Crystal Structure of Microbial Transglutaminase from *Streptoverticillium mobaraense*

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The crystal structure of a microbial transglutaminase from *Streptoverticillium mobaraense* has been determined at 2.4 Å resolution. The protein folds into a plate-like shape, and has one deep cleft at the edge of the molecule. Its overall structure is completely different from that of the factor XIII-like transglutaminase, which possesses a cysteine protease-like catalytic triad. The catalytic residue, Cys64, exists at the bottom of the cleft. Asp255 resides at the position nearest to Cys64 and is also adjacent to His274. Interestingly, Cys64, Asp255, and His274 superimpose well on the catalytic triad “Cys-His-Asp” of the factor XIII-like transglutaminase, in this order. The secondary structure frameworks around these residues are also similar to each other. These results imply that both transglutaminases are related by convergent evolution; however, the microbial transglutaminase has developed a novel catalytic mechanism specialized for the cross-linking reaction. The structure accounts well for the catalytic mechanism, in which Asp255 is considered to be enzymatically essential, as well as for the causes of the higher reaction rate, the broader substrate specificity, and the lower deamidation activity of this enzyme.

Transglutaminase (TGase); protein-glutamine γ-glutamyltransferase, EC 2.3.2.13) catalyzes an acyl transfer reaction in which the γ-carboxyamide groups of peptide-bound glutamine residues act as the acyl donors. The most common acyl acceptors of TGase are the ε-amino groups of lysine residues within peptides or the primary amino groups of some naturally occurring polyamines (1, 2). When lysine residues in proteins serve as acyl acceptors, intermolecular or intramolecular ε-(γ-glutamyl)lysine bonds are formed, resulting in the polymerization of proteins.

TGases are widely distributed in various organisms, including vertebrates (3–7), invertebrates (8, 9), mollusks (10), plants (11), and microorganisms (12). Among these TGases, the human blood coagulation factor XIII has been most characterized (13–18). By catalyzing the cross-linking between fibrin molecules, factor XIII forms fibrin clots for hemostasis and heals a wound. The crystal structure of human factor XIII has been determined, revealing that it consists of four domains with a cysteine protease-like active site (19–22). Many TGases are homologous to human factor XIII and share the common feature of Ca2+-dependent catalytic activity (9–8). A tissue-type TGase from red sea bream liver (fish-derived TGase (FTG)) is an example of such factor XIII-like TGases and shows 33% sequence homology to human factor XIII (7). The crystal structure of FTG has also been determined (23). The overall and active site structures of FTG are essentially similar to those of human factor XIII.

A microbial TGase (MTG) has been isolated from the culture medium of *Streptoverticillium sp.*, S-8112 (24), which has been identified as a variant of *Sv. mobaraense*. This enzyme is the first TGase obtained from a nonmammalian source. Thus far, few TGases have been identified from microorganisms, particularly from *Streptoverticillium* species (25). Although the physiological role of MTG is still unknown, this protein is secreted from the cytoplasm membrane as a zymogen and is activated by proteolytic processing (26). In contrast to many other TGases, the MTG activity is Ca2+-independent (24). A sequence analysis of MTG by Edman degradation revealed that the protein consists of 331 amino acids with a molecular mass of 37.9 kDa (12). The molecular weight of MTG is nearly half that of the factor XIII-like TGases. The amino acid sequence of MTG bears little significant homology to the factor XIII-like TGases or to any other sequences in the current protein sequence databases, except for the apparently homologous TGases from *Sv. Cinnamomeum*, etc. (25). In contrast to the factor XIII-like TGase, which possesses an active site region consisting of the consensus sequence motif of thiol proteases, thus far only a single cysteine (Cys64) has been identified as the catalytic residue in the sequence of MTG (12). A recent NMR study revealed that the reaction rate and the substrate specificity for the acyl donor of MTG are higher and lower than those of the factor XIII-like TGases such as guinea pig liver TGase and FTG, respectively (27). On the other hand, the deamidation activity of MTG is weaker than that of FTG, etc., implying that it is difficult for the water molecule to play the role of an acyl acceptor (28). Therefore, it is anticipated that MTG has a novel three-dimensional structure and that its catalytic mechanism is different from that of the factor XIII-like TGase.

