

Crystal Structure of Microbial Transglutaminase from *Streptovorticillum mobaraense**

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Tatsuki Kashiwagi, Kei-ichi Yokoyama, Kohki Ishikawa, Kunio Ono, Daisuke Ejima,
Hiroshi Matsui, and Ei-ichiro Suzuki‡

From the Central Research Laboratories, Ajinomoto Company Inc., 1-1 Suzuki-cho, Kawasaki-ku, Kawasaki,
Kanagawa 210-8681, Japan

The crystal structure of a microbial transglutaminase from *Streptovorticillum 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 (TGase¹; protein-glutamine γ -glutamyl-transferase, EC 2.3.2.13) catalyzes an acyl transfer reaction in which the γ -carboxamide 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 (3–8). 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 *Streptovorticillum 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 *Streptovorticillum* 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,

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The atomic coordinates and structure factors (code 1IU4) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (<http://www.rcsb.org/>).

‡ To whom correspondence should be addressed. Tel.: 81-44-210-5832; Fax.: 81-44-210-5872; E-mail: eiichiro_suzuki@ajinomoto.com.

¹ The abbreviations used are: TGase, transglutaminase; MTG, microbial TGase; FTG, fish-derived TGase; MIR, multiple isomorphous replacement.

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 pUC-TRPMTG-02 (33). pMTGF01(5'-cgatccatc gaagtcgtg attctgacga tcgtgttact cc-3') was the sense primer with the *Bam*HI site and the IEGR coding sequence upstream of the MTG gene. pMTGR01 (5'-caatttcgca gctcattacg gccaacctcg) was the antisense primer with the *Sac*I site. The PCR product was cloned into the pGEM-T Easy Vector (Promega, Madison, WI). Its sequence was confirmed, and the plasmid was designated as pGEMMTGXa, containing the *lacZ* promoter and the MTG gene in the same orientation. pET5a (Promega) was digested by *Eco*RI, blunt-ended, and digested by *Bam*HI. pGEMMTGXa was digested by *Sal*I, blunt-ended, and digested by *Bam*HI. The small fragment containing the MTG gene was integrated into the above prepared pET5a, and the new plasmid was designated as pETMTGXa-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 urea using 20 mM sodium acetate, pH 4.0. After 2 h of incubation at 5 °C, the pH was shifted from 4.0 to 6.0.² 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 mg 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-Sephacrose 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.

Crystallization—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 μ l and was composed of 25% (w/v) polyethylene glycol 1000, 100 mM cacodylate-HCl buffer, pH 5.0, and 25 mM CaCl_2 . Two μ l of MTG solution (15 mg/ml) and 2 μ l 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% (w/v) polyethylene glycol 1000, 120 mM cacodylate-HCl buffer, pH 5.0, and 35 mM CaCl_2 . The data collection statistics of the native MTG crystal are summarized in Table I. The diffraction data of the native crystal were collected using a macromolecular-oriented Weissenberg camera (35) installed on beam line (BL) 6B at the Photon Factory (PF) of the National Laboratory for High Energy Physics, Tsukuba, Japan. 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 (Fuji 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 $a = 78.41$ Å, $b = 117.12$ Å, $c = 85.74$ Å, and $\beta = 112.80^\circ$. The crystal contains four MTG molecules/asymmetric unit with a solvent content of 48.7%.

The crystallographic analysis of MTG 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 CuK_α radiation revealed that soaking with ethyl mercurithiosalicylate, K_2OsCl_6 , and K_2IrCl_6 yielded good heavy atom derivatives. The data collection statistics of the derivative crystals used for the phase calculation are also summarized in 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 Simulation 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_γ atom of Cys⁶⁴. 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 MLPHARE 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. The MIR electron density map (40.0–2.7 Å resolution) was of a poor 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 α -helices. At this stage, it was revealed that the crystal contains four MTG molecules (molecules A, B, C, and D)/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_α atoms on the longest α -helices of molecules B, C, and D onto those of molecule A. The noncrystallographic symmetry parameters were refined during the molecular averaging procedure performed 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 → 0.876). The map was further improved so that almost the

² K. Yokoyama, K. Ono, T. Ohtsuka, N. Nakamura, K. Seguro, and D. Ejima, manuscript in preparation.

