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# Comparison of the $Ca^{2+}$ binding properties of the γ-carboxyglutamic acid-containing module of protein Z in the intact protein and in N-terminal fragments

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Protein Z is a vitamin K-dependent plasma protein of unknown function. Its modular structure is identical with those of factors VII, IX, X, and protein C. These proteins have an N-terminal γ-carboxyglutamic acid (Gla)-containing module which binds six to ten $Ca^{2+}$. In factors IX, X, and protein C, the adjacent epidermal growth factor (EGF)-like module binds one $Ca^{2+}$ whereas the EGF-like module in protein Z does not. We have compared the $Ca^{2+}$ binding properties of a fragment of protein Z comprising the Gla and N-terminal EGF-like modules (pZ-GlaEGF$_N$) with those of intact protein Z and the isolated Gla module by measuring the $Ca^{2+}$-induced quenching of the intrinsic protein fluorescence. The similar $Ca^{2+}$ affinities of pZ-GlaEGF$_N$ and protein Z indicate that pZ-GlaEGF$_N$ has a native conformation and normal $Ca^{2+}$ binding properties. A comparison of the $Ca^{2+}$ binding to pZ-GlaEGF$_N$ with those to the corresponding fragments of factors IX, X, and protein C indicate that $Ca^{2+}$ binding to the N-terminal EGF-like modules in the latter proteins does not influence the folding and $Ca^{2+}$ binding properties of their Gla modules. Furthermore, the $Ca^{2+}$-induced fluorescence enhancements of GlaEGF fragments from factors IX, X, and protein C appear to be caused by $Ca^{2+}$ binding to the site in the EGF-like modules since it is not observed for pZ-GlaEGF$_N$.

Protein Z; Vitamin K-dependent; $Ca^{2+}$ binding; Gla module; EGF-like module

## 1. INTRODUCTION

Protein Z is a vitamin K-dependent plasma protein with a modular assembly identical with those of factors VII, IX, X, and protein C (Fig. 1) [1–4]. These homologous proteins have several postribosomal modifications in common. For protein Z this includes γ-carboxylation of the first 13 Glu residues [1,5,6], as well as β-hydroxylation of an Asp residue [1,7] and *O*-glycosylation of a Ser residue [8] in the N-terminal of two epidermal growth factor (EGF)-like modules. The C-terminal region of protein Z is homologous to the serine protease modules of factors VII, IX, X, and protein C. In contrast to these four proteins, however, protein Z can not be converted to an active enzyme, as the Asp and His residues in the catalytic triad are replaced by Thr and Ala, respectively [1]. Although protein Z was isolated several years ago [5,9,10], its function remains unknown.

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*Abbreviations:* EGF, epidermal growth factor; Gla, γ-carboxyglutamic acid; Hya, β-hydroxyaspartic acid; pZ, bovine protein Z; pZ-GlaEGF$_N$, residues 1–86/88 of bovine protein Z; pZ-Gla, residues 1–45 of bovine protein Z; fX-GlaEGF$_N$, residues 1–86 of the light chain of bovine factor X; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Factors IX, X, and protein C all have one or two γ-carboxyglutamic acid (Gla)-independent $Ca^{2+}$ binding sites [11–13], one of which is located in the N-terminal EGF-like module [14–18]. The sequence preceding the first Cys residue in the N-terminal EGF-like modules of these proteins is Asp-Gly-Asp-Gln, corresponding to residues 46 to 49 in bovine factor X. Recently, the sidechains of Asp-46, Gln-49, and the β-hydroxyaspartic acid (Hya) residue in position 63 were identified as $Ca^{2+}$ ligands in factor X [19]. Studies of synthetic factor IX EGF-like modules [20] and mutant factor IX molecules [21] have also shown the Asp and Gln residues, corresponding to residues 46, 48, and 49 in factor X, to be important for $Ca^{2+}$ binding and biological activity. Protein Z, which appears not to have a Gla-independent $Ca^{2+}$ binding site [22], has the sequence Gly-Gly-Ser-Pro in the positions corresponding to residues 46–49 of factor X, and thus lacks residues identified as $Ca^{2+}$ ligands [1,19]. The $Ca^{2+}$affinity of the site in the EGF-like module of factor X is higher in a fragment consisting of the Gla module linked to the N-terminal EGF-like module (fX-GlaEGF$_N$, $K_d \approx 100\ \mu M$) than in the isolated EGF-like module ($K_d$ = 1–2 mM) [16,23]. Similar results have been obtained with fragments of factor IX and protein C [24,25]. This may be due to a more mobile N-terminus in the isolated EGF-like modules [26] or to a contribution to the $Ca^{2+}$ binding from the Gla module. Binding of $Ca^{2+}$to fX-GlaEGF$_N$ and to the corresponding region in intact factor X are indistinguishable, as judged by the