These characteristics, including Ca2+-independence, the higher reaction rate, the broader substrate specificity for the acyl donor, the lower activity for deamidation, and the smaller molecular size, are advantageous for industrial applications of MTG. Actually, MTG is widely used to improve the physical and textural properties of many protein-rich foods such as tofu.
boiled fish paste, and sausage (29–32). Because of the usefulness of the cross-linking reaction, applications of MTG to other protein-related fine chemicals are also expected. To investigate the structure/function relationship of MTG and to obtain basic information for protein engineering aimed at various industrial applications, we determined the structure of MTG by x-ray crystallography. Here, we describe the novel overall and active site structures of MTG determined at 2.4 Å resolution, and discuss the catalytic mechanism of this enzyme.

EXPERIMENTAL PROCEDURES

Production and Purification of Recombinant MTG—Three types of recombinant MTG were prepared for the crystallization. One is an MTG variant that has an additional Met residue at the N terminus of the natural MTG amino acid sequence (Met-MTG). The second is an MTG variant in which the amino acid sequence lacks the N-terminal Asp residue of the natural MTG and has a Ser residue as the N terminus (Ser-MTG). Recombinant Met-MTG and Ser-MTG were expressed as described previously (33). The third recombinant MTG has the same amino acid sequence as the natural MTG and will be designated as Asp-MTG or simply MTG.

To produce Asp-MTG in Escherichia coli, the mature MTG sequence linked to the IEGR sequence (factor Xa recognition site) was expressed, and then the extra sequence was digested by factor Xa. The MTG expression plasmid, pETMTGXa-01, which has the IEGR coding sequence upstream of the MTG gene, was constructed. The base sequence corresponding to the IEGR sequence, was added by PCR using pUC19-cp (5′-ccgatccatcggaaggtcgtgattctgacgatcgt-3′) was the sense primer with the SalI site and the IEGR BamHI site and the IEGR coding sequence upstream of the MTG gene was amplified by PCR using pETMTGXa-01 (5′-ccgatccatcggaaggtcgtgattctgacgatcgt-3′) was the sense primer with the BamHI site and the SalI site. PCR product was cloned into the pGEM-T Easy Vector (Promega, Madison, WI). The plasmid was designated as pETMXa-01. The PCR product was digested by factor Xa, and then the plasmid was designated as pGEMMTGXa, containing the locz promoter and the MTG gene in the same orientation. pET5a (Promega) was digested by EcoRI, blunt-end-digested by BamHI, and digested by SalI, blunt-end-digested by BamHI. The small fragment containing the MTG gene was integrated into the above prepared pET5a, and the new plasmid was designated as pETMTGXα-01.

E. coli BL21(DE3) pLysS cells (Promega) harboring pETMTGXa-01 were cultivated as described previously (33) except that MTG expression was induced by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside when the optical density at 660 nm was 2.0. The cell pellets harvested from 800-ml cultures were disrupted by ultrasonication, and the inclusion bodies were obtained as described previously (33). MTG inclusion bodies were dissolved in 8 M urea containing 20 mM sodium phosphate, 1 mM EDTA, and 20 mM dithiothreitol, pH 7.5, and were incubated for 2 h at 37 °C. Precipitates were removed by centrifugation. The solubilized MTG solution (20 ml; about 20 mg/ml) was adjusted to pH 4.0 by adding HCl and was diluted 50-fold to a concentration of 0.16 M in urea using 20 mM sodium phosphate, pH 4.0. After 2 h of incubation at 5 °C, the pH was shifted from 4.0 to 6.0. Aggregates were removed by centrifugation after the dilution and the pH shift. The refolded MTG was concentrated about 10-fold and was applied to a gel filtration column (Sephadex G25M, Amersham Biosciences) equilibrated with 20 mM Tris(hydroxymethyl)aminomethane hydrochloride, pH 7.5. Five ml of bovine factor Xa (Hematological Technologies, Inc.) was added to 250 ml of the buffer-exchanged solution (143 mg of MTG), which was incubated for 16 h at 5 °C, adjusted to pH 5.8 by HCl, and diluted 10-fold by 20 mM sodium acetate, pH 5.8. It was applied to a cation-exchange column (CM-Sepharose FF, 2.6 × 10-cm internal diameter; Amersham Biosciences) equilibrated with 20 mM sodium acetate, pH 5.8. After the column was washed with 1 column volume of the same buffer, the MTG was eluted with a linear gradient of sodium chloride from 0 to 400 mM over 10 column volumes, at a flow rate of 5 ml/min. Fractions were collected and diluted 10-fold by 20 mM sodium acetate, pH 5.5. They were applied to a cation-exchange column (Resource 6 ml; Amersham Biosciences) equilibrated with 20 mM sodium acetate, pH 5.5. After the column was washed with 1 column volume of the same buffer, the MTG was eluted with a linear gradient of sodium chloride from 0 to 500 mM over 20 column volumes at a flow rate of 6 ml/min. Fractions that lacked impurities were assayed by analytical reverse-phase high pressure liquid chromatography, and the fractions of the low pI MTG variants, assayed by analytical cation-exchange chromatography, were pooled.

