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Substrate Specificity Analysis of Microbial Transglutaminase Using Proteinaceous Protease Inhibitors As Natural Model Substrates[1]

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The substrate specificity of microbial transglutaminase (MTG) from *Streptomyces mobaraensis* (formerly categorized *Streptoverticillium*) was studied using a *Streptomyces* proteinaceous protease inhibitor, STI2, as a model amine-donor substrate. Chemical modification and mutational analysis to address the substrate requirements for MTG were carried out around the putative reactive site region of STI2 on the basis of the highly refined tertiary structure and the solvent accessibility index of *Streptomyces* subtilisin inhibitor, SSI, a homolog of STI2. The results suggest that the P1 reactive center site (position 70 of STI2) for protease subtilisin BPN′ or trypsin may be the prime Lys residue that can be recognized by MTG, when succinylated β-casein was used as a partner Gln-substrate. It is characteristic in that the same primary enzyme contact region of STI2 is shared by both enzymes, MTG and proteases. For quantitative analysis of the TG reaction, we established an ELISA-based monitoring assay system using an anti-SSI polyclonal antibody highly cross-reactive with STI2. Site-specific STI2 mutants were prepared by an *Escherichia coli* expression-secretion vector system and subjected to the assay system. We reached several conclusions concerning the nature of the flanking amino acid residues affecting the MTG reactivity of the substrate Lys residue: (i) site-specific mutations from Asn to Lys or Arg at position 69 preceding the amine-donor 70Lys, led to enhanced substrate reactivity; (ii) amino acid replacement at 67Ile with Ser led to higher substrate reactivity, (iii) additive effects were obtained by a combination of the positive mutations at positions 67 and 69 as described above, and (iv) Gly at position 65 might be essential for MTG reaction. Moreover, the substrate specificity of guinea pig liver tissue transglutaminase (GTG) was compared with that of MTG using STI2 and its mutants. In contrast to MTG, replacement of Gly by Asp at position 65 was the most favorable for substrate reactivity. Also, 70Lys appeared not to be a prime amine-donor site for GTG-mediated cross-linking, suggesting a difference in substrate recognition between MTG and GTG.

Key words: amine-donor, ELISA-based assay, microbial transglutaminase, site-specific mutagenesis, *Streptomyces* protease inhibitor, substrate specificity.

Transglutaminases (termed TGs, EC 2.3.2.13) catalyze the acyl-transfer reaction between a γ-carboxyamide group of glutamine and ε-amino group of lysine or other primary amines, giving rise to the formation of γ-glutamyl-ε-lysine peptide chain bridges (*1*). This type of enzyme is widely distributed as a superfamily in archaea, bacteria and eukaryotes, and their enzymatic properties have been extensively studied (*2*). Recently, calcium-independent microbial TG (termed MTG) from *Streptomyces* (formerly *Streptoverticillium*) has been used as a powerful attractive catalyst for industrial applications, mainly in food processing (*3*, *4*). However, little is yet known of its physiological role, structure–function relationship, and, especially, substrate specificity as compared to its mammalian counterparts. For simplicity, synthetic oligopeptide substrates have often been used to analyze the substrate specificity of mammalian TGs and have led to useful findings about the substrate requirements of the TG reaction (*5–9*).

However, it should be noted that interactions between globular proteins such as enzyme/substrate (inhibitor), antigen/antibody or hormone/receptor are in general performed through molecular recognition at high-order structural levels. Therefore, more attention should be paid to the importance of the tertiary and/or quaternary structures of substrate proteins in TG reactions.

To date, no natural protein substrate for MTG has been

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Abbreviation: TG, transglutaminase (protein-glutamine:amine γ-glutamyltransferase, EC 2.3.2.13).

identified in MTG producing *Streptomyces* cells. For the present study, there are three main reasons for choosing *Streptomyces* protease inhibitors as candidates for the natural proteinaceous substrates for MTG. First, we have previously demonstrated that proteinaceous protease inhibitors that are homologs (*10–12*) of the *Streptomyces* subtilisin inhibitor, SSI (*13*), are distributed widely as a family (termed SIL proteins) in *Streptomyces* (*14*). In addition, a strong correlation was found between the structure around the reactive center region of SIL proteins and their inhibition specificities toward proteases (*15–18*). The structure–function relationships of SIL proteins are expected to provide useful insights into the molecular recognition of target protein substrates by MTG. Second, evidence of similar chromatographic behaviour on ion-exchange or gel-exclusion columns between MTG and an SIL protein, termed SIL-V6, during the purification of MTG from *S. mobaraensis* (unpublished data), was also observed by another group (*19*). This suggests a strong affinity between MTG and SIL protein. Third, findings that eukaryotic TGs have evolved from an ancestral protease, as revealed by the tertiary structure-based molecular modeling of the human blood clotting factor XIIIa′ (*20, 21*) and the experimental demonstration using the *Methanobacterium* phage pseudomurein endopeptidase (*22*) support the use of *Streptomyces* protease inhibitors. The transglutaminase reaction is considered to be the reverse of the proteolytic reaction catalyzed by thiol proteases that possess the catalytic triad of three active residues, Cys, His, and Asp (*21*). These factrors suggest a close relationship between MTG and protease inhibitor proteins such as SSI or SIL-V6.

Here we describe a substrate specificity analysis of MTG using a SIL proteins, STI2 from *S. longisporus* (*23*), which has been found to be the best natural model amine-donor substrate by chemical modification and mutational analysis. For quantitative analysis of the MTG reaction, we established an ELISA-based monitoring assay system using an anti-SSI antibody highly cross-reactive with STI2. Using this system, the requirements of STI2 as the substrate for MTG were investigated, especially from the tertiary structural point of view, based on the highly refined tertiary structure data for SSI (*24*), a homolog of STI2. Furthermore, a comparative study of substrate specificity was performed between MTG and a guinea pig liver tissue TG (GTG) using STI2 and its site-specific mutants. The findings obtained by this approach suggest some micro-environments related to the substrate recognition of MTG whose tertiary structure has not yet been established.

MATERIALS AND METHODS

Materials and Bacterial Strains—A polyclonal antibody against *Streptomyces* subtilisin inhibitor (SSI) was raised in rabbits and its reactivity with the antigen was checked at our laboratory. The anti-SSI antibody cross-reacted effectively with STI2 protease inhibitor. All of the restriction enzymes and modifying enzymes for genetic engineering were purchased from TaKaRa Shuzo. After careful exploration of the coating ability of the antibody, of more than ten microtiter plates with 96-wells produced by four companies, only a polystyrene microtiter plate produced by Nunc (Immuno Plate MaxiSorp F96; Nunc A/S, Roskilde, Denmark) was found to be suitable for the immuno-reaction between subtilisin BPN′ and its antibody. All other chemicals were of reagent grade and used without further purification.