TABLE I
 Statistics of data collection and MIR phasing

Data	Native	EMTS(1)	EMTS(2)	K ₂ OsCl ₆	K ₂ 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	1 mM EMTS 28 h	5 mM K ₂ OsCl ₆ 5 days	2 mM K ₂ IrCl ₆ 5 days
Device	PF BL-6B	SMART6000	PF BL-6B	PF BL-6B	PF BL-18B
X-ray wavelength (Å)	1.00	1.54	1.00	1.00	1.00
Collimator (mm)	0.2 × 0.2	1.0 × 1.0	0.2 × 0.2	0.2 × 0.2	0.2 × 0.2
Detector	Imaging plate	CCD	Imaging plate	Imaging plate	CCD ^a
Film distance (mm)	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)	40.0–2.4	40.0–2.7	40.0–2.7	40.0–2.7	100.0–2.7
No. of observations	187,008	82,979	127,046	140,702	140,603
No. of unique reflections	55,351	36,456	38,456	39,080	38,642
<I(hkl)/σ(I(hkl))>	12.0	10.7	5.9	10.9	11.4
Completeness ^b	0.980 (0.921)	0.910 (0.894)	0.991 (0.957)	0.996 (0.961)	0.979 (0.958)
R _{merge} ^{b,c}	0.040 (0.130)	0.097 (0.279)	0.081 (0.223)	0.048 (0.127)	0.048 (0.167)
R _{iso}		0.263	0.185	0.127	0.187
No. of heavy atom sites		5	7	5	8
R _{Cullis(cent)} /R _{Cullis(ano)} ^e		0.88/—	0.85/0.99	0.87/0.98	0.81/0.98
Phasing power ^f		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 DPS/MOSFLM (34).

^b The completeness and R_{merge} 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; K₂OsCl₆, 2.75–2.70 Å; K₂IrCl₆, 2.85–2.70 Å) are in parentheses. EMTS, ethyl mercurithio-salicylate.

^c $R_{\text{merge}} = \sum_{\text{hkl}} \sum_i |I(\text{hkl})_i - \langle I(\text{hkl}) \rangle| / \sum_{\text{hkl}} \langle I(\text{hkl}) \rangle$, where $I(\text{hkl})_i$ = measured diffraction intensity and $\langle I(\text{hkl}) \rangle$ = mean value of all intensity measurements of the (hkl) reflection.

^d $R_{\text{iso}} = \sum_{\text{hkl}} |F_{\text{PH}} - F_{\text{P}}| / \sum_{\text{hkl}} F_{\text{P}}$.

^e $R_{\text{Cullis(cent)}} = \sum_{\text{hkl}} |F_{\text{PH}} - |F_{\text{P}} \pm F_{\text{Hcalc}}|| / \sum_{\text{hkl}} |F_{\text{PH}} - F_{\text{P}}|$ for centric reflections, $R_{\text{Cullis(ano)}} = \sum_{\text{hkl}} ||F_{\text{PH}(+)} - F_{\text{PH}(-)}| - 2F_{\text{Hcalc}} \sin \varphi_{\text{P}}| / \sum_{\text{hkl}} |F_{\text{PH}(+)} - F_{\text{PH}(-)}|$.

^f Phasing power = $\langle F_{\text{Hcalc}} \rangle / (F_{\text{PH}} - |F_{\text{P}} \pm F_{\text{Hcalc}}|)$, where F_{PH} is the structure amplitude of a derivative crystal; F_{P} and φ_{P} are the structure amplitude and the phase of the native crystal, respectively; and F_{Hcalc} and F_{Hcalc} are the real and imaginary parts of the calculated heavy atoms structure amplitudes, respectively.

entire chains of all of the molecules could be traced. All of the residues of the MTG molecules could be assigned unambiguously to the electron density map.

Crystallographic Refinement—The constructed model of MTG was subjected to crystallographic refinement using the simulated annealing procedure of the program X-PLOR (38). The refinement was executed with the data corrected by the bulk solvent mask procedure of this program. During refinement, some manual rebuilding of the protein molecules was performed. After the R_{cryst} dropped to 0.246, water molecules were added to the model. Finally, the R_{cryst} and the R_{free} (5% test set) for the final model against 54,496 reflections (greater than $2\sigma(F)$) within 40.0–2.4 Å resolution were 0.199 and 0.266, respectively.

