Structure of the Ca\(^{2+}\)-free GLA domain sheds light on membrane binding of blood coagulation proteins

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Reversible membrane binding of \(\gamma\)-carboxyglutamic acid (Gla)-containing coagulation factors requires Ca\(^{2+}\)-binding to 10–12 Gla residues. Here we describe the solution structure of the Ca\(^{2+}\)-free Gla-EGF domain pair of factor X which reveals a striking difference between the Ca\(^{2+}\)-free and Ca\(^{2+}\)-loaded forms. In the Ca\(^{2+}\)-free form Gla residues are exposed to solvent and Phe 4, Leu 5 and Val 8 form a hydrophobic cluster in the interior of the domain. In the Ca\(^{2+}\)-loaded form Gla residues ligate Ca\(^{2+}\) in the core of the domain pushing the side-chains of the three hydrophobic residues into the solvent. We propose that the Ca\(^{2+}\)-induced exposure of hydrophobic side chains is crucial for membrane binding of Gla-containing coagulation proteins.

Several proteins involved in blood coagulation and anticoagulation bind to biological membranes, such as activated platelets, in a Ca\(^{2+}\)-dependent manner by \(\gamma\)-carboxyglutamic acid (Gla) containing domains\(^1,3\). This step is pivotal in the regulation of blood coagulation as a means of localising the response to the site of damage as well as increasing the local enzyme and substrate concentrations. Ca\(^{2+}\) is essential both for membrane binding and for the assembly and activity of the proteolytic complexes that constitute the coagulation cascade. Each enzyme in the coagulation cascade is virtually inactive against its physiological substrate in the absence of a suitable (phosphatidyl serine-containing) membrane surface and the appropriate cofactor-binding protein, which together enhance the activity of the enzyme approximately 10\(^3\)-fold (ref. 2).

Homologous Gla domains have been identified in coagulation factors VII, IX and X as well as in prothrombin and the anticoagulant proteins C and S (Fig. 1)\(^1,3\). The Gla domain binds 6–12 Ca\(^{2+}\) with low affinity, at least two of which bind cooperatively\(^2\). Gla is formed from Glu by a vitamin K-dependent carboxylase\(^1,3\). Vitamin K-antagonistic drugs (such as warfarin), which inhibit \(\gamma\)-carboxylation are used as anticoagulants in clinical medicine as they down regulate the coagulation cascade by reducing the essential Ca\(^{2+}\) binding and membrane interaction of Gla domains\(^1,3,5\).

The nature of the Ca\(^{2+}\)-induced transition in the Gla domain and the mode of interaction with the membrane has been enigmatic. It was long hypothesised that Ca\(^{2+}\) ions were sandwiched between Gla residues and negatively charged phosphate head groups\(^6\). This was questioned when the crystal structure was determined of the Ca\(^{2+}\)-free fragment of the prothrombin fragment 1 (consisting of the Gla domain and the first kringle domain) Gla domain, which consists of three short \(\alpha\)-helices with seven Ca\(^{2+}\) ions bound in an internal cluster between an amino-terminal loop and two adjacent helices\(^11\). In Ca\(^{2+}\)-free prothrombin fragment 1, no electron density was observed for residues 1–36 in the Gla domain,\(^13\). The Ca\(^{2+}\)-free form of the Gla domain was therefore assumed to be disordered with structure being induced by Ca\(^{2+}\) binding in a folding transition.

The Ca\(^{2+}\)-free Gla-EGF domain pair

To unravel the nature of the Ca\(^{2+}\)-dependent conformational change that affects membrane binding, we have used 2D NMR spectroscopy to determine the structure of the Ca\(^{2+}\)-free form of a domain pair from factor X, consisting of the Gla domain and the first epidermal growth factor-like domain (Gla-EGF domain; Fig. 2). The domain pair (residues 1–86) was obtained by selective proteolysis of coagulation factor X (refs. 14,15). The Ca\(^{2+}\)-affinity of the Gla domain in the domain pair is indistinguishable from that of intact factor X. We found that, in the absence of Ca\(^{2+}\), the Gla domain is not disordered when linked to the EGF domain, but has a helical structure. The structure of the EGF domain is very similar to that of the isolated EGF domain in factor X as determined previously\(^16,17\). Although the individual domains have a well-defined fold, the relative orientation of the two domains is poorly defined, indicating that they are joined by a flexible hinge region. Our results indicate one close contact between the Gla and EGF domains.
Ca\(^{2+}\)-binding exposes hydrophobic residues

The solution structure of the Ca\(^{2+}\)-loaded Gla-EGF domain pair was not obtainable by NMR spectroscopy due to aggregation at the high protein concentrations required\(^2\). To compare the structures of the Ca\(^{2+}\)-free and Ca\(^{2+}\)-loaded forms of the Gla domain we have modelled the Ca\(^{2+}\)-loaded structure of the factor X Gla domain from the crystal structure of the homologous prothrombin Gla domain, which can be performed with confidence due to the pronounced sequence similarity\(^{12,22,23}\).