Purification and Activity Measurement of MTG—The use of a hydroxyapatite column (Wako) in conjunction with an SP-sephadex C-50 column employed here was very effective in separating MTG and SIL-V6. A spore suspension of *Streptomyces mobaraensis* JCM4168 was inoculated into 100 ml of the medium developed by Zhu *et al.* (*25*), in each batch (total 10 Erlenmeyer flasks), and cultivated for 10 days at 30°C. The cells were removed by filtration through Whatman 3MM chromatography paper and then ammonium sulfate was added to approximately 1 liter of the filtrated culture medium to give 60% saturation. The precipitate was collected by centrifugation, dissolved in 50 mM sodium phosphate buffer (pH 6.0), and then dialyzed against the same buffer for 2 days. The dialysate was applied to an SP-sephadex C-50 (Pharmacia) column (3 × 45 cm). Subsequently, the protein was eluted by applying two step-wise column works using 0.1 M NaCl and 0.3 M NaCl in the same buffer. The fractions containing MTG were pooled, dialyzed against distilled water and then lyophilized. The lyophiliyed material was dissolved in 5 ml of 0.2 M sodium phosphate buffer (pH 6.8), applied to a hydroxyapatite column, and eluted with a linear gradient of 0.2 to 0.4 M sodium phosphate buffer (pH 6.8). For MTG assay, the hydroxamate formation of Cbz-L-glutaminylglycine was measured by a colorimetric procedure (*26*), using L-glutamic acid γ-monohydroxamate for the calibration curve. One unit caused the formation of one micromole of hydroxamic acid per min. Protein was measured using a Bio-Rad Protein Assay kit.

Purification of Protease Inhibitors—The sixteen protease inhibitors tested in this study are listed in Table I. Four mutants of SSI were kindly provided by Dr. S. Kojima, Gakushuin University. SSI and its eleven native homologs were purified by essentially the same procedure as reported previously (*15*). In the case of STI2 and SIL-V6, a two-step procedure involving chromatography on CM-cellulose and hydroxyapatite columns (described below) was added to the

TABLE I. **Cross-linking ability of various SIL protein.** SIL proteins cross-linked with β-casein by MTG were subjected to Western blotting. MTG reaction was performed in the presence or absence of dithiothreitol (DTT). [a]Cross-linking ability was estimated from Western blotting. NT, not tested. Degree of cross-linking: +++ > ++ > + > ± > –.

SIL protein	P1 site	Cross-linking ability[a]	
		DTT–	DTT+
SSI (*S. albogriseolus*)	M	±	±
SSI 73K (artificial mutant)	K	+	±
SSI 73Q (artificial mutant)	Q	±	±
SSI 71S101S (artificial mutant)	M	±	NT
SSI 71S73K101S (artificial mutant)	K	±	NT
STI2 (*S. longisporus*)	K	+++	+++
SIL1 (*S. cacaoi*)	R	±	NT
SIL4 (*S. lavendulae*)	K	–	NT
SIL5 (*S. fradiae*)	K	–	NT
SIL8 (*S. virginiae*)	M	±	NT
SIL10 (*S. thermotolerans*)	K	±	NT
SIL-V1 (*S. flavopersicus*)	K	±	±
SIL-V3 (*S. eurocidicus*)	K	±	NT
SIL-V6 (*S. mobaraensis*)	K	+	+ ±
SACI (*S. cinnamoneus*)	R	±	NT
SLPI (*S. lividans*)	R	±	NT

purification procedure established for SSI.

Chemical Modification of STI2 with 2,4,6-Trinitrobenzene Sulfonic Acid—To 100 μl of purified STI2 solution (2.2 mg/ml H_2O), 100 μl of 4% $NaHCO_3$ (pH 8.5) and 100 μl of 0.1% 2,4,6-trinitrobenzene sulfonic acid (TNBS) were added and the mixture was incubated at 40°C. After 2 h incubation, 100 μl of 10% SDS and 50 μl of 1 N HCl were added to terminate the reaction. Finally, the reaction sample was dialyzed against water and lyophilized.

Succinylation of β-Casein—To use β-casein as a Gln-substrate (substrate containing Gln, but not Lys with a free amine group), a chemically modified form was generated by succinylating the lysine residues of β-casein. β-Casein, 100 mg, was dissolved in 20 ml of buffer solution [0.06% $Ca(NO_3)_2 \cdot 4H_2O$, 1% KCl, 0.1% NaOH, 0.25% H_3BO_4, 0.3% Triton X-100; pH 9.16]. To this β-casein solution, 200 mg anhydrous succinate was gradually added while maintaining pH 8.5 with 2 N NaOH. After the completion of the succinylation, the reaction solution was brought up to 50 ml with distilled water, dialyzed against distilled water, and lyophilized.

Assay for Protein Cross-Linked by MTG—Various inhibitor protein samples (2 to 5 μg) were incubated with β-casein (20 μg) or succinylated β-casein (20 μg) in the presence of MTG (2 milliunits) in 50 mM phosphate buffer (pH 7.5) at 37°C for 24 h. If needed, 100 mM cystamine, MTG inhibitor, or 10 mM dithiothreitol (DTT) was added to the reaction mixtures. Prior to electrophoresis, the protein samples were precipitated with trichloroacetic acid at a final concentration of 8%. Subsequently, the polymerized products were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) after Coomassie Brilliant Blue staining. SDS-PAGE was carried out using 18.8% polyacrylamide gels as described by Laemmli (*27*). Western blot analysis was carried out using rabbit anti-SSI antibody diluted 5,000-fold, goat anti–rabbit immunoglobulin G diluted 7,500-fold conjugated to alkaline phosphatase and substrates, 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium, as reported previously (*28*).

