Vitamin K-dependent protein S, a cofactor of the anticoagulant enzyme-activated protein C, has four epidermal growth factor (EGF)-like modules, all of which have one partially hydroxylated Asp (EGF 1; \( \beta \)-hydroxyaspartic acid) or Asn (EGF 2, 3, and 4; \( \beta \)-hydroxyasparagine) residue. The three C-terminal modules have a typical Ca\(^{2+} \) binding sequence motif that is usually present in EGF modules with hydroxylated Asp/Asn residues. Using the chromophoric Ca\(^{2+} \) chelators Quin 2 and 5,5′-Br2BAPTA, we have now determined the Ca\(^{2+} \) affinity of recombinant fragments containing EGF modules 1–3, 1–4, 2–3, and 2–4. EGF modules 1–4 and 2–4 each contains two very high affinity Ca\(^{2+} \) binding sites, i.e., with dissociation constants ranging from \( \times 10^{-10} \) to \( \times 10^{-8} \) M in the absence of salt and from \( \times 10^{-8} \) to \( \times 10^{-6} \) M in the presence of 0.15 M NaCl. In contrast, in EGF 1–3 and EGF 2–3, the Ca\(^{2+} \) affinity is 2–4 orders of magnitude lower. EGF 4 thus appears to have the highest Ca\(^{2+} \) affinity, and furthermore it seems to influence the Ca\(^{2+} \) affinity of its immediate N-terminal neighbor EGF 3 by a factor of approximately 230. In addition, EGF 4 seems to influence the Ca\(^{2+} \) affinity of EGF 2 by a factor of approximately 25. The Ca\(^{2+} \) affinity of the binding sites in EGF modules 3 and 4 in fragments EGF 1–4 and EGF 2–4 is \( \times 10^{-10} \) to \( \times 10^{-8} \)-fold higher than in the corresponding isolated modules, implying important contributions to the Ca\(^{2+} \) affinity of each module from interactions with neighboring modules. This difference is much higher than the approximately 10-fold difference previously found in similar comparisons of EGF modules from fibrinogen. However, the modules studied in protein S and fibrillin appear to have the similar Ca\(^{2+} \) ligands. The structural basis for the difference in Ca\(^{2+} \) affinity is not yet understood.

Protein S is a vitamin K-dependent plasma protein that functions as a cofactor to activated protein C, a regulator of blood coagulation, by enhancing the activated protein C-mediated rates of degradation of factors Va and VIIIa (1–3). Protein S consists of an N-terminal γ-carboxyglutamic acid (Gla)\(^7\)-containing module that is followed by a module with a thrombin-sensitive peptide bond and four epidermal growth factor (EGF)-like modules, whereas the C-terminal half of the molecule is occupied by a module that is homologous to steroid hormone-binding proteins (4–7). Calcium binding to vitamin K-dependent clotting factors, including protein S, is complex, >10 calcium ions being bound to 2 or 3 types of sites that profoundly influence the function of these proteins (8). The Gla module binds Ca\(^{2+} \) and endows protein S with membrane affinity, whereas the EGF module-containing part of protein S appears to be involved in the interaction with activated protein C (9, 10).

The EGF modules in protein S are members of a large family of similar modules that are found in extracellular and membrane membrane proteins in species ranging from Caenorhabditis elegans to Drosophila melanogaster and humans (7, 11, 12). Several of these proteins are involved in cell differentiation, blood coagulation and fibrinolysis, and in the complement and fibrinolytic systems. The EGF modules are approximately 45 amino acids long and contain 6 cysteine residues that are paired in a characteristic manner: 1 to 3, 2 to 4, and 5 to 6, with a double-stranded \( \beta \)-sheet as the main structural feature. EGF modules provide a structural scaffold that supports protein-protein interactions, serves as spacer units, and orients adjacent modules relative to each other in a way that sustains biological activity.