A striking difference between the Ca\(^{2+}\)-free and Ca\(^{2+}\)-loaded structures of the Gla domain is observed in the N-terminal section (Fig. 4). In the Ca\(^{2+}\)-free form, the hydrophobic residues Phe 4, Leu 5 and Val 8 form a cluster that faces the interior of the structure, whereas in the Ca\(^{2+}\)-loaded form these three hydrophobic residues are exposed to the solvent. There is a concurrent change in orientation involving the Gla residues. In the apo form the Gla residues are exposed to the solvent and provide a negatively charged protein surface. On Ca\(^{2+}\) binding the Gla residues 6, 7, 16, 20 and 29 fold into the core where they ligate Ca\(^{2+}\) ions in the space previously occupied by Phe 4, Leu 5 and Val 8. The central role of these Gla residues in Ca\(^{2+}\)-binding has also been demonstrated in site-directed mutagenesis experiments\(^{22,23}\). It should be stressed that the structural change observed for these residues is found for every member of the family of structures, not only in the average structure.

The orientation of the C-terminal helix differs somewhat in the Ca\(^{2+}\)-free and Ca\(^{2+}\)-loaded forms, however, the Ca\(^{2+}\)-free form was modelled from prothrombin fragment 1 where the Gla domain is followed by a Kringle domain instead of an EGF domain. The C-terminal helix is involved in interactions with the neighbouring domain in both factor X and prothrombin fragment 1 which may affect its orientation, an effect the modelling procedure cannot account for.

A clue to the cooperativity of Ca\(^{2+}\) binding to the Gla domain emerges from a comparison of the apo and Ca\(^{2+}\) forms. In the Ca\(^{2+}\)-free form, all Gla residues are exposed to solvent. On Ca\(^{2+}\) binding, Gla residues 6, 7, 16, 20 and 29 turn inwards to coordinate Ca\(^{2+}\) ions. As several of the Gla residues are ligands to two or more Ca\(^{2+}\) ions, a correct positioning of a Gla residue by binding one

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**Fig. 1.** a, Schematic representation of the domain structure of coagulation factor X (identical with that of factors VII, IX and protein C). Disulphide bonds are denoted with thin lines. Gla residues are denoted with a Y. In prothrombin the Gla domain is followed by two kringle domains and a serine proteinase domain, whereas in protein S it is followed by four EGF domains and a domain homologous to steroid hormone binding proteins. b, Alignment of the amino acid sequences of some Gla domains. Gla residues are red, Cys residues are yellow whereas the hydrophobic patch residues, Phe 4, Leu 5 and Val 8, are highlighted in magenta, blue and green.
Ca\textsuperscript{2+} facilitates binding of the next. The binding of the first Ca\textsuperscript{2+} ion(s) to the interior sites will recruit one or more ligating Gla residues thus forcing hydrophobic residues into the solvent. The next Ca\textsuperscript{2+} ion(s) thereby have access to properly positioned ligands, and will bind with higher affinity. Any intermediates with only few ions bound will be highly disfavoured due to strong uncompensated repulsion between neighbouring Gla ligands, thus promoting cooperativity. Mutating any of Gla residues 7, 16, 20, 26 or 29 to Glu results in loss of membrane binding and/or biological activity \textsuperscript{22,23}. Four of these five essential Gla residues are ligands to the interior Ca\textsuperscript{2+} ions (Fig. 4).