STI2 Gene Cloning and Secretion Vector Construction for STI2 and Its Mutant Genes—A plasmid vector was constructed for the efficient secretory expression of STI2 in *E. coli* as follows (see Fig. 1). Basically, we used an *E. coli* high-level production vector system previously developed for SSI and SSI-fused proteins (*29*, *30*). Chromosomal DNA was prepared from *Streptomyces longisporus* ISP5166 according to the method of Saito and Miura (*31*). PCR primers for amplification of the DNA fragment encoding the STI2 mature region were synthesized based on the nucleotide sequence of the STI2 gene previously cloned by Strickler *et al.* (*23*). The two 25-mer oligonucleotides used for amplification were 5′-**GAATTC**GCCTCGCTCTACGCCCCCT-3′ (as a forward primer) and 5′-**GAGCTC**AGAACGCGAAGAGGCTGGT-3′ (as a reverse primer). Two restriction sites, *Eco*RI and *Sac*I, shown in bold letters in the oligonucleotide sequences, were created at both ends of the amplified fragment to be inserted into the pUC18 plasmid vector (*32*). PCR was carried out with cloned *Pfu* DNA polymerase (Stratagene) using a program of 30 cycles of 96°C for 3 min (denaturation), 50°C for 1 min (annealing), and 72°C for 3 min (elongation). After double digestion with *Eco*RI and *Sac*I, the amplified DNA fragment encoding the STI2 mature region was subcloned once into the pUC18 plasmid digested with the same enzymes. The resultant plasmid was termed pUSTI2. The DNA insert was sequenced on pUSTI2 using the double-stranded dideoxynucleotide sequencing technique (*33*). Finally, the relevant region was transferred from the pUSTI2 to the pOSΔB-AP1 vector for the SSI-fused gene (*30*) using *Eco*RI and *Bam*HI sites to generate a secretion plasmid vector, termed pOSTI2.

Generation of STI2 Mutants—Single mutations at four positions, 65, 67, 69, and 70 of STI2, and double mutations at two position pairs, 67/69 and 69/70, were carried out on pUSTI2 (for single mutations) and its plasmid derivatives (for double mutations) as follows using the mutagenic primers listed in Table II. (i) K70R mutant: For only this case, site-directed mutagenesis was carried out by the method of Morinaga *et al.* (*34*). (ii) G65A, G65D, G65E, G65N, G65S, G65T, and G65V mutants: Mutations were introduced using the primer pairs, MUT4 (TaKaRa Shuzo) and mutagenic primers (for the first PCR), and M13 primer RV

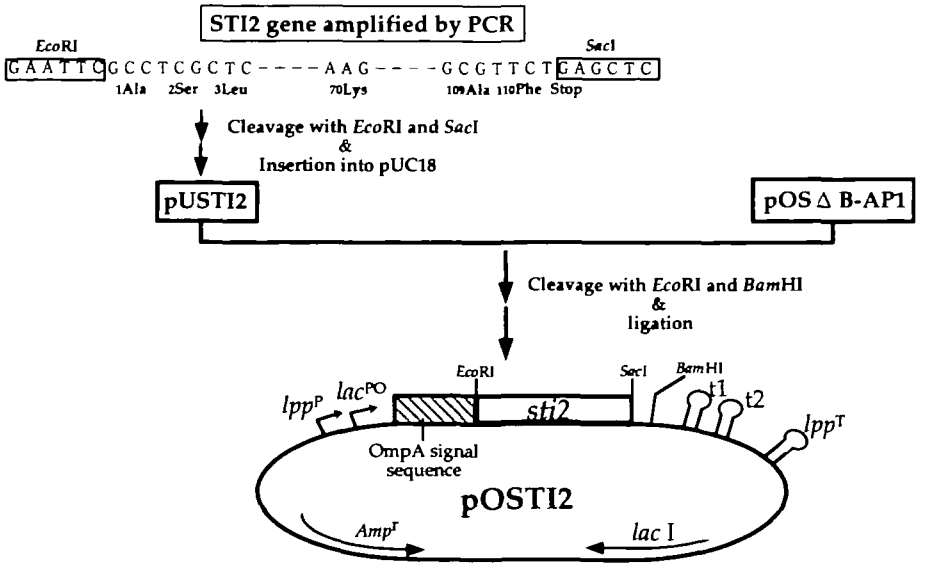

Fig. 1. **Secretory expression vector construction of the STI2 gene in *E. coli*.** The hatched and shaded boxes represent the OmpA signal sequence and the region encoding the mature part of STI2, respectively. *lpp*^{P}, lpp promoter; *lpp*^{T}, lpp terminator; *lac*^{PO}, lac promoter-operator; t1 and t2, SSI terminators 1 and 2; *Amp*^{r}, ampicillin resistance gene; *lacI*, lactose repressor gene.

(TaKaRa Shuzo) and M13 primer M4 (TaKaRa Shuzo) (for the first and second PCRs) *via* the heteroduplex formation between the two first PCR products (*35*). PCR was carried out using programs of 30 cycles of 94°C for 30 min (denaturation), 50°C for 1 min (annealing), and 72°C for 3 min (elongation) (for the first PCR), a linear mode of gradual cooling from 96°C (15 min) to 30°C (15 min) for 2 h (for heteroduplex formation), and 10 cycles under the same conditions as those for the first PCR (for the second PCR). The single strand region of the heteroduplex was filled in by the second PCR, followed by double digestion with *Eco*RI and *Bam*HI. The double stranded DNA fragment carrying the target mutation could, in principle, be selectively digested with both enzymes and subjected to cloning into the same restriction sites of the expression plasmid, pOSTI2. (iii) I67F, I67L, I67P, I67S, and I67V mutants, and (iv) N69A, N69F, N69K, N69L, N69P, N69R, N69S, and N69V mutants were prepared by almost the same method as for the G65 mutant series. (v) I67S-N69R and I67S-N69K mutants: Introduction of these double mutations was carried out on the pUSTI2 derivative carrying a I67S mutation using mutagenic primers for the N69K and N69R mutations. (vi) N69K-K70R mutant: In the same way, a double mutation was carried out on the pUSTI2 derivative carrying the N69K mutation using a mutagenic primer for the K70R mutation. All mutations were screened by colony hybridization (*36*) using the corresponding mutagenic primers as probes and confirmed by dideoxy sequencing of mutated plasmids.

Purification of Recombinant STI2 and Its Mutants from E. coli—STI2 and its mutants as substrate proteins for the MTG reaction were purified from *E. coli* JM109 harboring plasmid vector with the gene encoding each protein, by the procedure reported previously (*29*, *30*). Briefly, *E. coli* JM109 strain containing the pOSTI2 plasmid carrying the STI2 or mutant genes was cultivated aerobically at 37°C in 500 ml LB medium supplemented with 50 μg/liter ampicillin. To overproduce the STI2 or its mutant proteins, the lac promoter was induced with 1 mM isopropyl-β-D-thio-galactoside (IPTG) when the culture reached an absorbance of 0.5 at 610 nm. The bacterial cells were harvested by centrifugation 3 h after induction. Proteins in the culture supernatant of the transformant cells induced with 1 mM IPTG were concentrated by the addition of ammonium sulphate to 80% saturation. The periplasmic fraction containing STI2 or its mutant protein was prepared from the IPTG induced-transformant cells by osmotic pressure and combined with the culture supernatant fraction. The mixture was then salted out by the addition of ammonium sulphate to 80% saturation. The precipitate formed was collected by centrifugation, dialyzed against 20 mM sodium carbonate (pH 9.5), and applied to a DEAE-cellulose column. Elution was performed with a linear gradient of 0 to 0.4 M NaCl in the same buffer. Fractions showing protease inhibitory activity were combined, dialyzed against distilled water and lyophilized. The lyophilized material was dissolved in 5 ml of 0.2 M sodium phosphate buffer (pH 6.8), applied to a hydroxyapatite column, and then eluted with a linear gradient of 0.2 to 0.4 M sodium phosphate buffer (pH 6.8). The concentration of STI2 or its mutant protein was determined spectrophotometrically at pH 7.0 with an absorbance at 280 nm (1 mg/ml) of 0.95 calculated based on the molar absorption coefficient 19,100 $M^{-1}\cdot cm^{-1}$ of the SSI dimer form and its amino acid composition (*13*).

