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Engineering an auto-maturing transglutaminase with enhanced thermostability by genetic code expansion with two codon reassignments

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ABSTRACT: In the present study, we simultaneously incorporated two types of synthetic components into microbial transglutaminase (MTG) from *Streptoverticillium mobaraense*, to enhance the utility of this industrial enzyme. The first amino acid, 3-chloro-L-tyrosine, was incorporated into MTG in response to in-frame UAG codons, to substitute for the 15 tyrosine residues separately. The two substitutions at positions 20 and 62 were found to each increase thermostability of the enzyme, while the seven substitutions at positions 24, 34, 75, 146, 171, 217, and 310 exhibited neutral effects. Then, these two stabilizing chlorinations were combined with one of the neutral ones, and the most stabilized variant was found to contain 3-chlorotyrosines at positions 20, 62, and 171, exhibiting a 5.1-fold longer half-life than the wild-type enzyme at 60°C. Next, this MTG variant was further modified by incorporating the α-hydroxy acid analog of N^{ε}-allyloxycarbonyl-L-lysine (AlocKOH), specified by the AGG codon, at the

end of the N-terminal inhibitory peptide. We used an *Escherichia coli* strain previously engineered to have a synthetic genetic code with two codon reassignments, for synthesizing MTG variants containing both 3-chlorotyrosine and AlocKOH. The ester bond, thus incorporated into the main chain, efficiently self-cleaved under alkaline conditions (pH 11.0), achieving the autonomous maturation of the thermostabilized MTG. The results suggested that synthetic genetic codes with multiple codon reassignments would be useful for developing the novel designs of enzymes.

Keywords: chlorotyrosine, hydroxy acid, AGG codon, pyrrolysyl-tRNA synthetase, self-cleavage

Transglutaminases (TGase; EC 2.3.2.13) are enzymes that catalyze acyl transfer between the γ-carboxyamide group of a glutamine residue and the ε-amino group of a lysine residue, thus forming covalent bonds between polypeptides. While TGases occur in various organisms and play important physiological roles, microbial TGases are industrially useful in food processing.[1] Because of their specific activity being harmful to host cells, recombinant TGases are either expressed in the pro-enzyme form to be activated by the enzymatic removal of the N-terminal inhibitory peptide,[2,3] or expressed in an insoluble form to be refolded into the active conformation.[4] There have been attempts to isolate thermostable variants of microbial TGase from *Streptoverticillium mobaraense* (MTG); thousands of variants have been produced by random mutagenesis and DNA shuffling, and then examined for heat resistance.[5,6] On the other hand, we previously developed a different approach for thermostabilizing enzymes, based on

genetic code expansion including halogenated amino acids,[7] and were motivated to compare our approach and the conventional method.

In certain *Escherichia coli* strains, the UAG stop codon has been changed to a sense codon specifying non-natural components, to enable the incorporation of the synthetic components into proteins at multiple defined sites.[8—12] We previously reported on the engineering of thermostable variants of glutathione *S*-transferase (GST) and azoreductase (AZR), which contained halogenated tyrosines at multiple selected positions.[7] In the present study, we applied this approach and obtained MTG variants exhibiting a degree of thermostability comparable to the previously achieved ones. In addition, we incorporated a second non-natural component, a hydroxy acid, to remove the necessity of another enzyme for the activation of MTG. This is the first report on taking advantage of genetic code expansion for the self-maturation of an enzyme. The two different types of synthetic components were simultaneously incorporated into MTG by two codon reassignments.

RESULTS

Synthesis of MTG variants with 3-chlorotyrosines in place of tyrosines. MTG is a monomeric enzyme and comprises 331 residues with 15 tyrosines, which follow a 45-residue N-terminal pro-peptide. The phenolic rings of 7 tyrosine residues are buried inside the enzyme, and the 8 other residues protrude their side chains into the solvent (Figure 1A and Figure S1A).[13] We previously showed that the incorporation of 3-halogenated tyrosines increase the structural stability of proteins, when these derivatives are located at appropriate positions; no significant difference in the stabilizing effect was observed between 3-chloro-L-tyrosine and 3-bromo-L-tyrosine

(Figure S1B).[7] In this study, we first incorporated 3-chlorotyrosine into MTG, by using the *E. coli* B-95.ΔA strain.[10] This strain was transformed with a plasmid carrying the genes coding for UAG-reading tRNA and the variant of archaeal tyrosyl-tRNA synthetase specific to the halogenated tyrosines (iodoTyrRS-*mj*).[14] The resulting strain, designated as B-95.ΔA/ClY, was grown in a growth medium supplemented with 3-chlorotyrosine. The fifteen MTG variants, each with this amino acid in place of one of the 15 tyrosine residues, were synthesized in the pro-enzyme form, being N-terminally tagged with hexahistidine for purification.

