Crystal Structure of Red Sea Bream Transglutaminase*

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The crystal structure of the tissue-type transglutaminase from red sea bream liver (fish-derived transglutaminase, FTG) has been determined at 2.5-Å resolution using the molecular replacement method, based on the crystal structure of human blood coagulation factor XIII, which is a transglutaminase zymogen. The model contains 666 residues of a total of 695 residues, 382 water molecules, and 1 sulfate ion. FTG consists of four domains, and its overall and active site structures are similar to those of human factor XIII. However, significant structural differences are observed in both the acyl donor and acyl acceptor binding sites, which account for the difference in substrate preferences. The active site of the enzyme is inaccessible to the solvent, because the catalytic Cys-272 hydrogen-bonds to Tyr-515, which is thought to be displaced upon acyl donor binding to FTG. It is postulated that the binding of an inappropriate substrate to FTG would lead to inactivation of the enzyme because of the formation of a new disulfide bridge between Cys-272 and the adjacent Cys-333 immediately after the displacement of Tyr-515. Considering the mutational studies previously reported on the tissue-type transglutaminases, we propose that Cys-333 and Tyr-515 are important in strictly controlling the enzymatic activity of FTG.

Transglutaminase (TGase; protein-glutamine γ-glutamyltransferase) catalyzes the Ca²⁺-dependent acyl transfer between the γ-carboxamide groups of glutamine residues within peptides and the primary amino groups of various amines. A glutamine residue serves as the acyl donor, and the most common acyl acceptors are the e-amino groups of peptide-bound lysine residues or the primary amino groups of some naturally occurring polyamines, like putrescine or spermidine (1). When a protein-bound lysine residue acts as an acyl acceptor, inter- or intramolecular e-(γ-glutamyl)lysine bonds are formed, resulting in the polymerization of proteins (2–6). TGases are widely distributed in various organisms (7–18). Human blood coagulation factor XIII, which forms fibrin clots in hemostasis and wound healing by catalyzing the cross-linking of fibrin molecules, has been well characterized (19–28), and its crystal structure has been determined (22–24). However, the crystal structure is that of an inactive form, because the catalytic Cys is completely blocked. Human factor XIII is the enzyme that forms blood clots, and therefore its activation process is highly controlled so that its catalytic activity is not exhibited under normal conditions. It is assumed that the structural changes caused by substrate binding as well as the proteolysis of the N terminus by thrombin are necessary to activate human factor XIII (25, 26). Because the activation process is very complex, the structure-function relationship of human factor XIII remains unclear, even though the crystal structure has been reported. To investigate the structure-function relationships of the TGases, the crystal structures of other TGases will be very helpful. The red sea bream liver transglutaminase (fish-derived TGase, FTG) is a member of the tissue-type TGases (29). Although the biological function of FTG remains unknown, it plays an important role in gel formation in fish mince sols, by catalyzing a setting reaction that involves the cross-linking of myosin heavy chains in fish paste. Production and purification methods for recombinant FTG have already been established (29, 30), and the amino acid sequence identity between FTG and human factor XIII is ~33%. FTG has some advantages for studying the structure-function relationship because FTG works as a monomer, whereas human factor XIII works as a homodimer, and FTG does not require an initial proteolysis in its activation process. It is expected that the activation process of FTG is similar to, but simpler than, that of human factor XIII. We describe the crystal structure of FTG determined at 2.5-Å resolution and its potential substrate binding sites. We also propose a novel aspect of the activation process, which involves the regulation of the enzymatic activity by a Cys residue adjacent to the catalytic Cys.

EXPERIMENTAL PROCEDURES

Purification and Crystallization—Production and purification of recombinant FTG were performed as previously reported (29, 30). Crystallization experiments were performed using the method of vapor diffusion in hanging drops. The crystallization solution in the reservoir had a volume of 500 ml and was composed of 0.8 M ammonium sulfate, 1% polyethylene glycol 6000, 0.1 M Hepes, pH 7.0, and 5 mM DTT. Two µl of FTG solution (14 mg/ml) and 2 µl of the reservoir solution were mixed and then equilibrated against the reservoir at 4 °C for 2–4 weeks before crystals appeared.