Construction of an Assay System for the MTG Reaction—The assay system comprises the following processes. (i) To each well of a microtiter plate, 230 μl succinylated β-casein (10 μg/ml) dissolved in sodium carbonate buffer (pH 9.8) is added and the plate is stored at 4°C over night. (ii) The succinylated β-casein coated on each well is washed three times to remove unbound substrate with phosphate-buffered saline (pH 7.4) containing 0.05% Tween 20 (PBST). (iii) Then, 200 μl of the reaction mixture containing STI2 or its mutant (1–5 μg) and MTG (2 milliunits)/50 mM Tris-HCl (pH 7.5) is supplied to each well and the plate is incubated at 37°C for 1.5 h. When the Ca^{2+}-dependent enzyme, guinea pig liver tissue TG [GTG (Sigma)], is examined for comparison with MTG, a solution containing 50 mM Tris-HCl (pH 7.5) and 10 mM $CaCl_2$ is used as a reaction solution. (iv) After completion of the reaction, contaminants are removed from the cross-linked molecules with succinylated β-casein by washing as described above. (v) Two hundred microliters of 10^3-fold diluted anti-SSI antiserum dissolved in 100 mM PBST is added to each well and the immunoreaction is carried out by incubating at room temperature for various times. (vi) After washing, 200 μl of goat anti–rabbit

TABLE II. **Design for the generation of STI2 mutants.**

Original amino acid residue	Substituted amino acid residue	Designed mutagenic primer
65G	A, D, E, N, S, T, V	STI2 G65(–) 5′-CAGATCACG(AGT)(CT)GTCTCGCGCCTTC-3′ 5′-CAGATCAC(GC)TCGTCTCGCGCCTTC-3′ 5′-CAGATCACG(ACT)TGTCTCGCGCCTTC-3′
67I	F, L, P, S, V	STI2 I67(–) 5′-CTTGTTGCAG(AG)(ACG)CACGCCGTCTCG-3′
69N	A, F, K, L, R, V	STI2 N69(–) 5′-GTACAGCTTC(CT)TGCAGATCACGCC-3′ 5′-CGTACAGCTT(GA)(AG)(AC)GCAGATCACGCC-3′
70K	R	STI2 K70R(–) 5′-GTACAGCCTGTTGCAGATCAC-3′
(double)	I67S-N69R, I67S-N69K	STI2 I67S-N69RK(–) 5′-GTACAGCTTC(CT)TGCAGGACACGCC-3′
	N69K-K70R	STI2 N69K-K70R(–) 5′-CGTACAGCCTCTTGCAGATCA-3′

Listed are synthetic oligonucleotides used for site-directed mutagenesis. Underlined residues indicate base changes at the mutated sites at each position of STI2. Nucleotides in parentheses are mixtures that give the desired codons.

IgG diluted 1:7,500 conjugated to alkaline phosphatase is added and the reaction mixture is incubated at room temperature for 1 h. (vii) Finally, the cross-linking ability is estimated as a function of time for each STI2 substrate by measuring the absorbance at 405 nm corresponding to the release of *p*-nitrophenol due to enzymatic hydrolysis of 100 μM phosphatase substrate (*p*NPP) in a 200 μl reaction solution [0.1 M Tris-HCl (pH 8.5) and 5 mM $MgCl_2$] with a microplate reader (Model 550, BIORAD) according to the manufacturer's instructions. Data represent the means of triplicate samples. The standard deviations were <5% in all cases.

RESULTS

Examination of SIL Proteins as Potent Protein Substrates for MTG—As explained in "INTRODUCTION," we used proteinaceous protease inhibitor proteins, SIL proteins, as natural model substrates for the analysis of the substrate specificity of the MTG reaction. Among our huge collection of SIL proteins, including SSI mutants, the 16 purified SILs listed in Table I were used to test their potentiality as substrate for MTG. First, when each SIL protein was assayed as a sole molecular species, no cross-linking was detected for any SIL by Western blotting. This suggests no intramolecular homo-polymerization occurs for any inhibitor protein. Next, β-casein, frequently used a TG substrate, was added to the MTG reaction mixture containing each SIL protein in the presence or absence of DTT to examine the flexibility of the S-S bonds of the SIL proteins. As summarized in Table I, among the SIL proteins tested, STI2 was found to be the best substrate for the MTG reaction by Western blotting. A representative reaction pattern is shown in Fig. 2. The MTG-catalyzed heteromolecular cross-linking between STI2 and β-casein was detected as a mixture of reacted products with various molecular masses and regulated by the addition of the MTG inhibitor, cystamine. Next, chemical modification of the STI2 protein was performed with 2,4,6-trinitrobenzene sulfonic acid (TNBS), and the modified sample was added to the cross-linking reaction using β-casein. The MTG reaction failed to be detected with the chemical modification specific to Lys residue, strongly suggesting that STI2 can act as an amine-donor substrate with the Lys site(s) susceptible to MTG. SIL4, which is most homologous to STI2 (82% identity) (*14*, *15*), showed no cross-linking reaction. Therefore, for further investigation, STI2 was chosen as a model protein substrate for the MTG reaction.

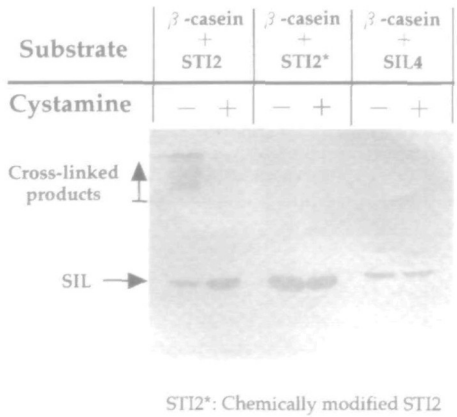

Fig. 2. **Substrate reactivity of STI2, chemically modified STI2, and SIL4 for MTG.** Western blotting patterns of the MTG-catalyzed polymerization of STI2, its chemically modified form, and SIL4. 2 to 5 μg of each SIL protein sample was applied to the MTG reaction with succinylated β-casein in the presence or absence of the MTG inhibitor, cystamine.