For buffer exchange, the pooled fractions (12 ml, 41 mg of MTG) were applied to a gel filtration column (Sephadex G25M) equilibrated with 20 mM sodium phosphate, pH 6.0. The Asp-MTG, as prepared above, was found to be highly purified as compared with the purified natural MTG analyzed by isoelectric focusing.

Crystalization—The crystallization experiments of Met-MTG, Ser-MTG, and Asp-MTG were executed under various conditions. However, we could not obtain good crystals from the Met-MTG and Ser-MTG samples. Only the Asp-MTG yielded crystals suitable for x-ray structural analysis, which were obtained under the following conditions.

The crystallization of Asp-MTG was performed at 20 °C with the hanging drop mode of the vapor diffusion method. The crystallization solution in the reservoir had a volume of 500 ml and was composed of 25% (v/v) polyethylene glycol 1000, 100 mM cacodylate-HCl buffer, pH 5.0, and 25 mM CaCl2. Two ml of MTG solution (15 mg/ml) and 2 ml of the reservoir solution were mixed and then equilibrated against the reservoir. After a few days, plate-like crystals emerged, which grew to a size (0.5 × 0.3 × 0.1 mm) sufficient for x-ray diffraction within about 10 days.

Data Collection and Processing—During the collection of the x-ray diffraction data, the MTG crystal was flash-cooled at 100 K after equilibration against a cryosolvent containing 35% (v/v) polyethylene glycol 1000, 120 mM cacodylate-HCl buffer, pH 5.0, and 35 mM CaCl2. The data collection statistics of the native MTG crystal are summarized in Table I. The x-ray wavelength was set to 1.00 Å, and the diffraction path was filled with helium gas to avoid air scattering. Diffraction intensities were recorded on imaging plates (Fujifilm Photo Film Co. Ltd.) using the oscillation method. The diffraction data were processed using the programs DENZO and SCALEPACK (36). The native crystal of MTG diffracted up to 2.4 Å resolution. It belongs to the space group P2₁, with unit cell dimensions of a = 78.4 Å, b = 117.12 Å, c = 85.74 Å, and β = 112.80°. The crystal contains four MTG molecules/ asymmetric unit with a solvent content of 48.7%.

The crystallographic analysis was performed by the multiple isomorphous replacement (MIR) method. The screening of several heavy atom compounds using the SMART6000 diffractometer (Bruker AXS) operated at 50 kV, 90 mA with CuKα radiation revealed that soaking with ethyl mercurithiosalicylate, K2[OeCl] and K[IrCl6] yielded good heavy atom derivatives. The data collection statistics of the derivative crystals used for the phase calculation are also summarized in Table I.

Table I.

MIR Phasing and Phase Improvement—The MIR analysis and the subsequent phase improvement were executed using the CCP4 program suite (37). The interpretation of the electron density map and the model building were performed using the program QUANTA (Molecular Simulations, Inc.) on an Octane graphics work station (Silicon Graphics Inc.).