The final model includes four MTG molecules, each consisting of 331 amino acid residues, and 550 water molecules. The root-mean-square deviations in bond length and bond angle are 0.020 Å and 3.655°, respectively. A Ramachandran plot analysis of the four MTG molecules in the unit cell using the program PROCHECK (39) revealed that 86.7% of the residues are in the most favored regions, 11.6% of the residues are in additional allowed regions, 1.6% of the residues are in generously allowed regions, and 0.1% of the residues are in disallowed regions.

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_α 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 Asp¹ nitrogen of molecule A is hydrogen-bonded and/or salt-bridged with the Gly¹⁰² oxygen and Glu¹⁰⁷ O_{e1} atoms of the symmetry-related molecule C. The Asp¹ O_{δ2} of

molecule B and the Asp¹ O_{δ1} of molecule D are salt-bridged with the Lys²¹⁶ N_ε of the symmetry related molecule D and the Arg⁴⁸ N_{γ1} of the symmetry related molecule C, respectively. The Asp¹ nitrogen and O_{δ2} atoms of molecule C are hydrogen-bonded with the Gly¹⁰² oxygen and Tyr²¹⁷ O_η atoms of the symmetry-related molecule A, respectively. The addition of the Met residue in Met-MTG or the deletion of Asp¹ 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. Cys⁶⁴, 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 α+β folding class, containing 11 α-helices and 8 β-strands. The α-helices and the β-strands are concentrated mainly at the amino and carboxyl ends of the polypeptide, respectively (Fig. 1D). These secondary structures are arranged so that a β-sheet is surrounded by α-helices, which are clustered into three regions. The central β-sheet forms a seven-stranded anti-parallel structure, although this β-sheet is severely twisted between the β₅ and β₆ strands and there is only one hydrogen bond between the main chains of these strands (Trp²⁵⁸ and Thr²⁷³). The first cluster of α-helices exists on the left side of the front view of the MTG molecule (Fig. 1B, left) and is composed of the α₁, α₂, and α₃ helices. Cys⁶⁴ resides on the loop between the α₂ and α₃ helices. The second cluster, comprising the α₄, α₅, and α₁₀ helices, and the third one, comprising the α₆, α₇, α₈, and α₉ 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

