Identification of the Calcium Binding Site and a Novel Ytterbium Site in Blood Coagulation Factor XIII by X-ray Crystallography*

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The biological importance of factor XIII (fXIII)1 (EC 2.3.2.13) lies in its ability to form new covalent bonds between protein chains. This activity was first recognized while studying blood coagulation; fXIII was required to form an insoluble clot (1). The activated form of fXIII covalently cross-links two fibrin molecules via an isopeptide bond between the side chains of a glutamine and a lysine located in the C-terminal region of the \( \gamma \)-chain. Over a longer time period in coagulation, it also forms cross-links between the \( \alpha \)-chains and the \( \gamma \)-chains of fibrin (2), and between \( \alpha \)-anti-plasmin and fibrin (3). fXIII has been shown to react with more than fibrin (4), and it has recently been found in brain tumors (5) and arthritic joints (6), and there are cases of fXIII deficiencies (7, 8). Factor XIII is a member in the family of transglutaminases (TGases), which have a wide range of biological functions (9).

Like most coagulation factors, fXIII is synthesized as a zymogen and then cleaved by a protease to become an active enzyme. The structure of fXIII zymogen was determined several years ago (10, 11). In this crystal form, the active site cysteine, Cys-314, is inaccessible to solvent and is not available for catalysis.

Physiologically, calcium ions are required for fXIII activation and for TGase activity. In the blood, activation of circulating fXIII requires thrombin cleavage, calcium ions (1.5 mM) (12–14), and fibrinogen (15). High levels of calcium (>50 mM) can activate fXIII without the use of thrombin (15), and it has recently been shown that platelet fXIII can be activated non-proteolytically in vivo (16).

Based on the amino acid sequence, the calcium binding site was predicted to be in a region (residues 468–479) with high similarity to the EF-hand motif (17, 18). The main calcium binding site, hypothesized to be the lanthanide inhibition site. We have found an additional ytterbium binding site at the dimer two-fold axis, near residues Asp-270 and Glu-272, and this site binds several angstroms removed. A novel ytterbium binding site is also found at the dimer two-fold axis, near residues Asn-436, Asp-438, Glu-485, and Glu-490. Calcium and strontium bind in the same location, while ytterbium binds several angstroms removed. All three ions bind to the same pocket. We have found an additional ytterbium binding site, hypothesized to be the lanthanide inhibition site.

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The atomic coordinates and structure factors (accession codes 1GOU, 1BL2, and 1GGY for the calcium-, strontium-, and ytterbium-bound factor XIII coordinates, respectively) have been deposited in the Protein Data Bank, Brookhaven National Laboratory, Upton, NY.

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1 The abbreviations used are: fXIII, factor XIII; TGase, transglutaminase; r.m.s.d., root mean square deviation; MES, 4-morpholineethanesulfonic acid.

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**Ion Binding in Factor XIII**

**TABLE I**

Data collection and refinement statistics

| Cation concentration (mM) | Calcium | Strontium | Ytterbium |
|---------------------------|---------|-----------|-----------|
| Unit cell dimensions      |         |           |           |
| a (Å)                     | 100.17  | 101.90    | 101.06    |
| b (Å)                     | 70.76   | 72.32     | 72.39     |
| c (Å)                     | 133.82  | 135.03    | 135.99    |
| β (°)                     | 106.1   | 105.9     | 106.1     |
| Data collection           | R-AXIS Iic | R-AXIS Iic | R-AXIS Iic |
| Temperature (°C)          | 25      | 25        | 25        |
| Completeness (%)          | 78      | 76        | 76        |
| Overall                   | 93      | 93        | 93        |
| R factor (%)              | 22.7    | 18.3      | 18.8      |
| R free (%)                | 31.3    | 27.5      | 28.1      |
| No. of reflections*       | 96,682  | 50,777    | 49,005    |
| No. of protein atoms      | 11,294  | 11,260    | 11,296    |
| Solvent B (Å²) (mean)     | 47.2    | 39.0      | 40.8      |
| Overall B (Å²) (mean)**   | 38.1    | 34.0      | 36.1      |
| Geometry (r.m.s.d.)       |         |           |           |
| Bonds (Å)                 | 0.017   | 0.012     | 0.011     |
| Angles (°)                | 2.0     | 1.7       | 1.7       |

* The calcium structure includes anisotropic scaling of the data and the bulk solvent model. If the structure is refined with data from 8.0 to 2.1 Å and includes the anisotropic scaling and same bulk solvent model, the R and Rfree are reduced to 20.9% and 29.6%, respectively.

