP-NH$^{\alpha}$-Ala-1-($N^{\epsilon}$-Ac-Lys)$_5$-F-1. *2*, TNP-NH$^{\alpha}$-Ala-1-($N^{\epsilon}$-Ac-Lys)$_5$-F-1 acetylated in the absence of metal ions. *3*, entry 2 incubated with 0.2 M hydroxylamine, pH 10, 50 °C, 1 h. *4*, entry 2 incubated with $N$-glycanase.

droxylamine on either the acetylated protein (entry 5) or the acetylated but deglycosylated protein (entry 6) led to partial restoration (24%) of the fluorescence transition.

Acylation of b-F-1 (entry 1, Table I) with acetic anhydride in the absence of Ca(II) or Mg(II) ions results in a fully acetylated protein, Ac-NH-F-1 (entry 9). The protein does not exhibit detectable PS/PC binding (Fig. 6*B*, *curve 2*), and the fluorescence transition is reduced to 8% (Fig. 6*A*, *curve 2*). Treatment of Ac-NH-F-1 with hydroxylamine partially restored the fluorescence transition (24%, entry 10 and Fig. 6*A*, *curve 3*). The protein (entry 5 and Fig. 6*B*, *curve 3*) did not exhibit PS/PC binding since under these conditions the *N*-acetyl groups at the $NH_2^\alpha$ of Ala-1 and the $NH_2^\epsilon$ of the lysyl residues are not removed. A control experiment (entry 8) indicated that the magnitude of the fluorescence transition of b-F-1 was not affected by 0.2 M hydroxylamine, pH 10.0, for 1 h at 50 °C.

In order to locate the second site of acetylation in $^{14}$C-acetylated TNP-NH-Ala-1-($N^\epsilon$-Ac-Lys)$_5$-F-1, we subjected the protein to a limited digest with $\alpha$-chymotrypsin in the presence of $\beta$-mercaptoethanol. Separation of the radiolabeled peptides (Fig. 8) by HPLC provided three $^{14}$C peptide peaks and a small peak of $^{14}$C-acetic acid (*peak 1*). Complete sequence analysis of *peak 2* (31% of the total radioactivity) indicated that this peptide represented the sequence Arg-75 to Arg-82. Partial sequencing of *peak 3* (40% of the total radioactivity) indicated the peptide resulted from cleavage of the Trp-90/Arg-91 bond. This peptide probably represents the Arg-91/Phe-114 section of the sequence and thus contains the $^{14}$C-acetylated Asn-101 residues identified by Welsch and Nelsestuen. *Peak 4* was a mixture of kringle peptides resulting from cleavage at Trp-42 and Tyr-45.

Since the radiolabeled peptide Arg-75/Arg-82 (*peak 2*, Fig. 8) appeared to result from cleavage of the Tyr-74/Arg-75 and the Arg-82/Ser-83 bonds we suspected the $\alpha$-chymotrypsin was contaminated with trypsin. Thus we conducted a tryptic digest of the $^{14}$C-acetylated TNP-NH-Ala-1-($N^\epsilon$-Ac-Lys)$_5$-F-1 protein. HPLC separation of the digest provided three radioactive peaks in addition to the $^{14}$C-acetic acid (*peak 1*, Fig. 9). *Peak 2* (21% of the radioactivity) was completely sequenced and proved to be the peptide, Gly-76/Arg-82. Partial sequencing of peak 3 (36% of the radioactivity) indicated a mixture of peptides resulting from cleavage at Arg-91, Arg-93, and Arg-148. *Peak 4* (28% of the total radioactivity) was a mixture of two peptides resulting from cleavage at Arg-55 and Arg-93.

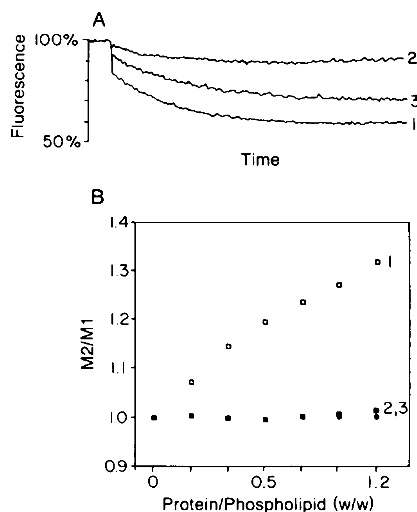



FIG. 6. *A*, Ca(II)-promoted fluorescence quenching, and *B*, phospholipid binding isotherms (7) of b-F-1 (*1*); Ac-NH-F-1 (*2*); Ac-NH-F-1 incubated with 0.2 M hydroxylamine, pH 10, 50 °C, 1 h (*3*).