EGF modules 2–4 of protein S belong to a large subgroup of such modules that has a Ca\(^{2+} \) binding sequence motif DI/VDE (or variants thereof) before the first Cys residue (13, 14), and the motif XD\(^9\)/N\(^9\)XXXXY/FX between the third and fourth Cys residue that is required for hydroxylation of Asp/Asn (denoted by an asterisk in the sequence) to \( \beta \)-hydroxyaspartic acid or \( \beta \)-hydroxyasparagine (12, 15, 16). The Asp*/Asn* and Tyr/Phe residues are adjacent in the major double-stranded \( \beta \)-sheet. The two sequence motifs appear to be coupled and phylogenetically conserved in Ca\(^{2+} \) binding EGF modules. There are however exceptions, such as the first EGF module of protein S, which contains the hydroxylation motif but lacks the Ca\(^{2+} \) consensus sequence before the first Cys residue.

Calcium binding to an EGF module was first observed in protein C and subsequently in factors IX and X (17–20). The Ca\(^{2+} \) affinity of isolated EGF-like modules is low and roughly equal (\( K_d = 0.5–8 \) mM at 0.15 M NaCl) (19, 20, 22), although the reported Ca\(^{2+} \) affinities for EGF sites in intact proteins vary from moderate (\( K_d = 0.1 \) mM) to very strong (\( K_d < 10 \) nM) (23–27). This is in accord with a large body of experimental data for different types of Ca\(^{2+} \)-binding proteins. These data...
show that the observed Ca\(^{2+}\) affinity of a specific site is not governed solely by local interactions between the calcium ion and its coordinating oxygens provided by the backbone and side chains of the protein as well as water molecules. Major contributions are provided by other parts of the protein, and long range interactions can have profound effects on affinity (28, 29). In coagulation factors IX and X, the adjacent Glu-containing module increases the affinity of the EGF site approximately 25-fold higher than the Ca\(^{2+}\) affinity found in the isolated third and fourth EGF modules from Protein S (38). Amino acid analysis after hydrolysis was found to be attributable to shielding of the calcium ion from solvent and the provision of a more defined binding site by the inter-domain interface.

To shed light on the effect of an adjacent EGF module on the Ca\(^{2+}\) affinity of its neighbor, we have expressed fragments containing two, three, or four Ca\(^{2+}\)-binding EGF modules from human Protein S in Spodoptera cells using baculovirus. The expression and characterization of the fragments are described in an earlier report.\(^2\) We have now determined the Ca\(^{2+}\) affinity of the recombinant modules using a Ca\(^{2+}\) complexation method and \(^1\)H NMR spectroscopy. EGF modules 3 and 4 each contain one very high affinity Ca\(^{2+}\)-binding site (\(K_d \approx 10^{-10} - 10^{-7} \text{ M}\)) whereas there is no site of comparable affinity in EGF 1 or 2. Moreover, the Ca\(^{2+}\)-binding site in EGF 3 is 10-fold to a 5,5-Y at each titration point corrected when necessary with 0.1 M NaCl, and the pH was checked and corrected with 0.1 M HCl or NaOH. Before using the pH meter, it was washed with 0.1 M EDTA, chelator, and finally 18 M water to avoid Ca\(^{2+}\) contamination. Before titration, a small aliquot of each EGF fragment solution was removed for determination of the protein concentration by amino acid analysis. To obtain the initial Ca\(^{2+}\) content in EGF 1–4 and EGF 2–4 samples, aliquots were removed for Ca\(^{2+}\) determination by atomic absorption spectroscopy. Titrations were performed by the sequential addition of \(2 \mu\)l aliquots from a 3.1 mM CaCl\(_2\) solution (determined by atomic absorption spectroscopy) followed by absorbance measurements at 263 nm in a Cary 4E spectrophotometer.