The difference Patterson map between the ethyl mercurithiosalicylate derivative and native crystals presented four strong peaks, corresponding to major mercury sites. After the structural determination, each site was found to lie near the S atom of Cys56. The heavy atom sites of the other derivative crystals were determined using the difference Fourier maps phased by the major mercury sites of the ethyl mercurithiosalicylate derivative. The phase refinement was iteratively performed using the program MHLPHARE in CCP4, with gradual inclusion of the minor heavy atom sites. The final figure-of-merit value became 0.458. The other statistics of MIR phasing are summarized in Table I.

Table I. The electron density map (40.0–2.7 Å resolution) was of a quality and difficult to interpret.

The MIR phases were improved using the program DM in CCP4. At first, the solvent flattening procedure was reiterated on the condition of 50% solvent content, using the 40.0–2.7 Å resolution data. The electron density map was improved and showed a clear protein–solvent boundary and many β-helices. At this stage, it was revealed that the crystal contained four MTG molecules A, B, C, and D in the asymmetric unit. In the next step, the phases were further improved by the molecular averaging procedure. The noncrystallographic symmetry parameters were initially determined by superimposing the positions of the mercury and C atoms on the longest β-helices of molecules B, C, and D onto those of molecule A. The noncrystallographic symmetry parameters were refined during the molecular averaging procedure at 40.0–2.7 Å resolution. The molecular averaging procedure was successfully finished, yielding improved values for the mean figure-of-merit of combined phases (0.732 → 0.816) and the correlation coefficient (0.492 → 0.576). The map was further improved so that almost the
Table I

| Data                      | Native | EMTS(1) | EMTS(2) | K$_2$O$_x$Cl$_6$ | K$_3$IrCl$_6$ |
|---------------------------|--------|---------|---------|------------------|--------------|
| Crystal size (mm)         | 0.4 × 0.2 × 0.1 | 0.4 × 0.2 × 0.1 | 0.3 × 0.2 × 0.1 | 0.3 × 0.2 × 0.1 | 0.4 × 0.2 × 0.1 |
| Soaking conditions        | 1 mm EMTS | 24 h | 30 h | 5 mm K$_2$O$_x$Cl$_6$ | 2 mm K$_3$IrCl$_6$ |
| Device                    | PF BL-6B | SMART6900 | PF BL-6B | PF BL-6B | PF BL-18B |
| X-ray wavelength (Å)      | 1.54 | 1.00 | 1.00 | 1.00 | 1.00 |
| Collimator (mm)           | 0.2 × 0.2 | 1.0 | 0.2 × 0.2 | 0.2 × 0.2 | 0.2 × 0.2 |
| Detector                  | Imaging plate | Imaging plate | Imaging plate | CCD$^a$ |
| Film distance (Å)         | 573.0 | 74.0 | 573.0 | 573.0 | 220.0 |
| Oscillation angle (°/frame)| 2.5 | 0.1 | 3.0 | 3.0 | 1.5 |
| Exposure time (s/frame)   | 150 | 60 | 180 | 180 | 80 |
| No. of frames             | 72 | 1800 | 60 | 60 | 120 |
| Resolution (Å)            | 40.9–2.4 | 40.9–2.7 | 40.9–2.7 | 40.9–2.7 | 100.0–2.7 |
| No. of observations       | 187,088 | 82,979 | 127,046 | 140,702 | 140,603 |
| No. of unique reflections | 55,551 | 36,456 | 38,456 | 39,080 | 38,642 |
| <1(hkl)<(d(hkl))          | 12.0 | 10.7 | 5.9 | 10.9 | 11.4 |
| Completeness$^a$           | 0.980 (0.921) | 0.910 (0.894) | 0.991 (0.957) | 0.996 (0.961) | 0.979 (0.958) |
| Rmerge                    | 0.263 | 0.263 | 0.185 | 0.127 | 0.187 |
| Rfree$^a$                 | 0.88–0.85/0.99 | 0.85/0.97 | 0.87/0.98 | 0.87/0.98 | 0.81/0.98 |
| Phasing power$^a$         | 0.95 | 0.84 | 0.68 | 0.76 |

$^a$ CCD, charge-coupled device. The diffraction data were collected using the Quantum 4R diffractometer (ADSC) and were processed using the program DPCS/SOSFLM (34).