Data Collection and Processing—X-ray diffraction data up to 2.5-Å resolution were collected on the beamline BL6B at Photon Factory (Tsukuba, Japan) using two differently aligned crystals. Because the crystals suffered from severe radiation damage at room temperature, they were flash-cooled at ~173 °C after equilibration against a cryosolvent containing 30% glycerol, 0.6 M ammonium sulfate, 8% polyethylene glycol 6000, 0.1 M Hepes, pH 7.0, and 5 mM DTT. The diffraction data were processed using DENZO and SCALEPACK (31). The crystals belong either to space group P6₁22 or P6₁22 with the following unit cell dimensions: a = b = 97.8 Å; c = 455.5 Å. A summary of the data collection is shown in Table I.
Molecular Replacement—The structure of FTG was solved by the molecular replacement method, based on the crystal structure of human factor XIII. The program AMORE (32) was used in the molecular replacement procedure using intensity data between 1.0 and 3.0 Å for the rotation search and between 1.0 and 4.5 Å for the translation search. There were single prominent solutions to both the rotation and translation searches. The solutions indicated that the correct space group is P6_22 and that the asymmetric unit has one FTG molecule, giving a calculated value of ~70% for the solvent content. Rigid body refinement of the orientation and the position of the molecule resulted in a crystallographic R factor of 0.525 at 3.5-Å resolution, and the crystal packing was realistic.

Structure Refinement—The side chains according to the FTG sequence were fitted into the initial 2Fobs − Fmap, if their electron densities appeared clearly. Otherwise, nonconserved residues were changed to alanine. The model was refined and completed by rounds of model building using QUANTA97 and simulated annealing using X-FLOR (33). After the Rcryst dropped to 0.294, water molecules were added to the model. Finally, the Rcryst and the Rfree against 37,856 reflections between 8.0- and 2.5-Å resolution were decreased to 0.196 and 0.246, respectively. The electron densities of five residues at the N terminus, eleven residues at the C terminus, and residues 462–471 and 569–571 were not observed. The final model includes 666 amino acid residues of a total of 695 residues, 382 water molecules, and 1 sulfate ion derived from the ammonium sulfate used in the crystallization procedure. The refined structure has two nonproline cis peptide bonds (Lys-268–Tyr-269 and Lys-384–Tyr-385), as suggested by Weiss et al. (24). The root mean square deviations in bond length and bond angle are 0.017 Å and 3.454°, respectively. A Ramachandran plot analysis reveals 85.7% of residues in most favored regions, 12.6% of residues in additional allowed regions, 0.5% of residues in generously allowed regions, and 1.2% of residues in disallowed regions. This result seems quite reasonable for a 2.5-Å resolution model.

RESULTS AND DISCUSSION

Overall Structure—The overall structures of FTG and the human factor XIII monomer are shown in Fig. 1, and the Ca trace of FTG is compared with that of the human factor XIII monomer in Fig. 2. The sequence alignment of FTG and human factor XIII, together with their secondary structural elements, is shown in Fig. 3. As shown in Fig. 1, the overall structures of FTG and human factor XIII resemble each other, although FTG lacks an activation peptide observed at the N terminus of human factor XIII. FTG, as well as human factor XIII, consists of four sequential domains named “β-sandwich,” “core,” “barrel 1,” and “barrel 2” by Yee et al. (22). In FTG, β-sandwich contains residues Gly-6 through Phe-134, core contains residues Asn-135 through Thr-461, barrel 1 contains residues Arg-472 through Ser-583, and barrel 2 contains residues Thr-584 through Lys-684. The secondary structures of β-sandwich, barrel 1, and barrel 2 are predominantly β-sheets, whereas core

| Crystal size (mm) | 1.1 × 0.3 × 0.3 | 0.3 × 0.4 × 0.3 |
|------------------|-----------------|-----------------|
| Wavelength (Å)   | 1.0             |                 |
| Collimator (mm)  | 0.2 × 0.2       |                 |
| Film distance    | 573.0           |                 |
| Oscillation angle ω (degrees/film) | 2.0 | |
| Rotation speed (degrees/s) | 2.0 | |
| Number of films  | 20              | 20              |
| Unique reflections | 46,507         |                 |
| Completeness (%) | 86.0            |                 |
| Rmerge (%)       | 6.3             |                 |

* Rmerge = Σ|I(h)| − 1 (I(h)) / Σ|I(h)|, where I(h), is the intensity of the jth measurement of the same reflection and <I(h)> is the mean intensity.