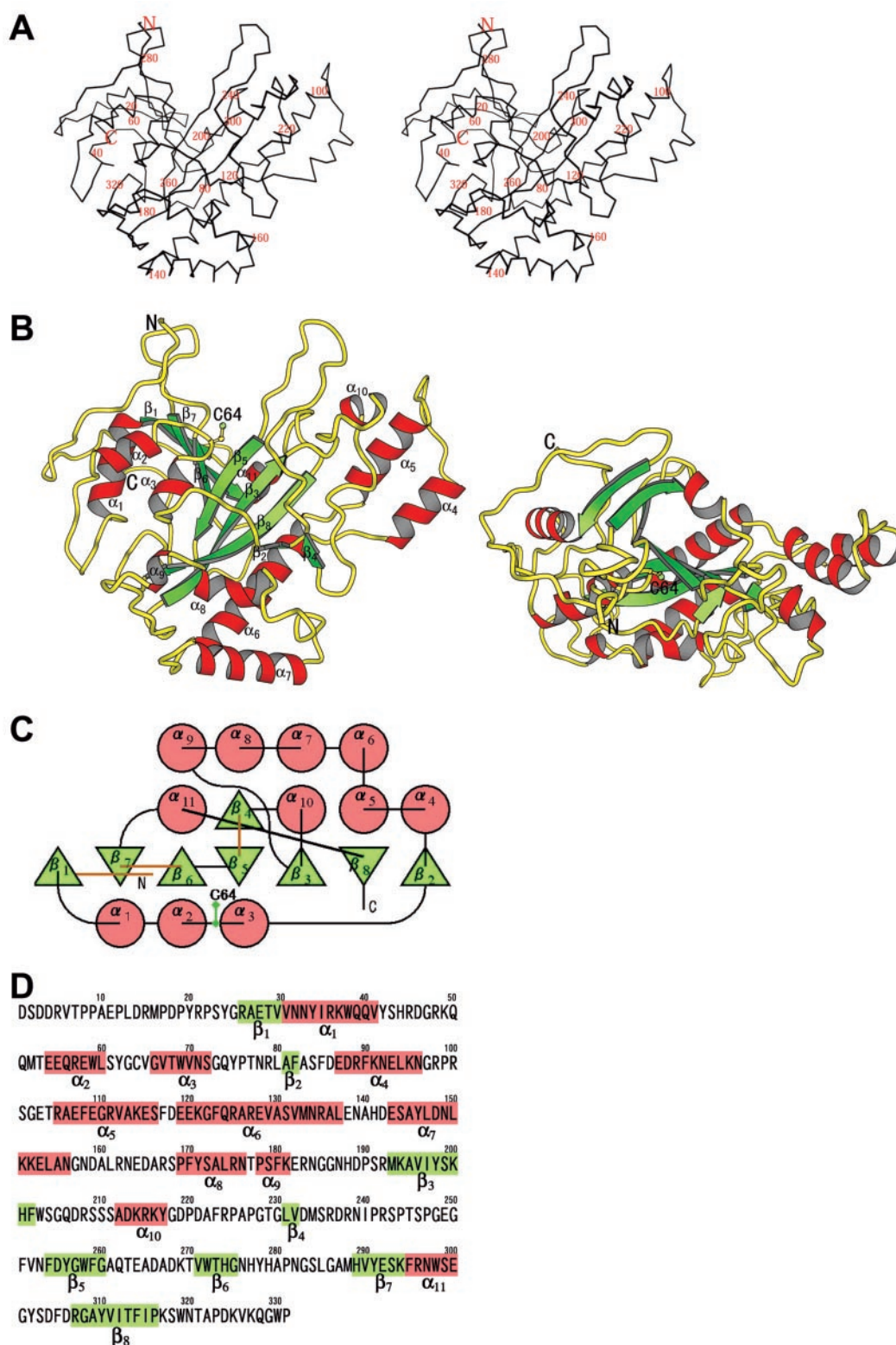


FIG. 1. **Overall structure of MTG.** A, the C α backbone trace (stereo view). The residue number is shown on every 20th residue. B, schematic ribbon drawings of the MTG molecule viewed from above the plate face (left) and from above the cleft (right). Hereafter we designate the left and right views as the front and top views, respectively. The numbering of the secondary structure is also represented. The side chain of Cys⁶⁴ is represented by a ball-and-stick model. The illustrations (A and B) were drawn with the program MOLSCRIPT (40). C, topology diagram. The circles and the triangles represent α -helices and β -strands, respectively. The loops that construct the side walls of the active site cleft are colored orange. D, secondary structure assignment onto the amino acid sequence of MTG. In B–D, α -helices and β -strands are colored red and green, respectively. The secondary structure assignment was performed with the algorithm of Kabsch and Sander as implemented in the program PROCHECK.

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

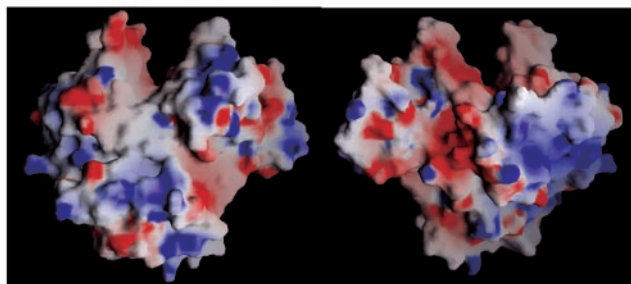


FIG. 2. **The electrostatic molecular surface of MTG.** The front and rear views are shown on the *left* and *right*, respectively. The surface, calculated using the program GRASP (41), is colored from *red* to *blue* in the range of -10.0 to $10.0 K_B T$, where K_B is the Boltzmann constant and T is the absolute temperature.

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 Asp¹, Asp³, Asp⁴, Glu²⁴⁹, Asp²⁵⁵, and Glu³⁰⁰, 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 Trp⁵⁹, Tyr⁶², Trp⁶⁹, Tyr⁷⁵, Tyr²⁷⁸, Tyr²⁹¹, and Tyr³⁰², on the surface around the active site cleft. These characteristic distributions of the acidic and aromatic residues would affect the substrate specificity of MTG.