**FIG. 1. Overall structure of the factor XIII dimer.** Panel A is the dimer looking down the two-fold axis. The domains, activation peptide (AP), and N and C termini of one monomer are labeled. Panel B shows one monomer, rotated 90° with the domains, N terminus, active site (AS), and ion sites labeled. For both views, three unique spheres are also shown: novel ytterbium site (light gray), active site (medium gray), and main ion site (dark gray).

tained by soaking zymogen crystals for 1–2 days in 90 mM CaCl₂, 120 mM SrCl₂ or 2 mM YbCl₃ in an artificial mother liquor of 1,2-propanediol (24%) and MES buffer at pH 6.2. Data for the calcium soaked crystal were collected at the Stanford Synchrotron Radiation Laboratory on beamline 9-1 with a MAR image plate detector at −170 °C. For the strontium and ytterbium crystals, data were collected in-house at room temperature on an R-AXIS IIc image plate detector equipped with a Rigaku RU200 rotating anode generator. The calcium crystal yielded data to 2.1 Å, and the strontium and ytterbium data sets diffracted to 2.5 Å (Table I).

**Structure Refinement**—Our previously refined P2₁, FXIII zymogen structural model was used to generate initial phases for all three ion-bound structures. The initial R (Σ [F_calc] − |F_obs|)/Σ |F_calc| was 48.2%, 36.8%, and 31.0% for the calcium, strontium, and ytterbium structures, respectively.

The calcium refinement began with a series of rigid body minimization steps at 8–3.5 Å resolution using X-PLOR (26). The first rigid body refinement used two monomers, while the next was composed of the eight domains; finally, a refinement with 193 secondary structure elements (helix, strand, loop) was performed. After those three steps, the R had dropped from 48.2% to 32.9%. This multistep rigid body procedure was used because of the cryogenic data collection and higher initial R factor. This rendered a 2.3% lower R factor than if a simple two-monomer rigid body step was used. From here, the resolution was expanded to 2.2 Å in six steps by alternating rigid body (with 193 groups) and positional least square refinements. The R dropped to 27.9% with an Rfree of 38.6%. Simulated annealing with slow cooling from 2000 to 300 K, followed by a round of positional and B value least squares minimization gave a drop in R of 0.4% and Rfree of 1.6%.

Refinement continued through several rounds of map fitting with the program XtailView (27) and least squares and dynamics minimization. The program DDQ (28) was used to assess model and map quality during the refinement procedure. Waters were added at positions with difference electron density peaks greater than 3.0 σ, which also were within hydrogen bonding distance of the protein or other waters. In the end, the structure was refined with overall anisotropic scaling of the data along with a bulk solvent model and a resolution of 20.0–2.1 Å; this resulted in an R of 22.7% and Rfree of 31.2%, with a total of 1001 waters (Table I).

The strontium and ytterbium refinements were carried out over the resolution range of 10–2.5 Å for all steps. The X-PLOR refinement began with a positional and individual B value minimization of the starting model, which lowered the R to approximately 27%, and was followed by adding two ions per dimer at the highest peaks in the
difference map (Table II). It then proceeded through more cycles of least squares minimization and manual map fitting in the program O (29). In the ytterbium refinement, three additional 10–12 \( \sigma \) difference peaks were observed near the non-crystallographic two-fold axis within the dimer, and three ions were added (sites 3, 4, and 5). Furthermore, one additional \( \text{Yb}^{3+} \) ion was added at a 10 \( \sigma \) difference peak (site 7), which is located at a crystallographic contact with another dimer. Following refinement of these four ions in the structure, one more difference peak appeared on the two-fold axis, so another ytterbium ion was added (site 6). After further refinement and map fitting, the \( R \) decreased to 19.8% (strontium) and 22.0% (ytterbium), and waters were placed at chemically reasonable 3 \( \sigma \) difference peaks. Additionally, a strong difference peak about 2 Å away from the main \( \text{Yb}^{3+} \) ion was identified, and a new \( \text{Yb}^{3+} \) ion (site 8) was added with an occupancy of 0.25, while the occupancy of the original \( \text{Yb}^{3+} \) ion was reduced to 0.75. Several more rounds of least squares refinement, map fitting, and water addition led to a final \( R \) of 18.3% and 18.8%, an \( R_{	ext{free}} \) of 27.5% and 26.1%, and 230 and 265 waters for the strontium and ytterbium complexes, respectively (Table I).