$^b$ The completeness and Rmerge of the outermost resolution shell (native, 2.44–2.40 Å; EMTS(1), 2.80–2.70 Å; EMTS(2), 2.75–2.70 Å; K$_2$O$_x$Cl$_6$, 2.75–2.70 Å; K$_3$IrCl$_6$, 2.85–2.80 Å) are in parentheses. EMTS, ethyl mercurithio-salicylate.

$^c$ Rmerge = ΣI(hkl) – Σ(hkl) / Σ(hkl), where Σ(hkl) = measured diffraction intensity and Σ(hkl) = mean value of all intensity measurements of the hkl reflection.

$^d$ Rmerge = Σ|Fcalc − Fobs| / Σ|Fobs|.

$^e$ Phasing power = (Fobs) / (|Fobs| + ε|Fcalc|), where Fobs is the structure amplitude of a derivative crystal; Fcalc and Fphi are the structure amplitude and the phase of the native crystal, respectively; and Fobs and Fphi are the real and imaginary parts of the calculated heavy atoms structure amplitudes, respectively.

RESULTS AND DISCUSSION

Crystallographic Analysis—The overall structures of the four independent MTG molecules are almost identical. The root-mean-square deviations of the C$_\alpha$ atom positions between the four MTG molecules range from 0.54 to 0.68 Å. In the crystal structure, molecules A and B and molecules C and D are related by noncrystallographic 2-fold axes, respectively. The interactions between them are slightly more extensive than the other intermolecular interactions in the crystal. However, the functional meaning of these dimers is questionable because there is no evidence supporting MTG dimer formation under physiological conditions.

The N-terminal Asp residue of each MTG molecule tightly interacts with adjacent residues of the symmetry related molecules. The Asp$^+$ nitrogen of molecule A is hydrogen-bonded and/or salt-bridged with the Gly$^{122}$ oxygen and Glu$^{203}$ O$_\alpha$ atoms of the symmetry-related molecule C. The Asp$^+$ O$_\alpha$ of molecule B and the Asp1 O$_\alpha$ of molecule D are salt-bridged with the Lys$^{216}$ N$_\alpha$ of the symmetry related molecule D and the Arg$^{48}$ N$_\alpha$ of the symmetry related molecule C, respectively. The Asp$^+$ nitrogen and O$_\alpha$ atoms of molecule C are hydrogen-bonded with the Gly$^{100}$ oxygen and Tyr$^{217}$ O$_\alpha$ atoms of the symmetry-related molecule A, respectively. The addition of the Met residue in Met-MTG or the deletion of Asp$^+$ in Ser-MTG would disturb the crystal packing. This situation may explain why Met-MTG and Ser-MTG did not yield good crystals.

Overall Structure—The overall structure of MTG is shown in Fig. 1. The MTG molecule forms a single, compact domain with overall dimensions of 65 × 59 × 41 Å. MTG adopts a disk-like shape and has a deep cleft at the edge of the disk. Cys$^{94}$, the residue essential for the catalytic activity, exists at the bottom of the cleft. Thus, we designate this cleft as the active site cleft.