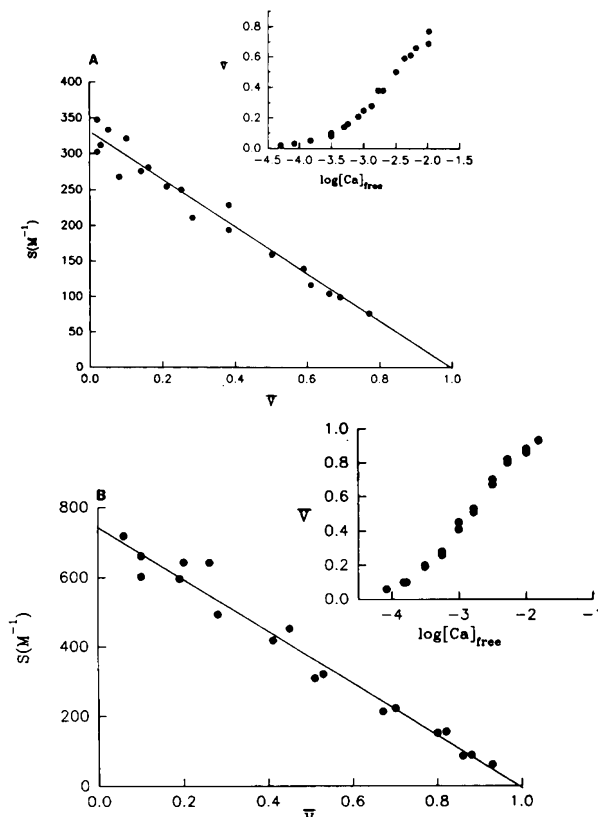



FIG. 7. *A*, Scatchard plot from the equilibrium dialysis data of the kringle peptide mixture (43–156/45–156) with Ca(II). *Inset*, plot of Ca(II) bound, $\bar{\upsilon}$ *versus* log Ca(II) free for kringle peptide mixture. *B*, Scatchard plot from the equilibrium dialysis data of 10-$\gamma$-MGlu-F-1 with Ca(II). *Inset*, plot of Ca(II) bound, $\bar{\upsilon}$, *versus* log Ca(II) free for 10-$\gamma$-MGlu-F1.

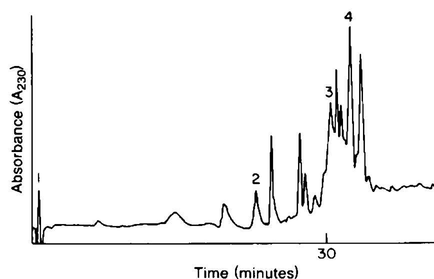



**FIG. 8. HPLC elution profile of limited $\alpha$-chymotryptic digest of TNP-NH$^\alpha$-Ala-1-($N^\epsilon$-Ac-Lys)$_5$-F-1 acylated with $^{14}$C-acetic anhydride in the absence of metal ions.** *Peak 1*, acetic acid (1080 cpm); *peak 2*, Arg-75/Tyr-81 (1550 cpm); *peak 3*, Arg-91/Leu-111 (2652 cpm); *peak 4*, mixture of Ala-43/Arg-156 and Thr-46/Arg-156 (2436 cpm), and Arg-82/Trp-90.

## DISCUSSION

The present study was initiated in order to establish the molecular consequences of the reaction of b-F-1 with acetic anhydride in the absence of Ca(II) or Mg(II) ions. In order to retain the Ca(II)-mediated phospholipid binding properties of the protein and at the same time block acylation of $NH_2^\alpha$ at Ala-1 or the $NH_2^\epsilon$ groups of the lysyl residues, we employed TNP-NH-Ala-1-($N^\epsilon$-Ac-Lys)$_5$-F-1 as the substrate for acetic anhydride. It is important to note that TNP-NH-Ala-1-($N^\epsilon$-Ac-Lys)$_5$-F-1 differs in several respects from b-F-1. As indi-

cated in Scheme 1, the conversion of b-F-1 to TNP-NH-Ala-1-($N^\epsilon$-Ac-Lys)$_5$-F-1 results in the loss of positive cooperativity in Ca(II) binding, a change in the number of Ca(II) ions bound, and a substantial decrease in the affinity of the protein for Ca(II) ions. Nevertheless the PS/PC binding properties and the metal ion-mediated fluorescence transition are retained by the modified protein.