The MTG variants synthesized in the present study are listed in Table S1. The yields of representative variants are listed in Table S2. The variant containing chlorotyrosine at position 20 (ClY20) was purifed in an amount of 13 mg from a 200-ml cell culture, which was comparable to the yield of the wild-type molecule (14 mg). An ESI-TOF-mass spectrometric analysis of this variant showed the major peak corresponding to the calculated mass (Figure S2B). The minor peaks with larger masses probably correspond to the chemically modified derivatives of the variant, because minor peaks were also detected with similar degrees of the shift in mass in the spectra of the wild-type MTG and other variants (Figure S2). If these minor peaks indicated the contamination of endogenous proteins, their masses would not vary among the different MTG samples. Together with the data of polyacrylamide gel electrophoresis (Figure S3), the sample of MTG(ClY20) was concluded to be >90% pure and homogeneous in term of the number of chlorinated sites. The other variants halogenated at one site should have comparable purity and homogeneity, as they were synthesized and purified in the similar manner.

We examined the 15 MTG variants matured by the enzymatic removal of the

N-terminal inhibitory sequence, and found many of them to be more active than the wild-type enzyme (Figure 1B). The highest activity, exhibited by the variant ClY75, was as 1.6-fold as that of the wild-type enzyme.

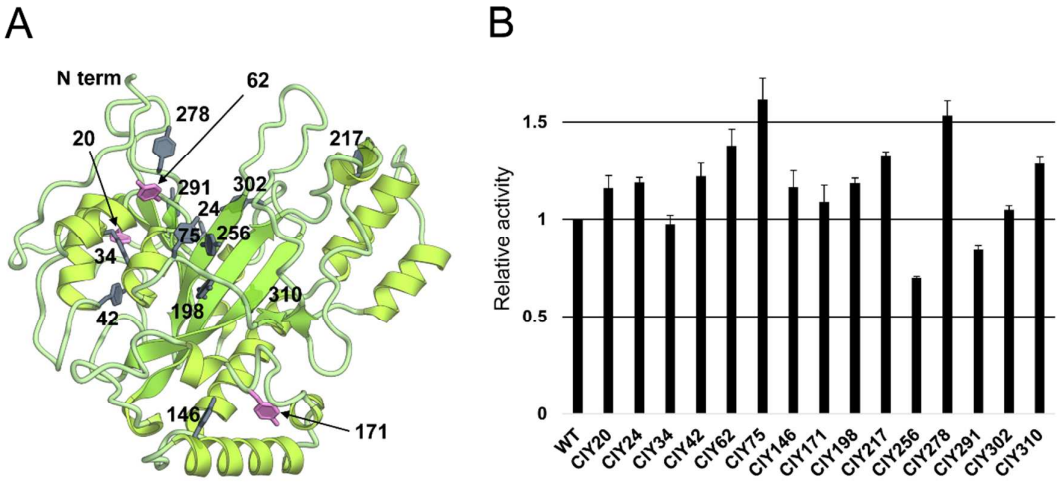

Figure 1. The 15 tyrosines in the tertiary structure of MTG (A) and the effects of the chlorination of these residues on the catalytic activity (B). (A) The tertiary structure of MTG (PDB ID: 1IU4)[13] is rendered in the ribbon representation using the software CueMol: Molecular Visualization Framework (http://www.cuemol.org/). The side chains of tyrosines at positions 20, 62, and 171 are colored purple, and those of the other tyrosines are colored grey. Tyr24, Tyr291, and Tyr302 are barely visible from this side. Tyr310 is located behind a β strand. (B) The activities of MTG variants with 3-chlorotyrosine at the indicated positions, relative to that of the wild-type molecule, are shown. The data were obtained from 3 or more experiments.