![FIG. 1. Schematic ribbon drawings of FTG (left) and human factor XIII (right). Helices and sheets are colored red and blue, respectively. Both TGases consist of four domains: β-sandwich, core, barrel 1, and barrel 2 from the N terminus. Human factor XIII has an activation peptide (green) at the N terminus. The active site in each TGase is marked with a yellow asterisk. This figure was produced using MOLSCRIPT (36).](image)

![FIG. 2. Structural comparison of FTG with human factor XIII. Stereoviews of the Ca traces of FTG and human factor XIII are shown. The superposition was done using QUANTA97. FTG, human factor XIII, and the activation peptide are colored blue, red, and green, respectively. The active site in each TGase is marked with a white asterisk.](image)
consists of almost equal amounts of α-helices and β-sheets (Fig. 3).

Three cysteine pairs are close enough to each other to form disulfide bridges. The distances between the β-carbon atoms of the cysteine pairs are 5.04 Å for Cys-272 and Cys-333, 4.82 Å for Cys-188 and Cys-285, and 4.82 Å for Cys-523 and Cys-552. Because DTT was added in the crystallization process, the electron densities between their sulfur atoms were not observed. However, in the absence of DTT, disulfide bridges would be formed, except between Cys-272 and Cys-333. The disulfide bridge formation between the two cysteine residues would still be prevented by a hydrogen bond between S$_g$ of Cys-272 and O$_h$ of Tyr-515. The special relationship between Cys-272 and Cys-333 will be discussed in a subsequent section (“Active Site Structure and Activation Process”).

**Domain Structure—** Both barrel 1 and barrel 2 consist of seven β-strands (Fig. 3). The relative positions of these β-strands in barrel 1 are quite similar to those in barrel 2 (Fig. 1).

The β-sandwich domain consists of two four-stranded anti-parallel sheets twisted about 50° with respect to each other. In addition, there is one α-helix in this domain. Although the topologies of the β-sandwich domains of FTG and human factor XIII resemble each other, the relative positions of the two β-sheets are somewhat different (Figs. 1 and 2).

The core domain, in which the active site is located, consists of 11 α-helices and 12 β-strands. Most of the α-helices of the enzyme are located in this domain. As compared with human factor XIII, there are distinct differences in the α-helix-rich region near β-sandwich and the region consisting of three β-strands between the active site and barrel 1.

**Structural Differences between FTG and Human Factor XIII—** As discussed in the previous section, there are some structural differences in the β-sandwich and core domains between FTG and human factor XIII. Because human factor XIII actually works as a homodimer, the dimer structure is shown in Fig. 4. The region between the active site and barrel 1 is adjacent to β-sandwich and the α-helix-rich region of the other half of the dimer; the activation peptide crosses the interface. This extensive region is thought to be an acyl acceptor binding site of human factor XIII (22).

The postulated activation process of human factor XIII is as follows. First, the activation peptide is cleaved by thrombin in the presence of Ca$^{2+}$, and then an acyl donor approaches the active site from the direction of the two barrel domains to form

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FIG. 3. **Structure-based sequence alignment of FTG and human factor XIII (FXIII).** The dashed lines represent incorporated gaps that bring the sequences into alignment. The asterisks represent identical matches. Residues constituting the catalytic triad are colored red. The red and blue bold lines represent α-helices and β-strands, respectively. The boxes colored magenta, orange, green, and blue represent β-sandwich, core, barrel 1, and barrel 2, respectively.

FIG. 4. **Dimer structure of human factor XIII.** Each domain is colored as follows: β-sandwich, red; core, blue; barrel 1, yellow; barrel 2, orange; and the activation peptide, green. Possible acyl acceptor binding sites are marked by magenta circles. This figure was produced using MOLSCRIPT (36).
an acyl-enzyme transition state. Finally an acyl acceptor accesses the active site from the above-mentioned region to make an e-(γ-Glu)Lys bond. If this hypothesis is correct, the structurally different regions between the two TGases are concentrated on the acyl acceptor binding sites. It is tempting to postulate that human factor XIII forms a homodimer to accept only a small subset of proteins as acyl acceptors by increasing the surface area of its binding site.