The Protein Data Bank codes for the calcium, strontium, and ytterbium structures are 1GGU, 1BL2, and 1GGY, respectively. Figures in this report were generated with Molscript (30), Raster3D (31), and XtalView (27).

RESULTS

Overall Structure—Each monomer of fXIII in the dimer has 731 residues and consists of four domains: the \( \beta \)-sandwich, the catalytic core, and barrels 1 and 2 (Fig. 1). The active site residues Cys-314, His-373, and Asp-396 are not accessible to solvent, as in thezymogen structure. The calcium binding site is located in the core domain, near the surface of the protein. The root mean square deviation (r.m.s.d.) between all atoms from the strontium- and ytterbium-bound structures compared with the starting ion-free fXIII zymogen structure is less than 0.85 Å. The calcium structure, on the other hand, differs from the two cation structures and the starting structure with an r.m.s.d. of 1.1 Å. This higher atomic coordinate deviation is likely due to the cryogenic data collection for the calcium structure.

Due to uninterpretable electron density, the monomers are missing various numbers of residues from the N terminus (residues 1–8), the linker between the activation peptide and the \( \beta \)-sandwich (residues 30–43), the region between the core and barrel 1 (residues 508–516), and the C terminus (residues 728–731). Most of the residues were well resolved, with 90% having average \( B \) values of less than 60 Å\(^2\).

Structure Quality—Less than 0.4% of the torsion angles are in the forbidden regions of the Ramachandran diagram, as reported by PROCHECK (32). Three peptide bonds in each monomer are in the cis conformation (10, 33). One involves a proline residue, 410–411, and the other two do not, 310–311 and 425–426. The structures are reasonable as judged by DDQ (28), Verify3D (34), ERRAT (35), and WHATIF (36).

Main Ion Site—The main ion binding site is near the interface between the catalytic core and barrel 1 (Fig. 1), and the ion binding helix (residues 485–501) is in contact with the other monomer. The calcium and strontium atoms superimpose within this site, while the main ytterbium ion is 2.7 Å from their location (Fig. 2). The mean peak sizes in an early difference map were about 4, 7, and 11 \( \sigma \) for the calcium, strontium, and ytterbium ions, respectively (Table II). When water molecules replaced the calcium ions at these positions, their \( B \) values refined to about 14–23 Å\(^2\). However, for the strontium and ytterbium sites, the \( B \) values of the replaced water molecules refine down to 2 Å\(^2\), the minimum allowable value. This analysis implies that a water molecule in this location does not have enough electrons to adequately match the strontium and ytterbium x-ray diffraction data.

Fig. 2. The main calcium binding site. The calcium (red), strontium (yellow), ytterbium (blue), and zymogen (gray) structures from monomer B are superimposed in this figure. Both positions of the \( \text{Yb}^{3+} \) ion are shown, with the weaker ion represented by the smaller sphere and labeled with parentheses. The water molecules in this region have been omitted for simplicity.

| Ion       | Site no. | Initial peak height observed in \( F_o - F_c \).map (\( \sigma \)) | Final peak height for \( F_o - F_c \) omit* map (\( \sigma \)) | Refined \( B \) (Å\(^2\)) for ion | Occupancy of ion | \( B \) (Å\(^2\)) of water at same location |
|-----------|----------|-------------------------------------------------|-------------------------------------------------|---------------------------------|----------------|-----------------------------------|
| Calcium   | 1        | 3.8                                            | 9.1                                            | 59                              | 1.0            | 23                                |
|           | 2        | 4.6                                            | 11.1                                           | 45                              | 1.0            | 14                                |
| Strontium | 1        | 7.8                                            | 21.6                                           | 52                              | 1.0            | 2\(^b\)                           |
|           | 2        | 5.6                                            | 24.3                                           | 46                              | 1.0            | 2                                  |
| Ytterbium | 1        | 11.7                                           | 36.8                                           | 55                              | 1.0            | 2\(^b\)                           |
|           | 2        | 11.0                                           | 36.5                                           | 36                              | 0.75           | 2                                  |
|           | 3        | 12.2                                           | 19.3                                           | 17                              | 0.25           | 2                                  |
|           | 4        | 11.6                                           | 18.7                                           | 23                              | 0.25           | 2                                  |
|           | 5        | 10.1                                           | 15.8                                           | 23                              | 0.25           | 2                                  |
|           | 6        | 3.9                                             | 20.8                                           | 10                              | 0.25           | 2                                  |
|           | 7        | 10.0                                            | 16.2                                           | 64                              | 0.50           | 5                                  |
|           | 8        | 8.1\(^a\)                                      | 14.1                                           | 35                              | 0.25           | 6                                  |