The structure of MTG belongs to the $\alpha+\beta$ folding class, containing 11 $\alpha$-helices and 8 $\beta$-strands. The $\alpha$-helices and the $\beta$-strands are concentrated mainly at the amino and carboxyl ends of the polypeptide, respectively (Fig. 1D). These secondary structures are arranged so that a $\beta$-sheet is surrounded by $\alpha$-helices, which are clustered into three regions. The central $\beta$-sheet forms a seven-stranded anti-parallel structure, although this $\beta$-sheet is severely twisted between the $\beta_6$ and $\beta_9$ strands and there is only one hydrogen bond between the main chains of these strands (Trp$^{212}$ and Thr$^{220}$). The first cluster of $\alpha$-helices exists on the left side of the front view of the MTG molecule (Fig. 1B, left) and is composed of the $\alpha_1$, $\alpha_2$, and $\alpha_3$ helices. Cys$^{94}$ resides on the loop between the $\alpha_2$ and $\alpha_3$ helices. The second cluster, comprising the $\alpha_4$, $\alpha_5$, and $\alpha_9$ helices, and the third one, comprising the $\alpha_6$, $\alpha_7$, $\alpha_6$, and $\alpha_7$ helices, exist on the right and bottom sides of the front view of MTG, respectively. The three-dimensional structure of MTG was compared with the other proteins in the Protein Data Bank by the threading method and the method detecting the similarities with known active site structures. These searches were performed
using the “Seqfold” and “Binding Site Analysis” modules of the program Insight II (Molecular Simulation Inc.), respectively. However, any proteins similar to MTG could not be detected, suggesting that MTG has a novel three-dimensional structure. The electrostatic molecular surfaces of MTG are shown in Fig. 2. Generally speaking, the negative charges are localized...
primarily in the active site cleft, and the body of the MTG molecule is predominantly covered with positive charges. There are a lot of acidic residues, including Asp, Glu, Asp, and Glu, in the active site cleft. Fig. 3 demonstrates the distribution of the aromatic and hydrophobic residues of MTG. There are a lot of aromatic residues, including Tyr, Trp, Val, Leu, and Met, Cys, and the other residues are colored blue, red, and green, respectively. This illustration was drawn using the program QUANTA (Molecular Simulation Inc.).

**Comparison with Other TGases**—Thus far, the crystal structures of two other TGases, human factor XIII and red sea bream liver TGase (FTG), have already been determined (19, 23). The overall structures of FTG and human factor XIII resemble each other. Fig. 5 demonstrates structural comparisons between MTG and FTG. The overall structure of MTG is completely different from that of FTG, a difference that can be expected from the lack of sequence similarity and the different molecular sizes of these TGases. In contrast to the compact, single domain structure of MTG, FTG as well as human factor XIII consist of four sequential domains, named “β-sandwich,” “core,” “barrel 1,” and “barrel 2” by Yee et al. (19). The active site of FTG exists in the core domain, which comprises 334 amino acid residues. Although the three-dimensional structure of the core domain consists of 11 α-helices and 12 β-strands and belongs to the α + β folding class, the overall folding patterns of MTG and the core domain of FTG are considerably different (Fig. 5A).

However, the relation between the active site structures of MTG and FTG is quite noteworthy. As shown in Fig. 5B, the arrangements of the secondary structures around the active sites of MTG and FTG are very similar. The active site cysteines, Cys in MTG and Cys in FTG, both exist near the N terminus of the α-helices (α helix in MTG). This arrangement of the position of the nucleophiles is also observed in cysteine proteases, subtilisin proteases, and α/β hydrolases (22). Furthermore, this α-helix is flanked by the four-stranded β-sheet (β, β, β, and β strands in MTG) in each TGase. The catalytic triad of FTG consists of Cys, His, and Asp, and His, His, and Asp reside on the central two strands of this β-sheet. Factor XIII and some cysteine proteases, such as papain (42) and actinidin (43), also share a similar segment of α-helix and β-sheet containing the catalytic triad. On the other hand, in the
active site of MTG, there is no such cysteine protease-like catalytic triad as “Cys-His-Asp(Asn).” This situation is the most striking and important difference between MTG and the factor XIII-like TGases. Interestingly, in the MTG molecule, Asp$^{255}$ and His$^{274}$ occupy the positions corresponding to those of His$^{332}$ and Asp$^{355}$ in FTG, respectively (Fig. 5C). The three residues, Cys$^{64}$, Asp$^{255}$, and His$^{274}$, of the MTG molecules superimpose well on the catalytic triad of FTG (Cys$^{272}$, His$^{332}$, and Asp$^{355}$), respectively, with relatively small root-mean-square deviation values (1.37–1.76 Å for the main chain and the β- and γ-position atoms). Thus, in the MTG molecule, the relative positions of the catalytically important His and Asp...
seem to be reversed relative to the Cys residue.