Structure of the Active Site Cleft—The active site cleft exists at the top of the front view of MTG. The depth of the cleft is ~ 16 Å. The side walls of the active site cleft are created by two protrusions composed of several loops and are separated by a distance of about 13 Å between the tops of the protrusions (between Ser²⁸⁴ O γ and G²⁴⁸ O). In the front view of MTG, an N-terminal loop (Asp¹–Ala¹⁰) and a loop (Asn²⁷⁶–Met²⁸⁸) between the β_6 and β_7 strands jointly form the left side wall of the cleft. These loops are tightly associated with many polar and hydrophobic interactions and constitute a stable structure with relatively low B-factors (average value in main-chain atoms = 25.8 Å²). The N terminus of MTG resides in the entrance of the active site cleft. Natural MTG is secreted as a zymogen, which has an additional pro-sequence consisting of 45 amino acid residues at the N terminus of the mature sequence (26). The zymogen has no enzymatic activity. The position of the N terminus of the mature MTG makes it possible to suggest that the pro-sequence covers the active site cleft and prevents the substrates from accessing the active site. On the other hand, it has been reported that the reaction rate of Ser-MTG is higher than that of Asp-MTG (27). The crystal structure of MTG, in which the N-terminal residue and Cys⁶⁴ are relatively close to each other, is consistent with the fact that the N-terminal residue of MTG affects the catalytic activity. A loop ranging from Asn²³⁹ to Asn²⁵³ forms another side wall of the active site cleft. In contrast to the other side wall, this loop demonstrates a fairly flexible feature. The tip of this loop (from Arg²⁴² to Gly²⁵⁰) shows the highest B-factors (average value in main-chain atoms = 41.3 Å²) in the MTG molecule.

The bottom of the active site cleft is constructed of the β_5 , β_6 strands, the α_{11} helix, and the loop between the α_2 and α_3 helices. Cys⁶⁴ exists on the C-terminal side of this loop. The bottom surface of the MTG active site cleft is shown in Fig. 4. Although Cys⁶⁴ is surrounded by many residues, such as Phe²⁵⁴, Asp²⁵⁵, Asn²⁷⁶, and His²⁷⁷, the sulfhydryl group of Cys⁶⁴ has only the van der Waals contact with Phe²⁵⁴ O (3.7 – 4.0 Å) and is sufficiently exposed to the solvent (accessible surface area of S γ = 17 – 21 Å²).

Comparison with Other TGases—Thus far, the crystal structures of two other TGases, human factor XIII and red sea

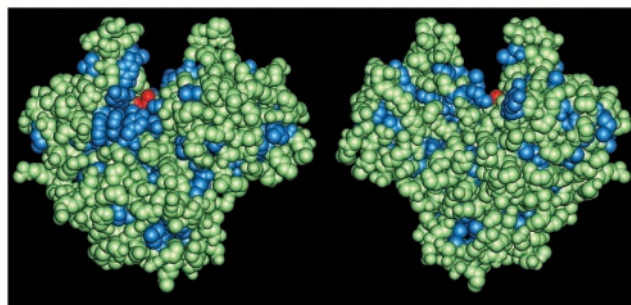


FIG. 3. **The van der Waals model of MTG.** The front and rear views are shown on the *left* and *right*, respectively. Aromatic and hydrophobic residues (Phe, Tyr, Trp, Val, Ile, Leu, and Met), Cys⁶⁴, and the other residues are colored *blue*, *red*, and *green*, respectively. This illustration was drawn using the program QUANTA (Molecular Simulation Inc.).

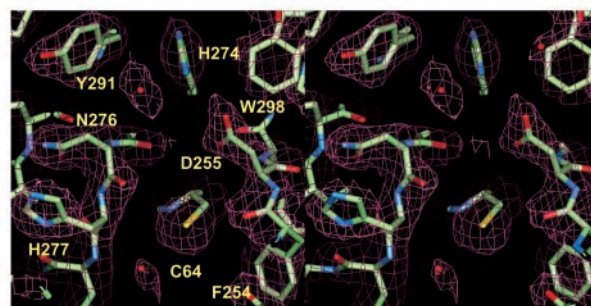


FIG. 4. **The bottom surface of the active site cleft of MTG (stereo view).** The $2F_o - F_c$ difference Fourier electron density map contoured at 1σ is superimposed on the model. Although Asn²⁷⁶ and His²⁷⁷ comprise the terminal part of the side wall protrusion, they overhang to Cys⁶⁴ and form the bottom surface of the active site. This illustration was drawn using the program QUANTA (Molecular Simulation Inc.).