Moreover, SIL-V6 of *S. mobaraensis* was found to be a better MTG substrate under reduced conditions (Table I). It is of interest to investigate the molecular interaction between the cognate pair, MTG and SIL-V6, in *S. mobaraensis* in terms of the physiological significance of MTG and a protease inhibitor such as SIL-V6 (to be discussed later).

Identification of Amine-Donor Lys Residue of STI2 Involved in the MTG Reaction—The highly refined tertiary structure of SSI at 1.8 Å resolution (*24*), a homolog of STI2, would enable us to analyze the substrate requirements of STI2 for the MTG reaction. The tertiary structure of plasminostreptin (PSN), an SIL protein, has also been determined at 2.8 Å resolution, and the effects of amino acid substitutions to the SSI framework were explored by Sugino *et al.* (*37*). The root mean squre deviation between the C_α positions of PSN and SSI was 1.4 Å when the dimers of these inhibitors were superimposed. This strongly indicates that PSN resembles SSI not only in the structure of their monomers but also in their quaternary structure, even though the overall amino acid homology between the two inhibitors is not so high (69%) (*38*). Thus, the SSI type dimeric structures, at least within a family of SIL proteins, seem to have been preserved during evolution. Figure 3A shows the sequence comparison around the already determined reactive center sites of two inhibitor proteins, SSI (*13*) and STI2 (*23*). This sequence information prompted us to identify the amino acid residue(s) of STI2 responsible for the MTG reaction. STI2 possesses four Lys residues and one Gln residue. From the structural basis of SSI (*13*), 84 Gln of STI2 (corresponding to 87Gln of SSI) may be located in the lower part of the hydrophobic core formed by β_4 strand and buried in the interior of the protein structure. Therefore, it is quite less possible that STI2 could function as a Gln substrate for the MTG reaction. Among four Lys residues in STI2, 100Lys (103Met for SSI) is supposed to be located in the α_2-helix. In the case of SSI, its electron density is quite clear, and the calculated relative solvent accessibility is nil (*39*), strongly suggesting that 100Lys for STI2 is also well fixed and buried in the center of the core. The other three Lys residues are all occupied at the molecular surface of STI2 as can be predicted from the data of SSI. 61Lys, 70Lys, and 86Lys of STI2 correspond to 64Thr at the flexible loop region, 73Met of the P1 reactive center site and 89Lys at the interface of β_4-β_5 strands of SSI, respectively.

To address which Lys residue is mainly involved in the MTG reaction, the concept of "solvent accessibility" is useful. This has been proposed as a good measure of the degree of exposure to solvents, of individual atoms, residues or proteins as a whole, and is easily calculated from the coordinates obtained by X-ray crystallography (*40*). The calculation was made for the whole dimeric molecule of SSI using the coordinate set including all the atoms of residues 6Leu to 113Phe (*39*). The relative accessible surface area for all side chain atoms (from C_β to the end) in each residue

is shown in Fig. 3B, by normalizing the P1 reactive center site (73Met) as 100% exposed to solvent. This definition of solvent accessibility is also expected to be a good criterion when considering the molecular recognition of MTG by the STI2 substrate. Both primary structure and total conformation appear to determine whether proteins can act as TG substrates, with reactive lysines often located on solvent-exposed or flexible areas of the protein (*5*, *6*). In order to identify the Lys residue of STI2 required for the MTG reaction, we first focussed on 70Lys, which corresponds to 73Met at the P1 reactive center site of SSI, and seems to be located in the exposed loop with the most solvent accessibility. Amino acid replacement with Arg drastically converted STI2 to a non-reactive substrate for MTG, as revealed by polymerization assay linked to Western blotting (data not shown). This result coincides well with evidence that TNBS-modified STI2 loses its inhibitory activity toward the intrinsic target proteases, subtilisin and trypsin (*23*), suggesting that 70Lys of the P1 reactive center site of STI2 is exposed to the solvent water, and thus sensitive to the chemical modifier molecule. Consequently, it was concluded that 70Lys is a main amine donor residue crucial for the MTG cross-linking reaction with succinylated β-casein. As for the involvement of 61Lys or 86Lys in the MTG reaction, site-directed mutagenesis at these positions would provide direct information about this possibility.

Establishiment of a Monitoring Assay System for the MTG Reaction—For quantification of the MTG reaction, we established a rapid and simple system based on enzyme-linked immunosorbent assay (ELISA) using anti-SSI polyclonal antibody highly cross-reactive with STI2. As illustrated in Fig. 4, the assay system consists basically of four experimental processes, (i) coating a microtiter plate with succinylated β-casein, (ii) adding culture supernatant samples containing each recombinant STI2 mutant substrate highly secreted by *E. coli* JM109 and then initiating the reaction by adding MTG, (iii) conducting a first specific immunoreaction with anti-SSI polyclonal antibody to the STI2 mutants linked with the succinylated β-casein, and (iv) spectrophotometrically measuring the reaction using anti–rabbit IgG conjugated to alkaline phosphatase. The minimal concentration of succinylated β-casein that can saturate the surface of a well was determined to be 10 μg/ml. Next, in wells coated with 10 μg/ml of succinylated β-casein, the absorbance at 405 nm was measured for various concentrations ranging from 0.1 to 30 μg/ml of authentic STI2. Over 10 μg/ml of STI2 gave practical saturation of

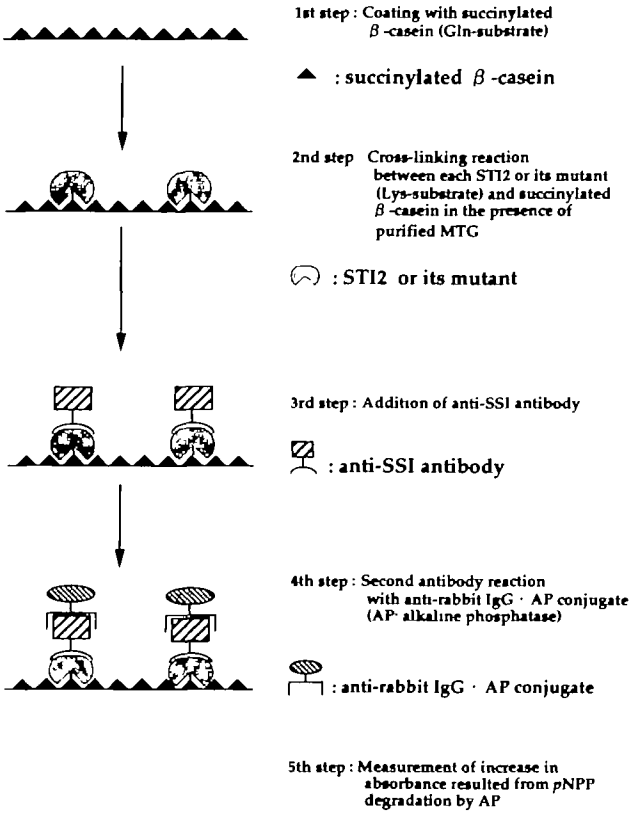

Fig. 4. **Schematic flow diagram for the quantification of cross-linking reactivity based on ELISA.** A detailed explanation is given in "MATERIALS AND METHODS." *p*NPP, *p*-nitrophenylphosphate.