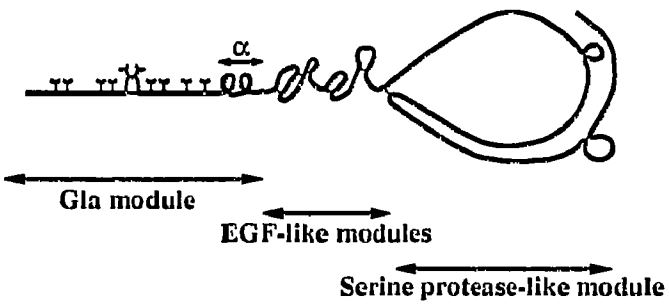



Fig. 1. Schematic representation of the modular structure of bovine protein Z. α denotes the α-helical region in the C-terminal part of the Gla module which contains a cluster of aromatic residues. The pairing of six cysteines in the serine protease-like module was made based on homology with factor X [30], and four cysteines were left unpaired.

$Ca^{2+}$-induced fluorescence changes, indicating that fX-GlaEGF$_N$ has a native conformation [23].

Not only the isolated N-terminal EGF-like modules but also the isolated Gla modules of factors IX and X bind $Ca^{2+}$ with lower affinity than when they are part of the intact proteins or of fragments consisting of the Gla plus EGF-like modules [23,24]. It is conceivable that $Ca^{2+}$ binding to the N-terminal EGF-like module influences folding and $Ca^{2+}$ binding of the adjacent Gla module. As factors IX and X contain a $Ca^{2+}$ binding site in the N-terminal EGF-like module whereas protein Z does not, we have characterized the $Ca^{2+}$ binding properties of a fragment comprising the Gla and N-terminal EGF-like modules of protein Z (pZ-GlaEGF$_N$). The $Ca^{2+}$-induced changes in intrinsic fluorescence emission of pZ-GlaEGF$_N$ were compared to those of intact protein Z, its isolated Gla module (pZ-Gla), and the corresponding fragments of factors IX, X, and protein C. The results show that the Gla module in pZ-GlaEGF$_N$, as opposed to pZ-Gla, binds $Ca^{2+}$ with the same affinity as intact protein Z. Moreover, the presence of the (non-$Ca^{2+}$-binding) N-terminal EGF-like module increases the $Ca^{2+}$affinity of the Gla module approximately to the same extent as the $Ca^{2+}$-binding EGF-like modules do in factors IX, X, and protein C. This implies that $Ca^{2+}$ binding and folding of the Gla modules of factors IX, X, and protein C are not affected by the $Ca^{2+}$ binding site in their N-terminal EGF-like modules. Instead the hydrophobic stack and/or EGF-like module *per se* appear to be important to stabilize the Gla module.