**Implications for membrane binding**

The three hydrophobic residues Phe 4, Leu 5 and Val 8, which are exposed on Ca\textsuperscript{2+} binding, are conserved in all Gla domains, except for that of factor IX where the hydrophobic cluster has been reversed in sequence (residues 5, 8 and 9; Fig. 1). We propose that these conserved hydrophobic residues are inserted into the membrane and interact with one face of the membrane bilayer. A

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**Fig. 2.** a, Stereo ribbon representation of the backbone of the energy minimised average structure. Residues Phe 40, Ile 65 and Gly 66 in the interdomain contact area are coloured red. b, The family of NMR structures superimposed by minimising the r.m.s.d. for the backbone atoms of residues 45–86 (the EGF domain) to the average structure. Details of the structure determination are given in Tables 1 and 2. c, The family of NMR structures superimposed on the Gla domain (residues 4–44, omitting residues 1–3 as there were no NOE constraints to these residues). d, The family of NMR structures superimposed on the entire domain pair (residues 4–86). Residues 1–19 are coloured orange, 20–31 red, 32–44 magenta, 45–55 pink, 56–65 green, 66–75 blue and 76–86 dark blue.
recent mutation of Leu 5 to Gln substantiates our proposal, as the mutant protein bound Ca\(^{2+}\) with affinities comparable to the wildtype whereas membrane binding was severely impaired\(^{14}\). The observation that Gla domain binding to neutral phosphatidylinositol-containing membranes requires unsaturation in the hydrocarbon side chains suggests the presence of a direct hydrophobic contact between the protein and the hydrophobic part of the fatty acids\(^{25}\). Prothrombin binds to membranes containing phosphatidyl serine in an ionic strength-independent manner, which further strengthens the idea that membrane binding is not merely the result of an unspecified electrostatic interaction\(^{26}\). The partially exposed Ca\(^{2+}\) ions may, however, contribute to membrane attachment through electrostatic interaction with the negatively charged phosphatidyl-serine carboxyl in addition to the hydrophobic contacts provided by residues 45 and 8. The conserved Lys/Arg in position 9 (position 4 in factor IX) is also conveniently positioned to interact with phosphate head groups.

**Ca\(^{2+}\)-induced membrane-binding**

The structural change caused by Ca\(^{2+}\)-binding to the Gla domain resembles that of annexin V, where Ca\(^{2+}\) binding induces the exposure of a certain Trp residue by taking its position within the structure. The Trp is then inserted into a membrane in close proximity to phospholipid headgroups\(^{27-28}\). Another example is recoverin, which binds to photoreceptor membranes in a Ca\(^{2+}\)-dependent manner. Here, biochemical experiments suggest that the N-terminal hydrophobic myristoyl moiety, essential for membrane binding, is constrained in the protein in Ca\(^{2+}\)-free recoverin and liberated by Ca\(^{2+}\) binding to penetrate the membrane\(^{29,30}\). Ca\(^{2+}\) can thus mediate a regulated exposure of hydrophobic residues for membrane interactions in various ways. The Ca\(^{2+}\)-induced exposure of hydrophobic side chains in Gla-containing proteins provides a particularly dramatic example of this mechanism, both in terms of the number of Ca\(^{2+}\) ions involved, and in the extent of the structural reorganization.

**Methods**

**Protein purification.** The Gla-EGF domain pair (residues 1-86) from bovine factor X was isolated as described previously\(^2\). The Gla domain at higher temperatures and the line broadening observed at lower temperatures. The assignment of H resonances was obtained with standard techniques as described in detail elsewhere\(^4\). The initial cross-relaxation rates were derived from NOESY spectra collected with four different mixing times (30, 60, 120 and 250 ms) in H\(_2\)O and D\(_2\)O, and distance constraints were derived using the isolated spin pair approximation. Upper and lower bounds for the distance constraints were set 10% above and 30% below the NOE-derived distance, and error margins were added corresponding to the scaling of equivalent and degenerate proton resonances. No pseudoatom corrections were added as distance constraints were incorporated using r\(^4\) averaging for non-stereospecifically assigned protons\(^3\). Backbone dihedral angle constraint intervals were \([-160°, -80°]\) for \(\psi\) and \([-80°, -40°]\) for \(\phi\) \(< 8\) Hz, and \([-80°, -40°]\) for \(\psi\) \(< 6\) Hz. Stereoscopic assignments were obtained from the structure of the isolated EGF domain for side chains where there were no significant shift differences\(^{17}\).