breast 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 $\alpha + \beta$ 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, Cys⁶⁴ in MTG and Cys²⁷² in FTG, both exist near the N terminus of the α -helices (α_3 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_3 , β_5 , β_6 , and β_7 strands in MTG) in each TGase. The catalytic triad of FTG consists of Cys²⁷², His³³², and Asp³⁵⁵. His³³² and Asp³⁵⁵ 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

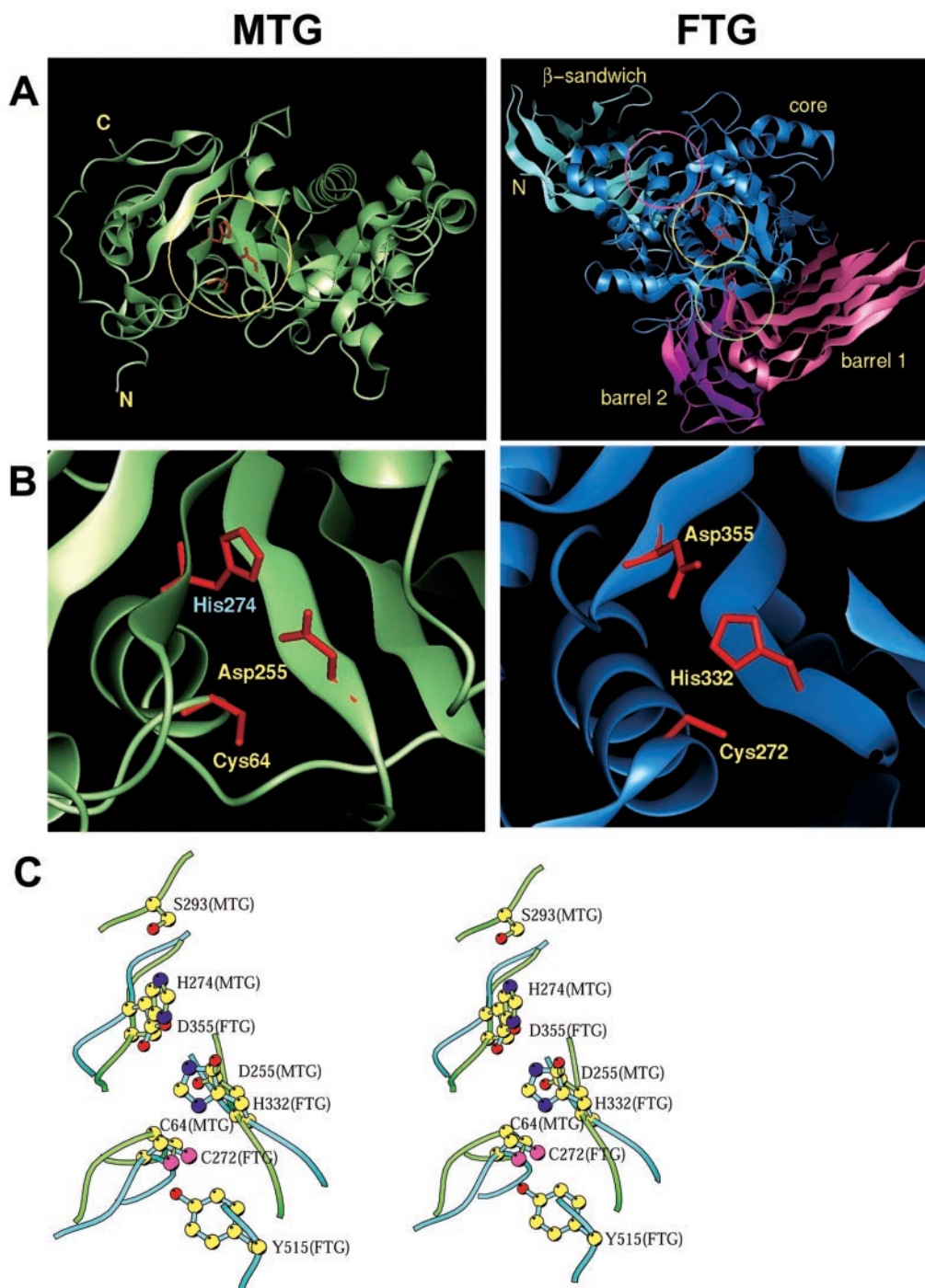


FIG. 5. **Structural comparison of MTG and FTG.** *A*, overall structures; *B*, structures around the active sites of MTG (left) and FTG (right). The top views of MTG are drawn with a green ribbon model. The four domains of FTG (β -sandwich, core, barrel 1, and barrel 2) are shown in light blue, dark blue, light purple, and dark purple, respectively. The catalytic triad of FTG (Cys272, His332, and Asp355) and the positionally corresponding residues of MTG (Cys64, Asp255, and His274) are represented by the red wire model. These illustrations were drawn using the program QUANTA (Molecular Simulation Inc.). In *A*, the regions enclosed by yellow circles, a green circle, and a purple circle represent active sites, a possible acyl donor binding site of FTG, and a possible acyl acceptor