Many proteases share similar tertiary structures of their active sites, including a catalytic triad and an oxianion hole, despite the diversity of their overall structures. This feature, first found in the structures of chymotrypsin and subtilisin, is regarded as a typical case of convergent molecular evolution (44). The similarity among the active site structures and the difference in the overall structures between MTG and the factor XIII-like TGases may imply that the relationship between these enzymes is a special case of convergent molecular evolution. However, if such relations are assumed, then Asp255 and His274 must perform the role of His and Asp in the catalytic triad of the factor XIII-like TGase, respectively. MTG may have developed a more unique catalytic mechanism specialized for the TGase reaction. The structure/function relationship of MTG will be discussed in a subsequent section.

MTG has a broader substrate specificity for the acyl donor and a higher reaction rate than the factor XIII-like TGases (27). In the three-dimensional structures of FTG and human factor XIII, the S/H9253 atoms of the catalytic Cys residues hydrogen bond with the O/H9257 atoms of Tyr residues (Tyr 515 in FTG and Tyr560 in factor XIII) and are inaccessible to the solvent (19, 23). The Tyr residue resides on the loop of the barrel 1 domain and covers the active site of the core domain. It is speculated that the binding of Ca2+ and the acyl donor causes the conformational change in which the Tyr residue is released from the catalytic Cys residue and the acyl-enzyme intermediate is formed. In contrast to the restricted solvent accessibility of the active site and the complicated activation process of the factor XIII-like TGase, Cys64 of MTG is sufficiently exposed to the solvent and can promptly react with substrates. Moreover, the flexibility of the right side wall of the active site cleft (Fig. 1B, left) may decrease the steric hindrance between the enzyme and substrates. These structural dissimilarities between MTG and the factor XIII-like TGases may be the reason for the differences in the substrate specificity and the reaction rate.

According to the superimposition shown in Fig. 5, B and C, the front and rear vestibules of the active site cleft of MTG correspond to the putative acyl donor and acyl acceptor binding sites (Fig. 5A), respectively, of factor XIII-like TGases (19, 23). The surface characteristics of these MTG regions are consistent with the properties of these substrates. As shown in the right side view of Fig. 2, the rear vestibule of the active site cleft is predominantly covered with the negative charges of Glu, Glu300, and Asp304, which would facilitate the access of a positively charged acyl acceptor. On the other hand, as shown in the left side views of Figs. 2 and 3, hydrophobic residues (Tyr62, Val65, Trp69, Tyr75, Ile240, and Phe254) and nonacidic hydrophilic residues (Lys200, Arg238, and Asn239) are concentrated in the front vestibule of the active site cleft. Interestingly, an experiment concerning the substrate specificity for the acyl donor of MTG demonstrated that synthetic peptides containing amino acid residues other than Gly and positively charged residues at the N-terminal side of Gln are good substrates of MTG, whereas MTG drastically loses its catalytic efficiency with the peptides containing residues other than Gly at the C-terminal side of Gln (45). These results suggest that the N-terminal side of Gln within the acyl donor binds to the front vestibule of the active site cleft of MTG and that MTG requires an acyl donor with large conformational flexibility and small side chains on the C-terminal side of Gln, to avoid steric hindrance with the enzyme itself.

**Speculation about the Catalytic Mechanism of MTG**—A cysteine protease-like catalytic mechanism of human factor XIII has been proposed on the basis of the structural similarity of the active sites between these enzymes (22). The catalytic triad

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**Fig. 6. A hypothetical catalytic mechanism of MTG.** Gln and Lys are the residues of substrate proteins. Although it has been shown that His274 is not essential for the catalytic activity (see footnote 3), we have included His274 in this figure for comparison with the catalytic mechanism of factor XIII, etc. Although the candidates for the oxanion hole-constructing residues are mentioned in the text, for clarity, they were omitted from this figure.
of factor XIII consists of Cys\textsuperscript{314}, His\textsuperscript{373}, and Asp\textsuperscript{396}. In the proposed mechanism, Cys\textsuperscript{314} and His\textsuperscript{373} mainly act in the acyl transfer reaction. Asp\textsuperscript{396}, which is hydrogen-bonded with His\textsuperscript{373}, functions to orient the conformation of the active site preferably and perhaps to stabilize the protonated form of His\textsuperscript{373}. Based on the structural comparison with the cysteine proteases, Trp\textsuperscript{279}, N\textsubscript{γ} and Cys\textsuperscript{314} nitrogen are considered to comprise the oxyanion hole as the hydrogen bond donors (22).