## 2. MATERIALS AND METHODS

### 2.1. *Proteins*

Bovine protein Z was purified according to Hashimoto et al. [27], but using DEAE-Sephacel (Pharmacia-LKB Biotechnology) in the first column chromatography step. Protein Z (0.7 mg/ml) in 50 mM Tris-HCl, pH 8.0, 0.1 M NaCl and 2 mM EDTA was digested with 10% (w/w) protease from *Staphylococcus aureus V8* (Sigma) at 37°C for 7 h to isolate pZ-GlaEGF$_N$. After concentration in a Filtron Novacell (cutoff level 5 kDa), the digest was chromatographed on a 1.6×9-cm column of Q-Sepharose Fast Flow (Pharmacia-LKB Biotechnology). The column was equilibrated with 50 mM Tris-HCl, pH 7.5, 0.1 M NaCl and eluted with a linear NaCl gradient (400 ml) from 0.1 to 0.8 M in the same buffer at 40 ml/h, 6.5-ml fractions being collected. The Gla module from bovine protein Z (pZ-Gla) was isolated as described by Morita et al. [22]. The absorption coefficients, $A^{1\%}_{1cm}$ at 280 nm, and molecular weights used to calculate protein concentrations were as follows: bovine protein Z 9.77 and 50,000 [22], pZ-GlaEGF$_N$ 19.3 and 10,457 (apoprotein), and pZ-Gla 20 and 6,094. The absorption coefficients of the two protein Z fragments were determined as previously described [16].

### 2.2. *Intrinsic protein fluorescence measurements*

Prior to spectrofluorimetry, protein Z, pZ-GlaEGF$_N$, and pZ-Gla were subjected to gel filtration on a 1.5 × 92-cm column of Sephadex G-75 or Sephadex G-50 (Pharmacia-LKB Biotechnology) equilibrated with $Ca^{2+}$-free 50 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl. Intrinsic protein fluorescence was measured at 25°C in an SLM 4800S spectrofluorimeter (SLM-Aminco Instr., Urbana, IL). Protein concentrations were 2 μM, and the exposure to exciting light was minimal to avoid photodecomposition. Fluorescence emission spectra were recorded between 300 and 400 nm with an excitation wavelength of 280 nm. The excitation and emission bandwidths were 2 and 4 nm, respectively. Spectra were recorded in the presence of 0.1 mM EDTA and after the addition of $CaCl_2$ to 3.1 mM (protein Z and pZ-GlaEGF$_N$) or to 10.1 mM (pZ-Gla), final concentrations. All spectra were normalized to values for the spectra in the absence of $Ca^{2+}$ of 1.00 at the wavelength of emission maximum.

The proteins were titrated by 1–10-μl additions of a 93.0 mM stock solution of $CaCl_2$ to 2 ml sample. The emission intensity was measured 30 s after each addition of $CaCl_2$ by averaging 30 signal readings of 0.25 s each. The excitation wavelength was 280 nm and the emission wavelengths were 340 nm for protein Z and 350 nm for pZ-GlaEGF$_N$ and pZ-Gla. The excitation and emission bandwidths were 2 and 8 nm, respectively. Corrections were made for successive dilution of the samples. All experiments were carried out in duplicate. The data were expressed as relative fluorescence intensities ($F/F_0$), where $F$ and $F_0$ are the emission intensities in the presence and absence of $Ca^{2+}$, respectively. No increase in light scattering was observed for pZ-Gla at 10 mM $Ca^{2+}$, which indicated that no aggregation took place. The fluorescence quenchings were reversed by the addition of excess EDTA.

### 2.3. *Susceptibility to chymotrypsin*

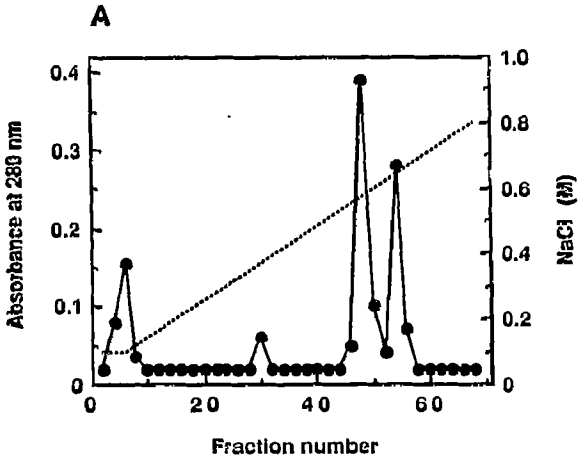
Bovine protein Z (0.8 mg/ml) in 50 mM Tris-HCl, pH 8.0, containing 0.1 M NaCl and 2 mM EDTA or 2 mM $CaCl_2$, was incubated at ambient temperature with 0.25% (w/w) α-chymotrypsin (Sigma). Aliquots of 20 μg were withdrawn at different times and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10–15% gradient gel.