\(^{a}\) The omit map is constructed from the final refined structure which contains all atoms except the ion being measured.

\(^{b}\) The refinement program (X-PLOR) was configured such that 2.0 Å\(^2\) is the lower limit on \( B \) values.

\(^{c}\) This peak was only observed after other \( \text{Yb}^{3+} \) ions (1–5 and 7) were included in the refinement and map calculation.

\(^{d}\) This was the value observed with \( \text{Yb}^{3+} \) ions (1–7) already included in the structure and map calculation.
The main cation site consists of a pocket with negatively charged side chains of Asn-436, Asp-438, Glu-485, and Glu-490 plus the main chain oxygen of Ala-457. All three ion-bound structures and the zymogen structure overlap quite closely in this region (Fig. 2). Table III lists the oxygen atoms that are potentially involved in coordinating each cation. The main chain oxygen from Ala-457 is the main protein ligand for the Ca$^{2+}$ and Sr$^{2+}$ ions, as indicated by its low B value and its distance from the metals. The Yb$^{3+}$ ion is farther from Ala-457 and closer to Glu-485 and Glu-490. The B values for the residues in the region from 484 to 490 are higher than average, and the electron density is weak.

The ion site in monomer B of the calcium structure has six putative ligands arranged in a distorted bipyramidal arrangement (Fig. 3). One protein atom (O–Ala-457) and up to five waters are within 4.0 Å of the ion. Residues Asp-438, Asn-436, and Ala-457 are hydrogen-bonded to several of these waters. Fewer waters were identified in the strontium and ytterbium structures due to the lower resolution data.

The ytterbium ion has one monodentate (Asn-436) and two bidentate (Glu-485, Glu-490) ligands provided by the protein and nearby water molecules. The ion site in monomer B of the ytterbium structure has two alternate positions for the Yb$^{3+}$ ion 1.8 Å from each other. The occupancies for each ion were

**Table III**

| Residue         | Atom | Monomer A (Å) | B (Å$^2$) | Monomer B (Å) | B (Å$^2$) |
|-----------------|------|---------------|-----------|---------------|-----------|
| Calcium         | Ala-457 | O             | 2.77      | 42.6          | 2.54      | 42.8      |
|                 | Glu-485 | OE1           | 3.94      | 75.5          |           |           |
|                 | Wat-62L  | O             | 2.26      | 54.1          |           |           |
|                 | Wat-402L | O             | 3.81      | 89.2          |           |           |
|                 | Wat-744L | O             | 2.89      | 46.5          |           |           |
|                 | Wat-745L | O             | 2.14      | 38.9          |           |           |
|                 | Wat-5L   | O             |           |               | 2.62      | 28.6      |
|                 | Wat-23L  | O             |           |               | 2.90      | 69.9      |
|                 | Wat-243L | O             |           |               | 2.83      | 47.1      |
|                 | Wat-300L | O             |           |               | 3.66      | 63.8      |
|                 | Wat-746L | O             |           |               | 2.52      | 30.4      |
| Strontium       | Asn-436 | O             | 3.94      | 21.9          | 3.48      | 41.7      |
|                 | Asn-436 | OD1           |           |               | 2.70      | 29.0      |
|                 | Ala-457 | O             | 2.57      | 36.1          | 2.86      | 71.2      |
|                 | Glu-485 | OE1           | 3.63      | 67.5          | 3.90      | 69.8      |
|                 | Glu-485 | OE2           |           |               |           |           |
| Ytterbium       | Asn-436 | OD1           | 3.68      | 33.1          |           |           |
|                 | Asp-438 | OD1           |           |               | 3.54      | 29.7      |
|                 | Ala-457 | O             |           |               | 3.62      | 29.9      |
|                 | Glu-485 | OE1           | 2.27      | 46.2          |           |           |
|                 | Glu-485 | OE2           | 2.13      | 33.8          | 2.25      | 63.4      |
|                 | Glu-490 | OE1           | 3.46      | 63.2          | 2.38      | 32.4      |
|                 | Glu-490 | OE2           | 3.63      | 65.4          | 2.29      | 28.0      |
|                 | Wat-3006V | O         | 2.21      | 36.9          | 3.68      | 32.4      |
|                 | Wat-5063Y | O        | 2.14      | 20.7          |           |           |