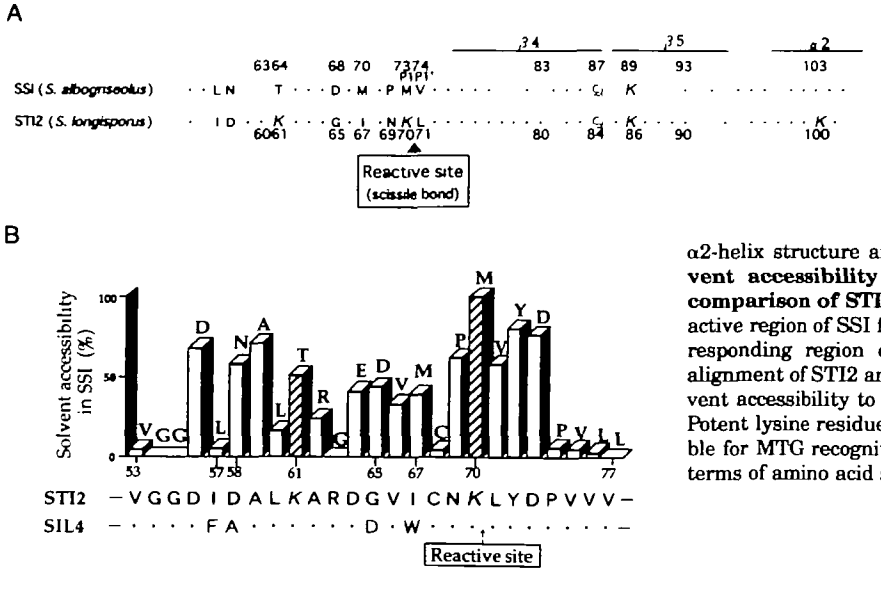

Fig. 3. **Panel A, sequence comparison of STI2 and SSI.** Residue numberings are those of SSI (upper) and STI2 (lower). Lysine and glutamine residues are indicated by large italic letters K and opened letters Q, respectively. The triangle indicates the reactive site for inhibition against subtilisin BPN' (scissile bond linked by P1 and P1' sites) determined previously for SSI (*13*) and STI2 (*23*). β-stranded structures, β4 and β5, and α2-helix structure are characterized in the SSI (*13*). **Panel B, solvent accessibility established in SSI along with sequence comparison of STI2 with SIL4.** The solvent accessibility in the reactive region of SSI for predicting the accessibility of MTG to the corresponding region of STI2 are shown along with the sequence alignment of STI2 and SIL4. The bars indicate the ratio of actual solvent accessibility to ideally exposure in SSI (modified from Ref. *13*). Potent lysine residues, 61K and 70K (numbering for STI2), responsible for MTG recognition are hatched. The critically different sites in terms of amino acid sequence between STI2 and SIL4 are shaded.

the immunoreaction with succinylated β-casein when the BSA blocking treatment often used for ELISA was omitted. Blocking reagents such as BSA disturb the MTG reaction. All of at least 0.4 μM of STI2 molecules are expected to react *via* the MTG reaction with the defined amount of succinylated β-casein. The secretory production level of recombinant wild-type STI2 by *E. coli* JM109 was estimated to be approximately 3 μM in the culture supernatant employed. In the culture supernatant samples, no contaminants linked non-specifically with the two amine-donor or amine acceptor substrates were detected by Western blotting. To confirm the adequacy of this assay system, authentic STI2 and its modified form (TNBS treated) were applied individually in equal amounts to the reaction system. As expected, a time-dependent increase in absorbance was observed for intact STI2, whereas the modified STI2 showed only the basal level of MTG reaction in accordance with the results of the polymerizing assay described above. Subsequently, using many STI2 mutant samples, this assay system was adopted for the systematic analysis of the sub-