Calculations of the Calcium Dissociation Constants from the Quin 2 and 5,5'-Br\(_2\)BAPTA Measurements—The macroscopic binding constants were determined by an iterative least squares fit directly to the measured data (i.e. to the absorbance at 263 nm as a function of total Ca\(^{2+}\) concentration), as described by Linse et al. (39). The reported macroscopic dissociation constants are the inverse of the binding constants (\(K = 1/\theta\)). Fits using two \((K_1, K_2)\) or three \((K_1, K_2, K_3)\) macroscopic binding constants were attempted for fragments EGF 1–3 and EGF 2–4 and three \((K_1, K_2, K_3)\) or four \((K_1, K_2, K_3, K_4)\) for fragment EGF 1–4. Fixed parameters in the fitting procedure were the Ca\(^{2+}\) dissociation constant of the chelator (KDP), the chelator concentration at each titration point \(i\) (\(C_{Q,i}\)), the total Ca\(^{2+}\) concentration at point \(i\) including initial and added Ca\(^{2+}\) (CATOT), and protein concentration at point \(i\) (\(C_{P,i}\)). The following dissociation constants were used: for 5,5'-Br\(_2\)BAPTA, 1.0 \(\times\) 10\(^{-7}\) M (low salt concentration) (37), 2.3 \(\times\) 10\(^{-6}\) (0.15 M NaCl) (34) and for Quin 2, 5.3 \(\times\) 10\(^{-8}\) M (low salt concentration) (36), 1.2 \(\times\) 10\(^{-7}\) M (0.15 M NaCl) (34). \(C_{Q,i}\), CP, and CATOT were corrected for the dilution due to the Ca\(^{2+}\) addition. Variable parameters in the fits are the macroscopic binding constants \(K_1, K_2, \ldots, K_n\) and the absorbances in the Ca\(^{2+}\)-free, AMAX, and Ca\(^{2+}\)-saturated solution, AMIN, respectively. For each set of values of the variables, the Newton-Raphson method was used to solve the free Ca\(^{2+}\) concentration \(Y\) at each titration point \(i\) using the following equation:

\[
Y = \text{CATOT} - Y \cdot \text{CQ} / (Y + \text{KDP}) - \text{CP} \cdot \sum_{k=1}^{n} A_k / (\text{CATOT} + \sum_{k=1}^{n} A_k) \quad (\text{Eq. } 1)
\]

where

\[
A_k = Y \cdot \prod_{j=1}^{k} K_j \quad (\text{Eq. } 2)
\]

This equation states that the free Ca\(^{2+}\) equals the total Ca\(^{2+}\) minus the chelator-bound Ca\(^{2+}\) and the protein-bound Ca\(^{2+}\). The absorbance could then be calculated as,

\[
A_{\text{calculated}} = \frac{\text{AMAX} - (\text{AMAX} - \text{AMIN}) \cdot Y}{(Y + \text{KDP})} \cdot \frac{\text{CO}}{\text{Q}} \quad (\text{Eq. } 3)
\]

where \(\text{CQ}\) is the initial chelator concentration. Thus, the changes in absorbance were due to Ca\(^{2+}\) binding to the chelator.

Minimization of the Error Square Sum—The error square sum, \(\text{ESS}\), was obtained by summing over all titration points (24),

\[
\text{ESS} = \sum (A_{\text{calculated}} - A_{\text{measured}})^2 \quad (\text{Eq. } 4)
\]

The variable parameters were iterated (in a separate procedure) to obtain an optimal fit of calculated to experimental data as deemed by the minimum in ESS.

Calcium-binding Measurement by \(^1\)H NMR—\(^1\)H nuclear magnetic resonance spectroscopy was used to determine the Ca\(^{2+}\) dissociation

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\(^3\) Y. Stenberg, B. Dahlbäck, and J. Stenflo, submitted for publication.
constant of fragment EGF 2–3. The Ca\(^{2+}\) titration was carried out on a Varian Unity Plus 600 spectrometer at 599.89 MHz. The standard one-pulse experiment was performed with 1.5 s presaturation pulse to reduce the water resonance. Each spectrum is made up of 4096 complex data points taken from 512 accumulated scans using a spectral width of 6410 Hz. The Ca\(^{2+}\) dissociation constant was determined by measuring changes in peak amplitudes induced by the addition of Ca\(^{2+}\). Only resonances in the methyl region (spectra not shown) were observed.