Acetylation of TNP-NH-Ala-1-($N^\epsilon$-Ac-Lys)$_5$-F-1 with $^{14}$C-acetic anhydride provided a $^{14}$C-labeled product containing 1.6 mol of $^{14}$C/mol of protein. The acetylated product did not bind within detection limits to PS/PC vesicles (Fig. 5) and exhibited a reduced fluorescence quench. Similar results were obtained upon acetylation of b-F-1 in the absence of metal ions (Fig. 6, *A* and *B*).

Hydrolysis at the $\beta$-amido carbonyl group of the Asn-101 residue was achieved using *N*-glycanase (15). Control experiments (Fig. 1) indicated that complete deglycosylation of TNP-NH-Ala-1-($N^\epsilon$-Ac-Lys)$_5$-F-1 could be achieved by incubation of the protein with *N*-glycanase at pH 7.4, 37 °C, for 18 h. The non-enzymatic hydrolysis of the $^{14}$C-labeled imide at Asn-101 is estimated at 12–18% under these conditions. The *N*-glycanase-catalyzed hydrolysis of the $^{14}$C-labeled protein provided approximately 50% of the total radioactivity as the 1-*N*-[$^{14}$C]acetylamino-2-deoxy-*N*-acetylamino carbohydrate (Figs. 2 and 4). Approximately 35–40% of the total radioactivity of $^{14}$C-acetylated protein remained in the protein fraction. Thus, 60% of the 1.6 mol of [$^{14}$C]acetyl groups appear to be located at Asn-101 (0.96 mol of $^{14}$C) and 40% (0.6 mol of $^{14}$C) is attached to the second acylation site. However, examination of the PS/PC binding and the fluorescence transition of the protein hydrolyzed with *N*-glycanase (Scheme 1 and Fig. 5) indicated that neither the PS/PC binding nor the fluorescence transition were restored by the enzymic conversion of Asn-77 and 101 to Asp residues.

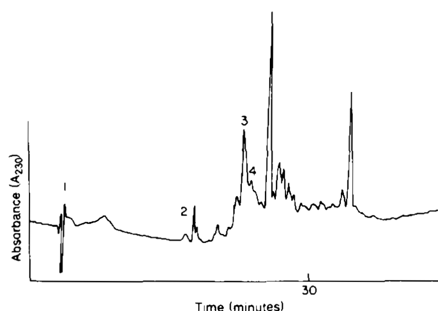


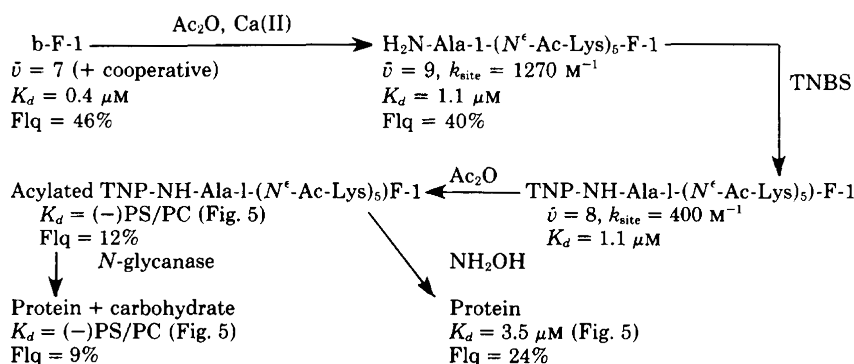

FIG. 9. **HPLC elution profile of tryptic digest of TNP-NH$^\alpha$-Ala-1-($N^\epsilon$-Ac-Lys)$_5$-F-1 acylated with $^{14}$C-acetic anhydride in the absence of metal ions.** *Peak 1*, acetic acid (1120 cpm); *peak 2*, Gly-76/Thr-81 (1776 cpm); *peak 3*, mixture of Tyr-94/Leu-110, Ser-92/Leu-110, Val-149/Arg-156 (2976 cpm); *peak 4*, mixture of Tyr-94/Arg-111 and Glu-56/Arg-75 (2364 cpm).