**Structure calculations.** Structural calculations were performed with the X-PLOR program (Brüger, A. T. 1992; X-PLOR, version...
Fig. 4. Location of side chains in the NMR structure family (left). The Ca²⁺ form of factor X as modelled from bovine prothrombin fragment-1 is shown to the right. The backbone of the Ca²⁺-free and Ca²⁺-loaded structures are matched on helix 1 and 2 (residues 13-30). The family of NMR structures was superimposed onto the average backbone structure, residues 4-12 in (a) and 12-29 in (b). Only the average backbone fold is shown as a coil following the Ca trace. a, Phe 4 (magenta), Leu 5 (blue) and Val 8 (green). b, Gla residues 6, 7, 14, 16, 19, 20, 25, 26 and 29. The essential Gla residues 7, 16, 20, 26 and 29 are red, whereas other Gla residues are pink. All side chain oxygens are white.

3.1. Yale University, New Haven, CT). Initial structures were obtained by distance geometry embedding using all atoms but without metrlzation. Subsequent simulated annealing refinement was made using standard X-PLOR dgsa and refinement protocols. Preliminary calculations were made on the individual domains to resolve ambiguities in the experimental data. Convergence rates were consistently high (50-70 %) using standard initial temperatures (2,000 and 1,000 K) and force constants (50 kcal mol⁻¹ for the NOE and 200 kcal mol⁻¹ rad⁻² for the dihedrals). Thereafter the domain pair was calculated as one unit. To increase the convergence (from 5 to 30 %) it was necessary to use initial temperatures of 4,000 and 2,000 K and higher force constants (100 kcal mol⁻¹ for the NOE and 300 kcal mol⁻¹ rad⁻² for the dihedrals). A converged set of 20 low-energy structures with distance violations lower than 0.36 Å and dihedral angle violations lower than 5° was derived from the final calculations. To check the effect of the higher force constants, the converged structures were subjected to a refine step using conventional force constants and temperatures. The resulting structures had slightly lower energies (~15 %, that is within experimental error) but average deviations from ideal geometry were the same as before. However, average r.m.s. deviations from experimental constraints were increased by 40 % (no individual violation larger than 0.36 Å / 5°). Therefore, the structure family calculated with the higher force constant was finally chosen to represent the structure. Hydrogen bonds were not included as constraints at any stage of the calculations. The conventional X-PLOR forcefield parameters (including hydrogen atoms) were used with square well NOE and dihedral angle potentials.

Table 2 Energetic parameters and r.m.s. deviations from experimental and structural constraints

<table>
<thead>
<tr>
<th>Energetic parameters (kcal mol⁻¹)</th>
<th>Ensemble</th>
<th>Minimised average structure</th>
</tr>
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<tbody>
<tr>
<td>total energy</td>
<td>316 ± 33</td>
<td>262</td>
</tr>
<tr>
<td>bonds</td>
<td>30 ± 4</td>
<td>26</td>
</tr>
<tr>
<td>angles</td>
<td>110 ± 13</td>
<td>98</td>
</tr>
<tr>
<td>impropers</td>
<td>22 ± 5</td>
<td>18</td>
</tr>
<tr>
<td>van der Waals</td>
<td>2 ± 1</td>
<td>0.4</td>
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<tr>
<td>dihedral restraints</td>
<td>105 ± 11</td>
<td>83</td>
</tr>
<tr>
<td>NOE restraints</td>
<td></td>
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</tr>
</tbody>
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Deviations from experimental constraints

| r.m.s.-NOE (Å) | 0.039 ± 0.002 | 0.035 |
| r.m.s.-dih (deg) | 0.62 ± 0.19 | 0.30 |

Deviations from ideal geometry

| r.m.s.-bond (Å) | 0.005 ± 0.001 | 0.004 |
| r.m.s.-angles (deg) | 0.56 ± 0.04 | 0.31 |
| r.m.s.-impropers (deg) | 0.45 ± 0.05 | 0.41 |

Modelling. The bovine factor X Gla domain was sequence aligned with the prothrombin Gla domain using the computer program MALIGNS to define the conserved regions. The three dimensional model was constructed by the computer program QUANTA (Molecular Simulations, Inc) using the crystal structure of the Gla domain of prothrombin as a template. The backbone was copied. The single deletion was effected by deleting Lys 3. Side chains were copied where identical, if not, side-chain coordinates were copied as far as applicable and for added atoms positions were generated in ideal positions from a structure library. One glycine residue was changed, Gly 4, but this did not cause difficulty. The computer program CHARMM ver 21 (ref. 32) was used to energy minimise the completed model to remove local strain. In view of the high similarity between the two domains, conformational refinement by molecular dynamics or Monte Carlo simulation was not deemed necessary. Structures are being deposited in the Brookhaven PDB databank.

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