The titration was carried out at +27°C and pH 7.5. Lyophilized EGF 2–3 fragment was dissolved in 99.9% D\(_2\)O containing 10% trifluoroethanol to a protein concentration of 93.5 \(\mu\)g/ml, and the pH was adjusted to 7.5 ± 0.05 with NaOD or DCI. To determine the fragment concentration, an aliquot was removed before titration for determination of the protein concentration by amino acid analysis. The initial Ca\(^{2+}\) concentration was determined from the changes in the absorption at 263 nm in a Ca\(^{2+}\) titration in the presence of 5,5'-Br\(_2\)BAPTA. Stock solutions of CaCl\(_2\) were made in 99.9% D\(_2\)O, and the pH was adjusted to 7.5 with NaOD or DCI. The titration was performed by 14 sequential additions of 4–5 \(\mu\)l aliquots of CaCl\(_2\) from 1 to 200 mM stock solutions (as determined by atomic absorption spectroscopy). After each addition, the pH was adjusted to its original value by adding 0.5–5.0 \(\mu\)l of NaOD or DCI. The Ca\(^{2+}\) sites in EGF 2–3 were saturated to approximately 90% when 5 fragment equivalents of Ca\(^{2+}\) had been added.

### Calculation of the Calcium Dissociation Constant from the \(^1\)H NMR Measurement

A one-site binding equation was used when calculating the Ca\(^{2+}\) binding to EGF 2–3.

\[
I_{calc} = (p_0 \times Ia + (1 - p_0) \times Ib) \cdot \frac{V_o}{V} \quad \text{(Eq. 5)}
\]

where \(Ia\) and \(Ib\) are the signal amplitudes in the Ca\(^{2+}\)-bound and Ca\(^{2+}\)-free form, respectively. \(V_o\) is the initial volume, \(V\) is the volume at titration point \(t\), and \(p_0\) is the fraction of the sample in the Ca\(^{2+}\) bound form. The Ca\(^{2+}\) dissociation constant was calculated by iterative fitting of the calculated intensities to the experimental ones as a function of total Ca\(^{2+}\) concentration. Equal weight was given to all titration points, and \(V/V_o\) corrects for the dilution due to the addition of Ca\(^{2+}\) and pH. Dissociation constant and intensities at zero and saturating concentrations of Ca\(^{2+}\) were allowed to vary during the iterative fit (see Fig. 4). The error square sum, ESS, was obtained by summing over all points in the titration (24),

\[
ESS = \sum (I_{calc} - I_{obs})^2 \quad \text{(Eq. 6)}
\]

### RESULTS

#### Calcium-binding Measurements

To determine the affinity of the Ca\(^{2+}\)-binding sites in the EGF modules of protein S, methods such as equilibrium dialysis were deemed unsuitable due to difficulties encountered in preventing the high affinity sites from picking up Ca\(^{2+}\) from the environment (24). Instead, the Ca\(^{2+}\) was allowed to partition between the protein and a Ca\(^{2+}\) chelating chromophore, Quin 2 and 5,5'-Br\(_2\)BAPTA, a range of dissociation constants from \(~10^{-10}\) to \(~10^{-5}\) \text{m} could be covered, Quin 2 covering those of 10–8–10–7 \text{m} and 5,5'-Br\(_2\)BAPTA covering those of 10–5–10–3 \text{m} (38). The dissociation constants were obtained directly from the data, i.e. the absorbance measurements, as a function of the total Ca\(^{2+}\) concentrations by performing iterative least squares fit to the data. The titrations of EGF 1–3 and EGF 1–4 (Quin 2 at low salt concentration) were performed twice. However, due to lack of material, the titrations of the remaining constructs were only performed once.

In a previous report, qualitative 5,5'-Br\(_2\)BAPTA blotting experiments suggested EGF 4 to be crucial for high affinity Ca\(^{2+}\) binding. 5,5'-Br\(_2\)BAPTA was used in titrations of EGF 1–4 and 2–4 at low ionic strength and Quin 2 at both low ionic strength and 0.15 \text{m NaCl}. The results of the 5,5'-Br\(_2\)BAPTA titrations performed at low ionic strength are shown in Fig. 1 together with the optimal curve obtained from least squares fit to the data.
only a weak band, whereas EGF 2–3 was not seen at all, and EGF 1–4 and EGF 2–4 gave intensely stained bands. The site(s) in EGF 1–3 and EGF 2–3 also proved to have too low affinity for Ca\(^{2+}\) to allow titration with Quin 2. In titrations with 5,5'-Br\(_2\)BAPTA, a fragment concentration almost double that of the chelator was required to obtain accurate measurements. Titrations were made both with and without 0.15 M NaCl in the buffer. The initial Ca\(^{2+}\) concentration in EGF 1–3 was approximately 0.14 mol of Ca\(^{2+}\)/mol of fragment. One of the titrations performed at low ionic strength is shown in Fig. 3 together with the optimal curve obtained by least squares fit to the data points. The optimal fit to two titrations with low salt was equally good, as judged by the error square sums, and yielded two dissociation constants (Table I). Due to a larger decrease in Ca\(^{2+}\) affinity of the fragment as compared with the chelator, no dissociation constants from titrations performed in the presence of 0.15 M NaCl were obtained, as the Ca\(^{2+}\) affinity was below the detection limit of the method.