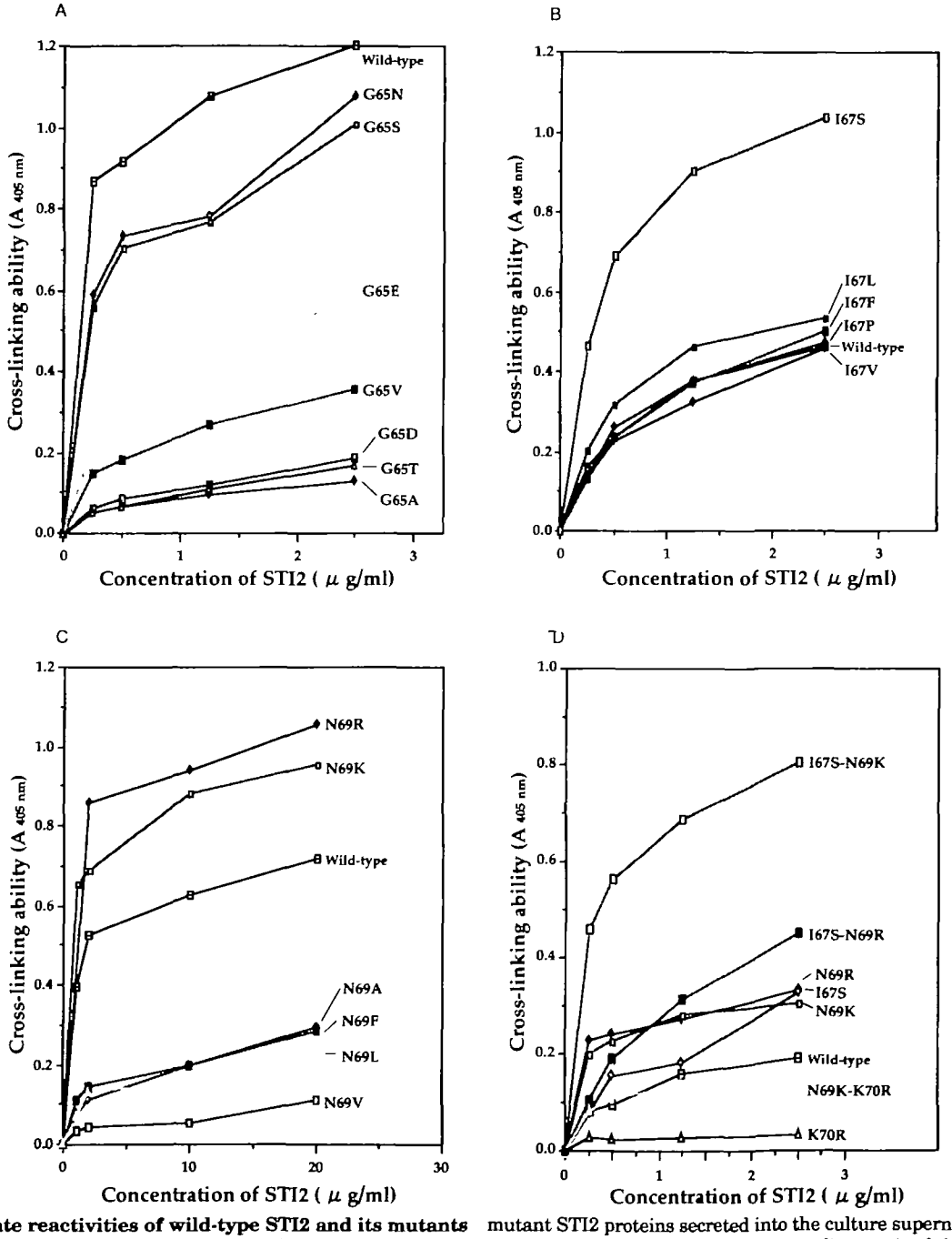

Fig. 5. **Substrate reactivities of wild-type STI2 and its mutants for MTG using culture supernatant samples.** Panels A–D show substrate reactivities of mutants at three positions. A, G65, mutants; B, I67 mutants; C, N69 mutants; D, double mutants. The amount of mutant STI2 proteins secreted into the culture supernatants were determined by scanning the corresponding stained bands following SDS-PAGE and equally normalized mutant proteins were applied to the ELISA-based system for MTG.

strate specificity of MTG.

Mutational Analysis of Other Substrate Requirements for MTG—As shown in Fig. 3B, two Lys residues are located at the same positions around the reactive site beween two inhibitor homologs, STI2 and SIL4. Nevertheless, SIL4 is not a substrate for MTG (Fig. 2). The critical difference in the MTG reaction between STI2 and SIL4 was considered to result from sequence diversity, that is, only four restricted amino acid residues, 57Ile (Phe for SIL4), 58Asp (Ala for SIL4), 65Gly (Asp for SIL4), and 67Ile (Trp for SIL4), in the molecular surface region involving the putative reactive site, ranging from 53Val to 77Val of STI2. Among these four positions, amino acid substitutions were introduced into positions 65 and 67 in close proximity to the P1 reactive site 70, along with other two positions, 69 and 70, in the STI2 molecule.

(1) Mutation at position 65: All seven mutations at this position led to a reduced cross-linking reaction with succinylated β-casein (Fig. 5A). From mutation of this position, glycine was found to be an important residue for the MTG substrate. Therefore, the positioning of Asp at the corresponding site in SIL4 might be a negative factor(s) rendering the inhibitor protein unsusceptible to MTG.

(2) Mutation at position 67: Out of five mutants, four exhibited almost the same (I67F,P,V) or slightly higher (I67L) substrate reactivities compared to the wild-type STI2. Interestingly, the I67S mutant showed a significantly increased reactivity (Fig. 5B). These results suggest that substitution at position 67 do not seriously affect the MTG reaction.

(3) Mutation at position 69: Substitution to positively charged amino acids, Arg and Lys, at this position enhanced the substrate reactivity, while reduced reactivity was observed by alterations to aliphatic (Ala, Leu, Val) or aromatic (Phe) amino acids (Fig. 5C). These results agree in part with the sequence preferences that basic polar or uncharged residues appear frequently at positions directly preceding the Lys-substrates in factor XIII TG (*6*).

(4) Double mutation at positions 67 and 69: To examine the additive effect of mutations positively contributing to much higher MTG reactivity, two double mutants, I67S-N69K and I67S-N69R, were subjected to the reaction system. As expected, drastic elevations in MTG reactivity were observed for both double mutants (Fig. 5D). The cooperativity of the double mutation at I67S-N69K was higher than that at I67S-N69R.

(5) Double mutation at positions 69 and 70: We constructed a double mutant, N69K-K70R, in order to examine whether a shift in the Lys residue from position 70 to the adjoining 69 maintains the ability of STI2 to function as a Lys-substrate. This mutant could actually act as an MTG Lys-donor substrate, although its reactivity was slightly lower (Fig. 5D). Enhanced substrate reactivities by single mutations, N69K and N69R, suggest that the presence of two adjacent basic amino acid residues at positions 69 and 70, which are the most exposed to the solvent, may be very effective for substrate recognition by MTG.

Using purified protein samples, the cross-linking abilities of eight representative STI2 mutants were assayed by the ELISA-based system employed here. As shown in Fig. 6A, almost the same profile was observed as for the measurements performed using culture supernatant samples (Fig. 5). From this, our assay system is demonstrated to be reliable and useful for the quantitative measurement of the MTG reaction and provides a versatile tool for screening MTG substrates.

Comparative Study of MTG and GTG—GTG is known to have a very broad substrate specificity in comparison with other members of the TG family (*41*). It is of interest to compare the difference in STI2 substrate recognition between MTG and GTG, which originate from different organisms. Quite different substrate specificity profiles were observed for the two enzymes using purified wild-type STI2 and its mutant proteins, as shown in Fig. 6, A and B.