binding site of FTG, respectively. *C*, stereo view of the superposition of the active site of MTG (green) on those of FTG (light blue). The catalytic triads of FTG and MTG, as well as the residues (S293(MTG) and Y515(FTG)) in which the side chains interact with the side chains of the catalytic triads (H274(MTG) and C272(FTG), respectively), are represented. The ball-and-stick representations were drawn using the program MOLSCRIPT.

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, Asp²⁵⁵ and His²⁷⁴ occupy the positions corresponding to those of His³³² and Asp³⁵⁵ in FTG, respectively (Fig. 5C). The three

residues, Cys⁶⁴, Asp²⁵⁵, and His²⁷⁴, of the MTG molecules superimpose well on the catalytic triad of FTG (Cys²⁷², His³³², and Asp³⁵⁵, 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

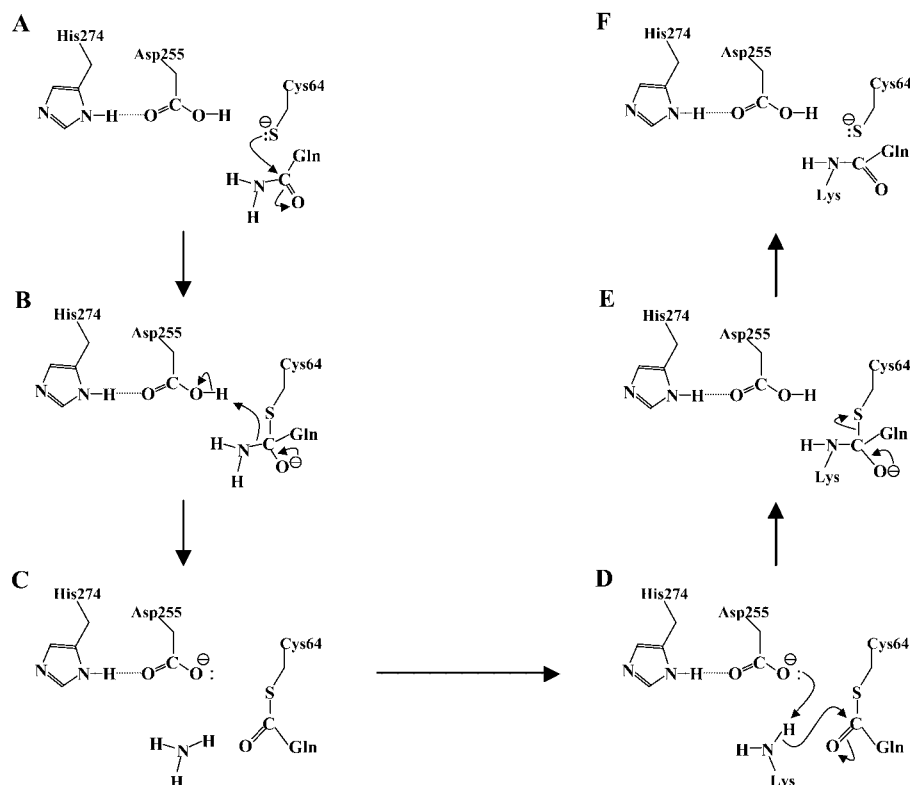


FIG. 6. **A hypothetical catalytic mechanism of MTG.** Gln and Lys are the residues of substrate proteins. Although it has been shown that His²⁷⁴ is not essential for the catalytic activity (see footnote 3), we have included His²⁷⁴ in this figure for comparison with the catalytic mechanism of factor XIII, etc. Although the candidates for the oxyanion hole-constructing residues are mentioned in the text, for clarity, they were omitted from this figure.