Titrations of EGF 2–3 with 5,5'-Br\(_2\)BAPTA demonstrated the Ca\(^{2+}\) affinity of the fragment to be below the detection limit of the method. The initial Ca\(^{2+}\) concentration in the EGF 2–3 fragment was approximately 0.15 mol of Ca\(^{2+}\)/mol of fragment. Calcium titrations were instead monitored by \(^{1}H\) NMR spectroscopy. Calcium binding induced amplitude changes in the methyl resonance region. The corresponding peaks were integrated and related to the Ca\(^{2+}\) concentration. The data was calculated using a one-site binding equation (Fig. 4). The dissociation constant was calculated and is shown in Table I.

### DISCUSSION

Protein S is the only vitamin K-dependent coagulation factor that is not a serine protease. Moreover, the N-terminal part of protein S has a modular structure that differs from that of factors VII, IX, and X and protein C. Whereas these proteins all have two EGF modules immediately C-terminal of the Gla module, protein S has a module with a thrombin-sensitive peptide bond between the Gla module and the four EGF modules. In factors VII, IX, and X and protein C, only the N-terminal part of the EGF module has the Ca\(^{2+}\) chelator, no dissociation constants from titrations performed in low salt or at 150 mM NaCl concentration.

### Table I

| Fragment | Chelator | NaCl | \(K_d^{1}\) | \(K_d^{2}\) | \(K_d^{3}\) |
|----------|----------|------|------------|------------|------------|
| EGF 1–4  | Q2, 5B   | 0    | \(\leq 7 \times 10^{-10}\) | \(3 \times 10^{-6}\) | \(3 \times 10^{-7}\) |
| EGF 2–4  | Q2, 5B   | 150  | \(2 \times 10^{-8}\) | \(2 \times 10^{-6}\) | \(5 \times 10^{-6}\) |
| EGF 1–3  | 5B       | 150  | \(7 \times 10^{-6}\) | \(8 \times 10^{-6}\) | \(1 \times 10^{-5}\) |
| EGF 2–3  | NMR      | 1.5  | \(1.5 \times 10^{-5}\) | \(1 \times 10^{-5}\) | \(1 \times 10^{-5}\) |

\(^a\) Q2, Quin 2; 5B, 5,5'-Br\(_2\)BAPTA.

\(^b\) The data reported in the table are from titrations performed with the chelator that has a \(K_d\) closest to the apparent \(K_d\) of the site in the protein. The values shown for \(K_d\) in EGF 1–4 under low ionic strength are weighted averages of titrations with 5,5'-Br\(_2\)BAPTA and Quin 2.
ones. The first EGF module of protein S is atypical, however, in that it has the \( \beta \)-hydroxylation motif but lacks the characteristic N-terminal motif required for \( Ca^{2+} \) binding (Fig. 5).