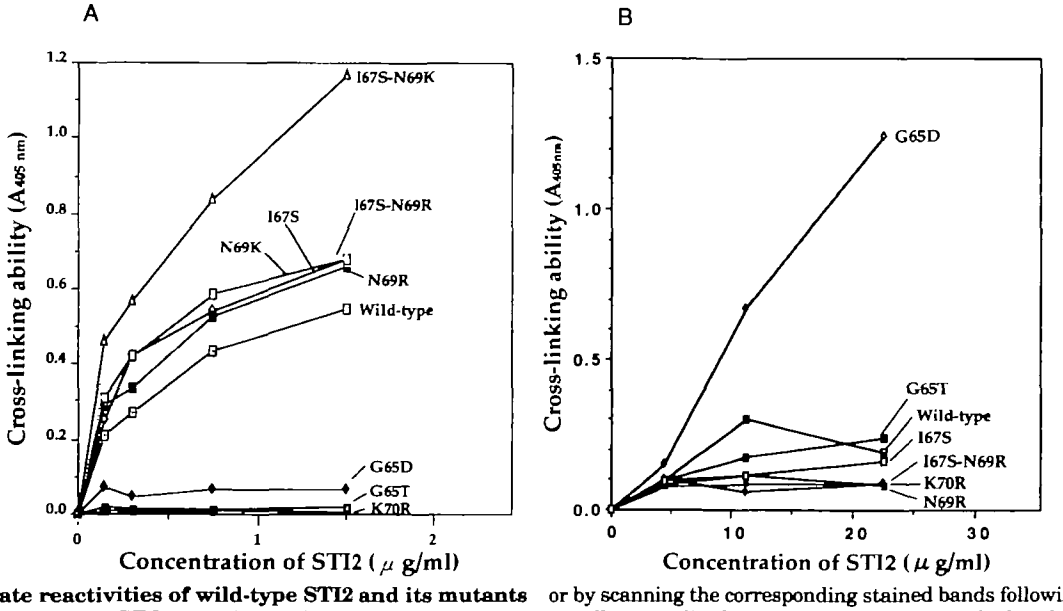

Fig. 6. **Substrate reactivities of wild-type STI2 and its mutants for MTG (panel A) and GTG (panel B) using purified samples.** The amounts of wild-type and mutant STI2 proteins purified from the culture supernatants were determined by photometric measurement or by scanning the corresponding stained bands following SDS-PAGE; equally normalized mutant proteins were applied to the ELISA-based system for MTG.

First, the GTG reaction demanded much higher amounts of STI2 substrate and showed a very slow rate during the initial stages as compared with the MTG reaction. Second, opposite results were obtained, in particular, by mutation at position 65, which has been shown to be important for maintaining the substrate reactivity of MTG. In contrast with MTG, mutation of G65D in GTG was the most effective. Also, mutations at positions 67 and 69 that were positive for MTG all exhibited less or the same cross-linking ability for GTG compared with wild-type STI2. Unexpectedly, the K70R mutant showed a less reduced substrate reactivity, suggesting that 70Lys is not a unique site for the recognition of GTG.

DISCUSSION

Although a few attempts have been made to characterize the substrate specificity of MTG using synthetic peptide derivatives (*3*, *42*), nothing has been done to characterize the substrate recognition of MTG taking tertiary structure into account. The protease inhibitor SSI is one of the most extensively characterized proteins produced by *Streptomyces* (*13*). The vast number of natural and artificial mutants (over 100 proteins isolated) of SSI are useful as model substrate proteins to investigate the substrate specificity of the MTG reaction. As expected, among SIL proteins, STI2 from *S. longisporus* was found to be an amine-donor substrate for MTG. In this study, based on the critical difference in the primary sequence between STI2 and SIL4, the substrate requirements of STI2 for the MTG reaction were systematically addressed.

From the present findings obtained by a combination of chemical modification and site-specific mutation as analyzed with an ELISA-based assay system, the following conclusions can be drawn. (i) STI2 is the best natural amine-donor substrate for the MTG reaction among the SIL proteins tested. (ii) 70Lys, the putative P1 reactive center site of STI2, is a prime site for the recognition of MTG, but possibly not a unique site for GTG. (iii) Alteration to a basic amino acid at position 69 enhances the MTG reaction. (iv) A Ser substitution at position 67 gives rise to greater substrate reactivity. (v) An additive effect in the enhanced substrate reactivity is brought about by a combination of positive mutations, N69K/R and I67S. (vi) Gly at position 65 might be essential for the MTG reaction, while an alteration to Asp at this position is the most effective for the GTG reaction.

Through the mutational analysis, 65G-66V-67S-68C-69K-70K has been chosen as the best STI2 sequence for MTG substrate reactivity. The STI2 mutant with this sequence could be efficiently used to screen the natural partner Gln-substrate(s) involved in the MTG reaction system or for designing a substrate analogue-type peptide inhibitor specific toward MTG. Possibly, the conversion of SIL4 to a good MTG substrate can be achieved by replacing the original sequence on SIL4 with this selected sequence motif. It is also important to address the substrate determinants for MTG of the partner amine-acceptor substrate, β-casein, and this project is currently underway.

Zeevwen *et al.* (*7*) showed that human epithelial protease inhibitor (SKALP/elafin) is a very efficient TG substrate for both GTG and epidermal TG, and acts as an amine-donor as well as amine-acceptor. Human elafin, a protease inhibitor with an anchoring sequence ('cementoin') rich in Lys and Gln residues, was also demonstrated to be a good substrate for GTG (*43*). The other type of serine protease inhibitor, plasminogen-activator inhibitor type-2, can serve as a substrate for both activated factor XIII and tissue TG (*44*). These findings suggest that protease inhibitors can also be substrates for MTG, as demonstrated in this study. In our case, it is characteristic in that the same primary enzyme contact region of STI2 is shared by two different enzyme types, MTG and proteases such as subtilisin or trypsin. Indeed, the inhibitory activity of STI2 against subtilisin is remarkably restricted by the addition of MTG to the reaction mixture, suggesting a competitive inhibition between the enzymes.

The reduced form of SIL-V6 was a substrate for MTG (Table I). This suggests that conformational factors as well as the primary structures of substrates may play an important role in the MTG reaction. Matsumura *et al.* reported that MTG-catalyzed protein polymerization is enhanced by conversion to the molten globule state caused by the removal of Ca^{2+} from holo-α-lactoalbumin (*45*). In this sense, structural analysis of the conformational intermediates of a substrate molecule are necessary for understanding the actual physiological process(es) mediated by TG. Considering the finding that an MTG-mediated linkage may be a factor causing the assembly of the spore coat proteins of *Bacillus subtilis* (*46*), an MTG-mediated protein cross-linking system might be involved in some physiological event(s) such as the morphological differentiation in MTG producing *Streptomyces*. In fact, we have immunological evidence for the presence of high molecular weight protein complexes linked with SIL-V6 in the membrane fraction of sporulating cells of *S. mobaraensis* (manuscript in preparation, Taguchi *et al.*). A distribution study of MTG is important for clarifying the physiological significance of this enzyme.

In the future, structural studies of MTG by X-ray chrystallography or NMR analysis will provide a useful rationale for understanding the molecular recognition of target substrates by MTG in comparison with mammalian TGs. At that time, the STI2 mutants prepared in this study will be very useful for studying the structure/function relationships based on the tertiary structure of MTG.

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