### 2.4. *Other methods*

SDS-PAGE and amino acid analyses were performed as described elsewhere [16]. N-terminal amino acid sequences were determined on an Applied Biosystems 475A pulsed liquid sequencer according to the manufacturer's instructions.

## 3. RESULTS AND DISCUSSION

The Glu residues in positions 86 and 88 [1], i.e. between the two EGF-like modules of protein Z, suggested that V8 protease digestion could be used to isolate pZ-GlaEGF$_N$. After incubation of protein Z with 10% V8 protease for 7 hours, pZ-GlaEGF$_N$ was isolated by ion-exchange chromatography (Fig. 2A). The first major peak (fractions 46–50) contained intact protein Z. The second peak (fractions 53–56), eluting with approximately 0.6 M NaCl, contained pZ-GlaEGF$_N$ which



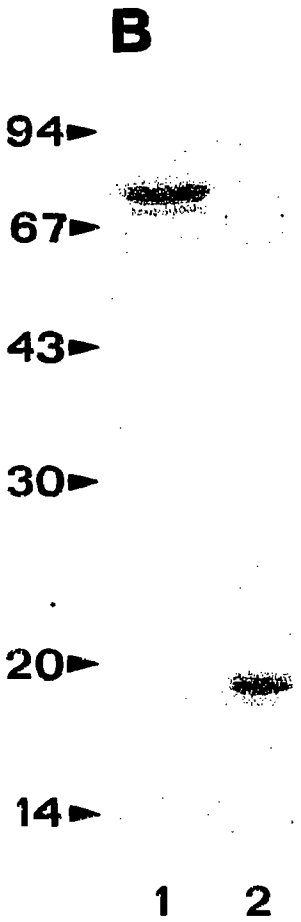




Fig. 2. (A) Ion-exchange chromatogram of bovine protein Z digested with V8 protease. The digest (11.7 mg) was chromatographed on a column of Q-Sepharose Fast Flow as described under Materials and Methods. Fractions 46–50 and 53–56 contained intact protein Z and pZ-GlaEGF$_N$, respectively. (B) SDS-PAGE of the pooled fractions 46–50 (protein Z, lane 1) and 53–56 (pZ-GlaEGF$_N$, lane 2) on a 10–20% gradient gel. The arrows show the positions of molecular mass markers (kDa).

appeared to be homogeneous as judged by SDS-PAGE (Fig. 2B). N-terminal sequence analysis of pZ-GlaEGF$_N$ gave the sequence Ala-Gly-Ser-Tyr, corresponding to residues 1–4 of bovine protein Z. No internal cleavage was observed. The amino acid composition and the Gla and Hya contents were in agreement with residues 1–86 or 1–88 of bovine protein Z or a mixture of both (Table 1). Approximately 1 mg of pZ-GlaEGF$_N$ was obtained from 11–14 mg of protein Z, corresponding to ≈ 35% recovery.

The effects of $Ca^{2+}$ on the intrinsic fluorescence emission of intact protein Z and protein Z fragments were characterized. The spectrum of pZ-GlaEGF$_N$ blue-shifted dramatically, with emission maxima at 354 nm in the absence of $Ca^{2+}$ and 340 nm in its presence, indicating a more hydrophobic environment around one or both Trp residues (positions 19 and 42) in the presence of $Ca^{2+}$ (Fig. 3). There was no significant $Ca^{2+}$-induced change in wavelength of emission maximum for protein Z and pZ-Gla. The fluorescence emission was monitored during titrations of protein Z and the protein Z fragments with $Ca^{2+}$. The $Ca^{2+}$ affinity of the Gla module was estimated from the half-maximal quenching. For protein Z and pZ-GlaEGF$_N$, half-maximal fluorescence quenching occurred at approximately 0.8 and 0.6 mM $Ca^{2+}$, respectively (Figs. 4A and B), indicating that the Gla module of pZ-GlaEGF$_N$ retained its native $Ca^{2+}$ binding conformation. In contrast, for pZ-Gla half-maximum occurred at 2.5–3 mM (Fig. 4C). It is noteworthy that, unlike the corresponding fragments