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 oxyanion 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_γ atoms of the catalytic Cys residues hydrogen bond with the O_η atoms of Tyr residues (Tyr⁵¹⁵ 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 Glu²⁴⁹, 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

of factor XIII consists of Cys³¹⁴, His³⁷³, and Asp³⁹⁶. In the proposed mechanism, Cys³¹⁴ and His³⁷³ mainly act in the acyl transfer reaction. Asp³⁹⁶, which is hydrogen-bonded with His³⁷³, functions to orient the conformation of the active site preferably and perhaps to stabilize the protonated form of His³⁷³. Based on the structural comparison with the cysteine proteases, Trp²⁷⁹ N_{ε1} and Cys³¹⁴ 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 FTG 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 Asp²⁵⁵ 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 Cys⁶⁴ nucleophilically attacks an acyl donor, the side chain of the Gln residue. In steps B and C, Asp²⁵⁵ 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 Asp²⁵⁵, 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 Asp²⁵⁵ is supported by the following facts. First, among the amino acid residues with polar side chains, Asp²⁵⁵ exists at the position nearest Cys⁶⁴. Second, a mutant protein in which Asp²⁵⁵ is replaced by Ala (D255A) drastically decreases its catalytic activity to a background level, suggesting that Asp²⁵⁵ is essential for the enzymatic reaction.³ Third, to fulfill this mechanism, Asp²⁵⁵ must be neutral at the initial state. So far, there is no direct evidence for the neutral state of Asp²⁵⁵. However, the neutralization of Asp²⁵⁵ may be possible because this residue is fairly well buried in the protein molecule, with small accessible surface areas of the O_{δ1} and O_{δ2} atoms (4.1 and 1.2 Å², respectively). Fourth, the negatively charged state of Asp²⁵⁵ 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 than the neutral species such as water molecules by the electrostatic interaction with Asp²⁵⁵. In fact, the deamidation activity of MTG is weaker than that of FTG, etc. (28).

On the other hand, the role of His²⁷⁴ in the catalytic reaction does not look so important. In the MTG molecules A, B, C, and D, the distances between the Asp²⁵⁵ O_{δ1} and His²⁷⁴ N_{δ1} atoms are 3.1, 3.0, 4.1, and 3.1 Å, respectively. Therefore, the γ-carboxyl group of Asp²⁵⁵ and the imidazole group of His²⁷⁴ 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, Asp²⁵⁵ and His²⁷⁴ 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 Asp²⁵⁵ in reaction step D. Actually, an MTG mutant in which His²⁷⁴ is replaced by Ala (H274A) still retains about 50% activity relative to the wild type, suggesting that His²⁷⁴ is not essential for the enzymatic reaction.³ The antagonistic effects of the hydrogen bond between Asp²⁵⁵ and His²⁷⁴, 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 His²⁷⁴.

In the case of the factor XIII-like TGases, the candidates for the residues that construct the oxyanion hole are Trp²⁷⁹ (factor XIII) and Trp²³⁶ (FTG). In the structure of MTG, Asn²⁷⁶ exists near the position corresponding to Gln¹⁹ of papain (42) or actinidin (43), which is the residue constructing the oxyanion hole. Asn²⁷⁶, as well as Cys⁶⁴, Asp²⁵⁵, and His²⁷⁴, is conserved in the amino acid sequence of the MTG homologue (25). Therefore, in the case of MTG, Asn²⁷⁶ may construct the oxyanion hole.

Conclusions—In this research, we have determined the three-dimensional structure of a novel type of TGase from a microorganism. Although its overall structure is completely different from those of the factor XIII-like TGases and the cysteine proteases, the secondary structure framework of the MTG catalytic center is very similar to them, implying a functional relationship between these enzymes. Based on this structural similarity around the active site, we have proposed that Asp²⁵⁵ of MTG plays the role of the His residue in the cysteine protease-like catalytic triad. Some serine proteases have been known to utilize the Ser-Lys diad mechanism (47), whereby Ser and Lys residues serve as the nucleophile and the general base, respectively. If our assumption is true, then MTG is the first enzyme that utilizes a Cys-Asp diad mechanism. Although direct evidence for the proposed mechanism is not available yet, further studies, including the determination of the three-dimensional structure of an MTG-substrate or -inhibitor complex, would allow us to inspect this novel catalytic scheme of MTG.

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PROTEIN STRUCTURE AND FOLDING:
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