*These refer to the strong and weak ytterbium ions, which are modeled as 0.75 and 0.25 occupancy, respectively.

**Fig. 3.** Stereoview of the electron density for the calcium site. Shown is the final $2F_o - F_c$ electron density (1.2σ contour) for the calcium binding site in monomer B of the calcium structure. The residues are labeled with the single-letter code for amino acid and residue number. Waters from Table III are labeled, and all waters are shown as crosses. Two waters (300L and 23L) have visible electron density at a lower contour level (0.5σ; not shown).
chosen such that they refined to equal $B$ values and summed to 1. It is unlikely that the weaker Yb$^{3+}$ ion (site 8) is a negative chloride ion because it is very close to the position of the positive calcium ion from the calcium structure, which implies that its location is suitable for only positive ions. The ligand distances between the Yb$^{3+}$ ion and the oxygen atoms are as low as 1.98 Å, but this is consistent with other Yb$^{3+}$-oxygen distances, as observed in the Protein Data Bank (codes 1CNT (37), 1NCG and 1NCH (38), 1YTT (39), and 2BOP (40)).

**Novel Ytterbium Site**—Four large electron density peaks near the non-crystallographic dimer two-fold axis form a nearly perfect tetrahedron with an edge length of approximately 3.6 ± 0.1 Å (Fig. 4 and Table II). Furthermore, a similar tetrahedron is observed in the ytterbium soak of the orthorhombic crystal form (10) of fXIII (data not shown). After modeling four ytterbium ions at 0.25 occupancy into the electron density, the $B$ values refined to 17, 23, 23, and 10 Å$^2$. Placing waters at full occupancy in these locations gave $B$ values that refined to 2 Å$^2$.

No ions or solvent molecules exist in the same location in the Ca$^{2+}$- or Sr$^{2+}$-bound crystal structures. Residues Asp-270, Asp-271, and Glu-272 of each monomer are in this region, but the electron density does not specify one distinct conformation for the side chains, and the $B$ values are quite high.

**Water Analysis**—After the refinement of the three structures, waters were grouped into subsets based on their occurrence in each crystal. If waters from different structures were located within 2.4 Å from each other, they were considered equivalent, and given the same chain identification and residue number in the Protein Data Bank files. In some cases, the determination of equivalent waters was made on the basis of their $B$ values and local protein differences between structures. A Venn diagram was constructed (Fig. 5) showing the seven different subsets from the three independent groups. If a water from one structure had no equivalent waters from another structure, it was given a particular chain identifier (L for calcium, R for strontium, Y for ytterbium). If a water molecule was in only two different structures, it was given the same residue number and chain identification (U for calcium and strontium, V for calcium and ytterbium, W for strontium and ytterbium). Finally, water molecules that exist in all three structures were given the chain identification of S along with the same residue number.

The average and standard deviation of $B$ values and $C_B$ density for the water molecules are presented in Table IV. The $C_B$ density (41) estimates the amount of burial at each water site by counting the number of $C_B$ atoms within 10 Å of the water site. It has a numerical range of 2–30 atoms, which qualitatively ranges from highly exposed to deeply buried.

Many of the water molecules are located in sites common to all three structures. Water molecules that are present in all three structures (subset S) have $B$ values about 10 Å$^2$ lower than unique waters. Likewise, these common waters are more buried, as shown by the 50% larger number of nearby $C_B$ atoms.