Table 1

Amino acid composition of pZ-GlaEGF$_N$

| Amino acid | Found | Calculated |
|---|---|---|
| Asp | 6.7 | 6 |
| Thr | 4.1 | 4 |
| Ser | 5.5 | 6 |
| Glu | 18.3 | 18 |
| Pro | ND[a] | 4 |
| Gly | 7.6 | 8 |
| Ala | 7.2 | 7 |
| Cys[b] | 5.3 | 8 |
| Val | 2.4 | 2 |
| Met | 0.9 | 1 |
| Ile | 2.3 | 2 |
| Leu | 5.1 | 5 |
| Tyr | 4.6 | 5 |
| Phe | 3.9 | 4 |
| His | 0.9 | 1 |
| Lys | 1.0 | 1 |
| Arg | 2.7 | 3 |
| Trp | ND | 2 |
| Gla | 10.4 | 13 |
| Hya | 0.9 | 1 |

The calculated numbers refer to the sequence data for residues 1–88 of bovine protein Z [1].

[a] Not determined

[b] Determined as cystine.

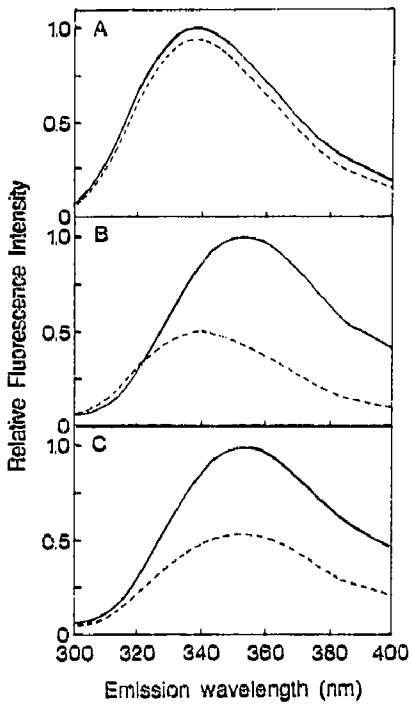



Fig. 3. Calcium-induced changes in the intrinsic fluorescence spectra of bovine protein Z and protein Z fragments. Normalized emission spectra of protein Z (A), pZ-GlaEGF$_N$ (B) and pZ-Gla (C) were acquired at 25°C in 50 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl, in the presence (- - - - -) and absence of $Ca^{2+}$ (——). Protein concentrations were 2 $\mu$M and the total $Ca^{2+}$ concentrations were 3 mM for protein Z and pZ-GlaEGF$_N$ and 10 mM for pZ-Gla.

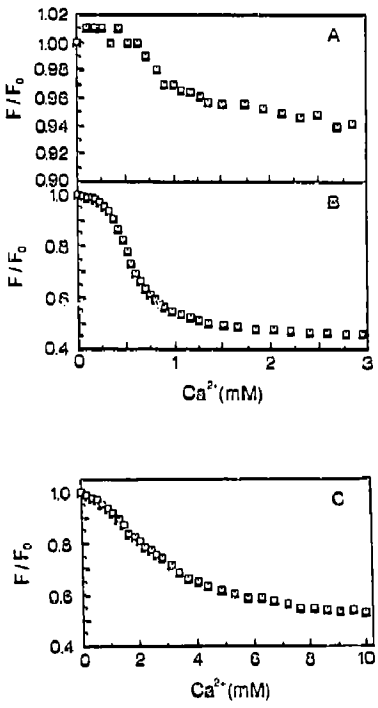



Fig. 4. Calcium dependence of the intrinsic fluorescence of intact and fragments of bovine protein Z. Protein Z (A), pZ-GlaEGF$_N$ (B) and pZ-Gla (C) were titrated with $CaCl_2$ as described under Materials and Methods. $F$ and $F_0$ are the emission intensities in the presence and absence of $Ca^{2+}$, respectively.