On the other hand, there seem to be few structural differences in the acyl donor binding site in terms of the Ca traces. However, if we take a closer look at the site, there are distinct differences in the surface structure and the charge distribution around the acyl donor binding sites between the two TGases (Fig. 5). In human factor XIII, the acyl donor binding site forms a deep and wide groove. On the other hand, the corresponding groove of FTG is shallow and narrow. In addition, there are some basic regions on the surface of the groove of FTG, whereas the groove of human factor XIII is scarcely charged. These structural and electrostatic differences in the substrate binding sites are plausible causes for the different substrate specificities of FTG and human factor XIII.

**Active Site Structure and Activation Process**—Fig. 6 shows a comparison of the active sites of FTG and human factor XIII. The catalytic triad reminiscent of the cysteine proteases consists of Cys-272, His-332, and Asp-355 in FTG and Cys-314, His-373, and Asp-396 in human factor XIII (Fig. 6). Because FTG and human factor XIII have similar active site structures and both require Ca\(^{2+}\) to exhibit their catalytic activity, their activation processes are thought to be similar. The catalytic Cys residue is situated between a Tyr residue (Tyr-515 in FTG and Tyr-560 in human factor XIII) and another Cys residue (Cys-333 in FTG and Cys-374 in human factor XIII). The Tyr belongs to barrel 1 and is located on the loop projecting over the core. It seems that O\(_{\text{H}}\) of the Tyr and S\(_{\text{g}}\) of the catalytic Cys form a hydrogen bond, which suppresses the enzymatic activities of the TGases. Therefore, the Tyr must be removed to activate the enzymes.

It has been reported that either the thrombin cleavage or the binding of one calcium ion alone does not cause the conformational changes of human factor XIII (23). At present, the activated structure of human factor XIII has not been elucidated, because substrate binding is probably required to cause the large conformational changes necessary for the enzymatic activity.

If the Tyr covering the catalytic Cys were simply removed, then it is predicted that the Cys would form a disulfide bridge with the adjacent Cys, and then the enzyme would lose the...
catalytic activity immediately. Therefore, we suppose that these two TGases cannot be activated in the absence of substrates. In other words, the formation of an acyl-enzyme intermediate is essential to prevent the formation of the disulfide bridge. There is good evidence to support this hypothesis; i.e. the catalytic activity of human factor XIII was reduced to 22% by the proteolysis of the linker peptide joining the core and barrel 1 domain (28), whereas TGase 3, in which the noncatalytic Cys in the active site is replaced with Val, was activated 15-fold by the same treatment (35). We can explain these phenomena by assuming that the catalytic Cys of human factor XIII forms a disulfide bridge with the neighboring Cys following the removal of the barrel domains containing the Tyr covering the catalytic Cys, whereas in the case of TGase 3, the catalytic Cys was exposed and became more reactive. The remaining activity (22%) of human factor XIII is accounted for by the presence of DTT in the activity measurement. Consequently, the activation processes of FTG and human factor XIII are considered to be as follows. 1) Ca\(^{2+}\) binds to its site in TGase. 2) An acyl donor is led to the active site. 3) The Tyr covering the catalytic Cys is removed by a conformational change caused by the acyl donor binding. 4) An acyl-enzyme intermediate is formed between the acyl donor and the catalytic Cys (in the case of human factor XIII, proteolysis of the activation peptide by thrombin is necessary between steps 1) and 2)). In this activation process, not only the Tyr covering the catalytic Cys, but also the other Cys near the catalytic Cys, is considered to play an important role in selecting only appropriate substrates.

Because human factor XIII is the enzyme that forms blood clots, it is assumed that its activation process is highly controlled to prevent its activity in the absence of specific substrates, such as fibrin. Although the physiological role of FTG remains to be determined, its activation process is also thought to be ingeniously controlled so that it is activated only under specific conditions. In future studies, the three-dimensional structure of an FTG-substrate complex should be determined to demonstrate the activation processes of TGases.

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