The present studies performed on recombinant human EGF modules from protein S demonstrated the presence of very high affinity \( Ca^{2+} \) binding sites in the EGF module region of the protein. The studies also demonstrated the high \( Ca^{2+} \) affinity to be independent of the Gla module. This is in good agreement with previous \( Ca^{2+} \) blotting experiments performed on bovine and human protein S (24). The affinity of one of the \( Ca^{2+} \) sites in EGF 1–4 and 2–4 was too high to allow an accurate estimate of the binding constant with the Quin 2 method, i.e. \( K_d < 10^{-10} \) M (in the absence of NaCl). This site, as well as a second high affinity site (\( K_d \approx 2 \times 10^{-8} \) M in the absence of NaCl), appeared to be located in the third or fourth EGF module, as judged by the \( Ca^{2+} \) titrations. In the presence of 0.15 M NaCl, the \( Ca^{2+} \) affinity of the sites in EGF 1–4 and 2–4 decreased 10–1000-fold, and the affinity of the site in EGF 1–3 became too low to be measured accurately with 5,5'-Br2BAPTA. This is consistent with all the binding sites in highly charged environments. Similar salt effects have been found in studies of \( Ca^{2+} \)-binding sites in calmodulin and calbindin \( \delta_9 \) (34, 39). In contrast, the effect of salt on the \( Ca^{2+} \) affinity of the isolated EGF 3 (\( K_d = 5.2 \) mM in the absence of salt and 6.1 mM in the presence of 0.15 M NaCl) was negligible and for EGF 4 (\( K_d = 0.6 \) mM in the absence of salt and 8.6 mM in the presence of 0.15 M NaCl) reduced the affinity by about 10-fold, as has previously been observed in the isolated EGF modules from factors IX and X (14, 19). The \( Ca^{2+} \) affinity of EGF 3 and 4 in EGF 1–4 and EGF 2–4 in the presence of 0.15 M NaCl is \( 10^{2} \) and \( 10^{-5} \)-fold higher than in the corresponding synthetic isolated modules. The present data show that a major part of the increase in \( Ca^{2+} \) affinity for EGF 3 and EGF 4, when going from isolated module to intact protein, stems from interactions with a neighboring EGF module in the protein.

A noteworthy result in this study was that EGF 3 has a \( Ca^{2+} \)-binding site manifesting higher \( Ca^{2+} \) affinity in fragment EGF 2–4 than it does in fragment EGF 1–3, (\( K_d \approx 2 \times 10^{-8} \) M versus \( K_d \approx 7 \times 10^{-6} \) M), i.e. EGF 3 appears to have an at least 350-fold lower \( Ca^{2+} \) affinity in EGF 1–3 than in EGF 2–4. It has been shown in several cases that the \( Ca^{2+} \) affinity of an EGF module is reduced if the protein segment N-terminal to it is removed. This may be an effect of stabilization of the site, since one or two of the \( Ca^{2+} \) ligands may be located N-terminal to the module. The data obtained for the site in EGF 3 demonstrates an influence on \( Ca^{2+} \) affinity from the module on the C-terminal site. However, we suspect that EGF 4 has higher affinity than EGF 3 because of extra negative charge in its N-terminal region (see below). If EGF 3 has the highest affinity site, then its affinity would have been increased by the addition of EGF 4. Also EGF 2 has ~25-fold higher \( Ca^{2+} \) affinity in EGF 1–4 than in EGF 1–3; this is also influenced by EGF 4. EGF 1 seems to influence EGF 2 but to have no influence on EGF 3 or 4. To clarify the interactions between modules in detail, access to recombinant EGF 3–4 would have been helpful. Unfortunately, at this stage we could not investigate the \( Ca^{2+} \) binding constants further, as attempts to express a construct encompassing modules 3–4 were fraught with difficulty because the recombinant protein yielded only a smear and high molecular weight oligomers when analyzed by SDS-polyacrylamide gel electrophoresis (attempts to express EGF 1–2 were beset with similar problems).

In this context, it should be borne in mind that EGF 4 in human protein S has the sequence EDIDE (positions 201–205; Fig. 5), whereas bovine protein S has the sequence DDVDE in the corresponding region, i.e. in both species, EGF 4 has an extra negative charge that is not found in most other \( Ca^{2+} \)-binding EGF modules. This may contribute to the very high affinity of the \( Ca^{2+} \) site but does not in itself suffice to explain the difference in \( Ca^{2+} \) affinity compared with for instance, fibrillin (\( K_d \approx 0.35 \) mM in the C-terminal module in a module pair) (27). The \( Ca^{2+} \) binding properties of other EGF modules with the extra negative charge have yet to be reported.