As discussed in the preceding section, the similarity of the secondary structure arrangement of the active site between MTG and FGT is very impressive, although the catalytic triad is not conserved in the active site of MTG. Therefore, we propose a cysteine protease-like catalytic mechanism for MTG in which Asp\textsuperscript{255} plays the role of the His residue in the factor XIII-like TGases. A hypothetical catalytic mechanism of MTG is shown in Fig. 6. In step A of Fig. 6, the thiolate ion of Cys\textsuperscript{54} nucleophilically attacks an acyl donor, the side chain of the Glu residue. In steps B and C, Asp\textsuperscript{255} donates a proton to the resultant oxyanion intermediate, and an ammonium is released. In step D, an acyl acceptor, such as the side chain of the Lys residue, approaches the active site, and the side chain of Asp\textsuperscript{255}, which is now negatively charged, nucleophilically attacks a proton of the acyl acceptor. In steps E and F, the product is released from the resultant oxyanion intermediate, and the catalytic reaction is finished.

The validity of assigning the above described roles to Asp\textsuperscript{255} is supported by the following facts. First, among the amino acid residues with polar side chains, Asp\textsuperscript{255} exists at the position nearest Cys\textsuperscript{54}. Second, a mutant protein in which Asp\textsuperscript{255} is replaced by Ala (D255A) drastically decreases its catalytic activity to a background level, suggesting that Asp\textsuperscript{255} is essential for the enzymatic reaction. Third, to fulfill this mechanism, Asp\textsuperscript{255} must be neutral at the initial state. So far, there is no direct evidence for the neutral state of Asp\textsuperscript{255}. However, the neutralization of Asp\textsuperscript{255} may be possible because this residue is fairly well buried in the protein molecule, with small accessible surface areas of the O\textsubscript{δ1} and O\textsubscript{δ2} atoms (4.1 and 1.2 Å, respectively). Fourth, the negatively charged state of Asp\textsuperscript{255} in step D seems to be advantageous for the substrate specificity of the acyl acceptor. That is, the positively charged amino groups are more attracted to the neutral species such as water molecules by the electrostatic interaction with Asp\textsuperscript{255}. In fact, the deamidation activity of MTG is weaker than that of FGT, etc. (28).

On the other hand, the role of His\textsuperscript{274} in the catalytic reaction does not look so important. In the MTG molecules A, B, C, and D, the distances between the Asp\textsuperscript{255} O\textsuperscript{δ1} and His\textsuperscript{274} N\textsubscript{γ} atoms are 3.1, 3.0, 4.1, and 3.1 Å, respectively. Therefore, the γ-carboxyl group of Asp\textsuperscript{255} and the imidazole group of His\textsuperscript{274} can form a hydrogen bond. This hydrogen bond may play a role in retaining the preferable conformation of the active site. However, in this hydrogen bond, Asp\textsuperscript{255} and His\textsuperscript{274} play roles as the hydrogen acceptor and donor, respectively. This hydrogen bonding pattern is completely opposite that of the factor XIII-like TGases and seems to decrease the nucleophilicity of Asp\textsuperscript{255} in reaction step D. Actually, an MTG mutant in which His\textsuperscript{274} is replaced by Ala (H274A) still retains about 50% activity relative to the wild type, suggesting that His\textsuperscript{274} is not essential for the enzymatic reaction. The antagonistic effects of the hydrogen bond between Asp\textsuperscript{255} and His\textsuperscript{274}, that is, the positive effect for the preferable conformation and the negative effect for the catalytic efficiency, may be compensatory and reduce the catalytic importance of His\textsuperscript{274}.

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