The location of the second acylation site within the protein was suggested by the isolation of $^{14}$C-labeled peptides from the chymotryptic hydrolysis (Fig. 8) and tryptic hydrolysis (Fig. 9) of $^{14}$C-acetylated TNP-NH-Ala-1-($N^\epsilon$-Ac-Lys)$_5$-F-1. Both enzymatic digests provided radiolabeled peptides corresponding to the 75–82 region of the protein. This region contains Asn-77 (the site of the second *N*-linked carbohydrate in b-F-1), Ser-79, and Thr-81. Presumably these side chains represent the only possible acylation sites in this sequence. Acylation of the $\beta$-amido nitrogen atom of Asn-77, in analogy to the same reaction that results at Asn-101, can be tentatively ruled out on the basis of the *N*-glycanase experiments. *N*-Glycanase hydrolysis of TNP-NH-Ala-1-($N^\epsilon$-Ac-Lys)$_5$-F-1 removes the carbohydrate side chains at both positions. However *N*-glycanase hydrolysis of the $^{14}$C-acetylated TNP protein leaves 35–40% of the radioactivity bound to the protein. Non-enzymatic hydrolysis at pH 8.5, 37 °C also leaves approximately 40% of the $^{14}$C-label bound after 6 h. If [$^{14}$C] acetyl imides were present at both Asn-77 and -101, these would be expected to exhibit similar chemical reactivity under hydrolytic conditions. Thus, the location of the acetyl group at Asn-77 appears unlikely.

*O*-Acetylation at the side chain of either Ser-79 or Thr-81 would appear to be the most likely possibility for the second acylation site. Since the [$^{14}$C]acetyl group is removed from the second site in 1 h by the action of 0.2 M hydroxylamine at pH 10.0, 50 °C (Fig. 4) the acetylation product could be the *O*-acetyl ester of either residue. The partial restoration of both the PS/PC binding and the fluorescence transition (Scheme 1 and Fig. 5) upon treatment of the TNP-protein with hydroxylamine suggest a fundamental role for the residues involved in this region of the protein molecule. These results suggested that treatment of Ac-NH-F-1 with 0.2 M hydroxylamine should restore the fluorescence transition of b-F-1 acetylated in the absence of metal ions. However, no effect on PS/PC binding would be expected since acylation at $NH_2^\alpha$ of Ala-1 would not be reversed by the action of hydroxylamine. This proved to be the case (Fig. 6, *A* and *B*) treatment of Ac-NH-F-1 with hydroxylamine provided a protein with a partially restored (24%) fluorescence transition.

Acetylation at Asn-101 and the second site is blocked by the presence of Ca(II) or Mg(II) ions. This could result from either a metal ion-binding site located in this region of the protein or from a Ca(II) or Mg(II) ion-promoted conformation change which somehow protects these residues from acylation. $^{45}$Ca(II) binding studies to the mixture of kringle peptides (43–156/46–156) produced by the limited $\alpha$-chymotryptic hydrolysis of b-F-1 (Fig. 7*A*) indicated a single, low affinity Ca(II) site to be present in the kringle region. This site should also be observed in a b-F-1 derivative in which the Gla residues were decarboxylated. Equilibrium dialysis studies on

SCHEME 1. **Summary of the modifications conducted on b-F-1.** $\bar{\upsilon}$ = [Ca(II)]$_{bound}$/[protein] · [Ca(II)]$_{free}$; $K_d$ = dissociation constant for lipid-protein; $k_{site}$ = site binding constant for Ca(II); Flq = percentage of intrinsic fluorescence quench.



10-γ-MGlu-F-1 (Fig. 7*B*) also revealed a single, low affinity Ca(II) site.

We were led to look for a metal ion-binding site in the kringle domain by the acetylation studies of Welsch and Nelsestuen and the initial inferences from the Patterson difference map of Sr(II)/b-F-1 crystals with Ca(II)/b-F-1 crystals. The Ca(II)/b-F-1 and Ca(II)/apo b-F-1 structures have now been refined at high resolution (2.2 Å) (9).[3] The differences between the Ca(II)/b-F-1 and Ca(II)/apo b-F-1 structures in the vicinity of Ser-79/Thr-81 and Asn101 appear to be secondary structural changes resulting from a major rigid body conformational shift of about 30° in the relative position of the 36–47 helix of the Gla domain and not due to an intrinsic Ca(II) site. The difference Patterson vectors arising from Asn-101 between Sr(II)/b-F-1 and Ca(II)/b-F-1 crystals are most likely also due to such changes. Thus, we find a low affinity Ca(II) site in the kringle domain by equilibrium dialysis that is not seen in the crystal structure. The differences in these observations might be due to: 1) intrinsic differences in the crystal and solution structures of b-F-1 or differences between b-F-1 and TNP-NH-Ala-1-($N^{\epsilon}$-Ac-Lys)$_5$-F-1; 2) the affinity of the site is too low to be confirmed by election density methods; or 3) the carbohydrate side chains at Asn-77 and -101 which are disordered in both crystal structures (with and without Ca(II) ions) may be involved in defining a disordered Ca(II)-binding site. Further studies will be required to probe these options.

[3] Soriano-Garcia, M., Padmanabhan, K., de Vas, A. M., and Tulinsky, A. (1991) manuscript submitted.

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