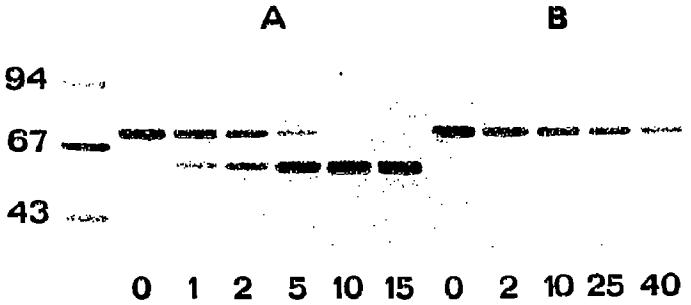



Fig. 5. The effect of $Ca^{2+}$ on the susceptibility of the peptide bond between Tyr-45 and Met-46 in bovine protein Z to chymotrypsin. Protein Z was digested with 0.25% (w/w) $\alpha$-chymotrypsin in the presence of 2 mM EDTA (A) or 2 mM $Ca^{2+}$ (B) as described under Materials and Methods. The digestion time in minutes is given below each lane. The outer left lane shows the positions of molecular mass markers (kDa).

from protein C and factors IX and X [23–25], pZ-GlaEGF$_N$ did not exhibit an initial increase in fluorescence emission at low $Ca^{2+}$ concentrations. This suggests that the fluorescence enhancement is correlated to the Gla-independent $Ca^{2+}$ binding site in the first EGF-like module.

There are several possible explanations of the higher $Ca^{2+}$ affinity of the Gla module when linked to the EGF-like module as compared to the affinity of the free Gla module: (1) the EGF-like module serves as a scaffold for the folding of the Gla module; (2) $Ca^{2+}$ binding to the site in the EGF-like module is required for the Gla module to attain a native conformation; and (3) the aromatic residues in the $\alpha$-helical hydrophobic stack in the C-terminal part of the Gla module (residues 38–45 in factor X) is a nucleation site for the folding of the Gla module. The $Ca^{2+}$ titration of pZ-GlaEGF$_N$ showed half-maximal quenching at a $Ca^{2+}$ concentration similar to that observed for intact protein Z but below that required by pZ-Gla. Similar results have previously been obtained with the corresponding fragments of factors IX and X, which contain a $Ca^{2+}$ binding site in the EGF-like module [23,24]. This suggests that $Ca^{2+}$ binding to the EGF-like module in factors IX, X and protein C is not a prerequisite for normal $Ca^{2+}$ binding to the Gla module. However, our $Ca^{2+}$ binding data do not resolve whether the EGF-like module is essential for the folding of the Gla module or whether cleavage in the $\alpha$-helical hydrophobic cluster precludes normal folding of the Gla module. The hydrophobic stack is in an $\alpha$-helix which may be a nucleation site for the folding of the Gla module [28]. It should be noted that the three-dimensional structure of prothrombin fragment 1 demonstrates a direct interaction between the Gla module and the disulfide loop that connects the Gla and kringle modules [28].

To investigate the possible role of the hydrophobic stack, the $Ca^{2+}$ effect on its accessibility was studied. In factors IX and X, chymotryptic cleavage in the hydro-

phobic stack at a position corresponding to the peptide bond between Tyr-45 and Met-46 in bovine protein Z is prevented in the presence of $Ca^{2+}$ [23,24,29]. Although protein Z lacks the $Ca^{2+}$ binding site in the N-terminal EGF-like module, the presence of the metal ion conferred resistance to cleavage of the peptide bond between Tyr-45 and Met-46 (Fig. 5). It thus appears as if the conformational change that prevents chymotrypsin from hydrolyzing in this position solely is a consequence of $Ca^{2+}$ binding to Gla-dependent site(s). Consistent with this hypothesis, no $Ca^{2+}$-induced protection against chymotryptic cleavage was observed for decarboxylated fX-GlaEGF$_N$ [23].

Our results with protein Z and factor X [16,23] suggest that a covalently intact hydrophobic stack (corresponding to residues 38–45 in factor X) and/or the N-terminal EGF-like module are a prerequisite for native conformation of and normal $Ca^{2+}$ binding to the Gla modules in the vitamin K-dependent plasma proteins. However, the $Ca^{2+}$ binding site in the N-terminal EGF-like modules appears not to be important in this respect.

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