EGF modules 2, 3, and 4 from protein S are similar to most other \( Ca^{2+} \)-binding EGF modules, e.g. from thrombomodulin and fibrillin, in that the sequence motif DI/VDE before the first Cys residue in the module is conserved. The other coagulation factors form a unique group in that they all have the sequence DGQ before the first Cys residue in the N-terminal EGF module (7, 12). Moreover, they have \( \text{erythro}-\beta\)-hydroxyaspartic acid/Asp where EGF 2, 3, and 4 from protein S have \( \text{erythro}-\beta\)-hydroxyasparagine/Asn. Yet, the \( Ca^{2+} \) affinity of the isolated

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**Fig. 4. EGF 2-3 titration curve.** Intensity (amplitude) changes of a \( ^1H \) NMR signal in EGF 2–3 as a function of \( Ca^{2+} \) to protein ratios. Protein concentration was 93.5 \( \mu \)M in \( D_2\)O, pH 7.5, 10% trifluoroethanol, and 0 M NaCl. Amplitudes not shown in the titration curve are 57, 59, and 58 and were monitored at \( Ca^{2+}/\)protein ratio 12.5, 20.3, 36.0, respectively.

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**Fig. 5. Comparison of EGF sequences in human protein S.** Alignment of the sequences of EGF 1–4 in human protein S is shown. The hydroxylation of Asp/Asn to \( \text{erythro}-\beta\)-hydroxyaspartic acid or \( \text{erythro}-\beta\)-hydroxyasparagine in protein S and the Tyr/Phe residue that is adjacent in the \( \beta\)-pleated sheet are denoted by asterisks in the sequence. The figure shows the typical pairing of disulfide in EGF modules. Residues that are \( Ca^{2+} \) ligands in factor X are denoted by dashes (side chain ligands) and dots (backbone ligands). The Met residue at position 210 was changed to Leu to avoid problems associated with oxidation of a Met residue.
EGF modules from factors IX and X is in the same range as those from protein S and fibrillin, i.e. $K_d \approx 1 \text{ mM}$ (14, 19, 30). The function of the Gln residue is not known. At this stage it can only be speculated that the adjacent C-terminal $\alpha$-helix in the Gla module can not accommodate the bulky side chain of an Ile/Val residue in this position for steric reasons but requires a small residue such as glycine.

The present and previous studies demonstrate that EGF modules can provide extremely versatile Ca$^{2+}$-binding sites in extracellular and membrane proteins. The wide span of Ca$^{2+}$ affinities, from $K_d$ values in the nanomolar to the millimolar range, is striking. How the structure accounts for the differences in Ca$^{2+}$ affinity, in addition to the apparent variations in net negative charge in the vicinity of the site, is not clear in the absence of a high resolution structure for protein S. The same Ca$^{2+}$ ligands were identified in the determination of the structure of the Ca$^{2+}$ form of the isolated EGF modules from factor X (NMR spectroscopy) and factor IX (x-ray crystallography) (21, 33). The EGF module pair from fibrillin binds Ca$^{2+}$ with approximately 10-fold higher affinity than does the isolated modules from factors IX and X (27, 30). The x-ray structure of the module pair did not lead to the identification of additional Ca$^{2+}$ ligands. Instead, Downing et al. proposed the increased Ca$^{2+}$ affinity to be attributable to shielding of the calcium ion from the solvent and the provision of a better-defined binding site by the intermodular surface (33). The present findings for protein S suggest that EGF 4 increases the Ca$^{2+}$ affinities of the sites in EGF 3 and EGF 2 in a manner that remains to be elucidated. In this context, it is also of interest that EGF 1–4 is 10-fold more active in this respect than EGF 1–3, suggesting extensive module-module interactions among all four EGF modules.

Determination of the solution structure of a pair of Ca$^{2+}$ binding EGF modules from fibrillin has shown them to be oriented in a near linear arrangement that is stabilized by Ca$^{2+}$ ligation. The orientation of the EGF modules in protein S is not known. Nor do we know the biological effects of the high variation in Ca$^{2+}$ affinity between the EGF modules in protein S. Elucidation of the importance of Ca$^{2+}$ binding in module-module interactions appears to be a prerequisite for an understanding of the function of these modules in coagulation factors as well as in the more complex receptors.

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