**DISCUSSION**

**Overall Structure**—Calcium and other cations have definite effects on fXIII behavior in solution. The ion soaking concen-
Structurally significant water molecule. Water 6059S is shown as a sphere with several secondary structure elements nearby. The residues near this water are also shown. The active site residue His-373 and calcium binding ligand Ala-457 are shown as a sphere with several secondary structure elements nearby. The root mean square deviation is shown in parentheses.

### Table IV
| Water subset | Ca, Sr, and Yb | Ca and Sr | Ca and Yb | Sr and Yb | Ca only | Sr only | Yb only |
|--------------|----------------|-----------|-----------|-----------|---------|---------|---------|
| PDB chain ID | S U            | V W       | L R       | Y        |
| Number of H2O| 79 68 118      | 13 736 70 | 55        |
| Ca$^{2+}$ structure: | | | | |
| (B) $\overline{A}^2$ | 36 (12)$^a$ | 42 (15) | 29 (12) | 51 (18) |
| (# $C_{B2}$) | 19.3 (4.6) | 16.0 (4.8) | 18.4 (5.3) | 14.4 (5.5) |
| Sr$^{2+}$ structure: | | | | |
| (B) $\overline{A}^2$ | 35 (14) | 42 (14) | 48 (21) | 44 (16) |
| (# $C_{B2}$) | 18.8 (4.3) | 15.4 (4.7) | 18.7 (5.2) | 11.4 (5.0) |
| Yb$^{3+}$ structure: | | | | |
| (B) $\overline{A}^2$ | 35 (14) | 42 (14) | 48 (21) | 12.8 (6.6) |
| (# $C_{B2}$) | 18.7 (4.3) | 18.2 (5.1) | 19.5 (5.5) | |
| Overall average: | | | | |
| (B) $\overline{A}^2$ | 35 | 42 | 39 | 41 |
| (# $C_{B2}$) | 19 | 16 | 18 | 19 |

$^a$ The root mean square deviation is shown in parentheses.
it difficult to model the protein ligands.

We postulate that this tetrahedron of partial occupancy ions is the lanthanide inhibition site. Achyuthan et al. (24) have observed that the addition of lanthanide ions above 40 \( \mu \text{M} \) to thrombin-cleaved \( \text{FXIII} \) results in the non-competitive inhibition of \( \text{FXIII} \). This inhibition cannot be reversed by a 200-fold molar excess of calcium ions. Furthermore, when the level of the lanthanide ion is between 10 and 40 \( \mu \text{M} \), \( \text{FXIII} \) can be activated by thrombin cleavage as in the presence of calcium. These results (24) could be explained by multiple lanthanide binding sites. The main ion site, observed in all three ion-bound structures, is required for proper thrombin activation, and the novel \( \text{Yb}^{3+} \) site, which has a slightly weaker affinity, is responsible for the inhibition of \( \text{FXIII} \). Given the soaking concentration of 2 \( \mu \text{M} \) in this data set, both ions sites are filled. Functionally, the location at the dimer interface could potentially destabilize the quaternary structure. Additionally, the residues that form the lanthanide inhibition site (Asp-270 to Glu-272) are only 5 residues away from Trp-279, which may play an important role in catalysis (11).

**Waters**—Grouping the waters into subsets based on their presence in multiple structures has been helpful in identifying structurally significant waters. One example is that of water 6059S (Fig. 6). This water in the core domain binds to several residues that are fully conserved in the TGase family (Thr-466, Lys-467, Arg-333, and Tyr-204). The water is located at the separation fork of a pair of \( \beta \) strands. One half of each strand forms a paired sheet, and the other half of each strand diverges nearly at a right angle. Furthermore, these strands are linked with functionally important parts of the protein. One strand pairs with the strand containing the active site His-373; and the other strand contains a ligand (Ala-457) for the calcium binding site. Monomer B also contains a water molecule in this location (6009S). We believe this water is structurally important for maintaining the unique conformation of this pair of strands.

Many other waters in this subset \( S \) are contacting conserved residues in this protein, and it is possible that some of these waters may help to explain natural \( \text{FXIII} \) deficiency mutations or other interesting structural and evolutionary features. In fact, of the 18 identified missense mutations (46–49)\(^2\) that are structurally significant and important for the function of \( \text{FXIII} \). Overall, these three structures positively identify the calcium binding site in factor \( \text{XIII} \).

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\(^{2}\) H. Mikkola, personal communication.