Evaluation of the thermostability of the chlorinated MTG variants. We wanted to find variants showing increased heat-stability at 60°C, to compare our results with the previous reports. The first screening was conducted at 57°C, to loosen the selection

stringency, and identify many favorable replacements with 3-chlorotyrosine. The 15 MTG variants, each chlorinated at a single tyrosine site, were heated at 57°C for 10 min, and residual activities were determined (Figure 2A). The replacement of tyrosine with 3-chlorotyrosine at position 20 was found to remarkably increase the heat-stability of MTG. This variant retained 84% of the activity after heating, whereas the wild-type enzyme retained only 17%. Another variant, chlorinated at position 62, showed an appreciable increase in the heat-stability, while chlorination at positions 24, 34, 75, 146, 171, 217, and 310 had neutral effects. The remaining variants were more vulnerable to heat than the wild-type MTG.

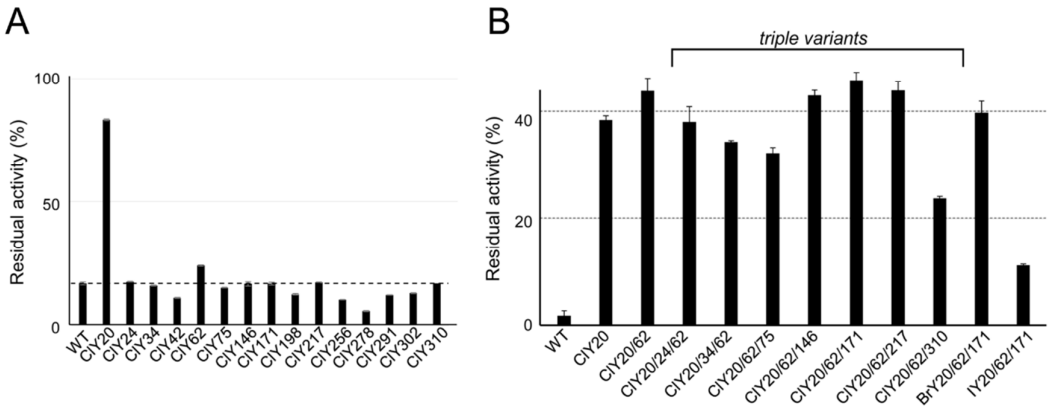

Figure 2. Heat resistance of chlorinated MTG variants. The residual activities of the wild-type MTG (WT) and the variants with 3-halogenated tyrosine at the indicated positions are shown. Heat treatment was conducted at 57°C for 10 min (**A**) and 60°C for 10 min (**B**). "ClY", "BrY", and "IY" represent 3-chloro-, 3-bromo-, and 3-iodotyrosines, respectively. The data were obtained from 3 experiments, except for the ClY146 variant, the residual activity of which is the average of two measurements.

Next, we examined the effects of combining chlorination at different positions, by synthesizing MTG variants halogenated at two and three sites simultaneously. The mass

spectra and PAGE data of the CIY20/62 and CIY20/62/171 variants are shown in Figures S2 and S3, respectively; these variants were thus nearly pure, and homogeneous in terms of the number of chloride atoms. We assumed similar qualities for the other variants. The activity of the wild-type MTG was reduced to 1.8% after heating at 60°C for 10 min, whereas the ClY20 variant retained 38% of the activity, and the double variant (ClY20/62) chlorinated at the two stabilizing positions retained 44% (Figure 2B). This observation showed a favorable effect of combining stabilizing positions. We then synthesized seven triple variants chlorinated at one of the seven neutral positions, in addition to these two stabilizing positions. MTG(ClY20/62/171) retained 46% of the activity, thus exhibiting a slightly higher degree of heat resistance than the double variant MTG(ClY20/62). The half-life of this triple variant was calculated to be 5.1-fold longer than that of the wild-type molecule. On the other hand, the additional chlorination at one of the four neutral positions (24, 34, 75, and 310) destabilized MTG(ClY20/62), indicating that there can be conflicting effects of halogenation between different positions.

The best combination of positions 20, 62, and 171, identified for chlorination, was then tested for bromination and iodination. Bromination at these positions achieved a similar degree of heat resistance, whereas a much less stability was observed for the iodinated variant (Figure 2B). The iodine atom, the bulkiest among the tested halogens, probably caused steric hindrance, which may have cancelled out much of the stabilizing effects.

Finally, the basal activity before heating was compared between the wild-type and the MTG variants halogenated at the stabilizing positions (20, 62, and 171). MTG(CIY20/62) and MTG(CIY20/62/171) showed higher activities than the wild-type

MTG, with the latter being 1.4-fold more active than the wild-type enzyme (Figure 3). This observation is consistent with the aforementioned increases in the activity, observed among many of the chlorinated MTG variants (Figure 1B). The bromination and iodination introduced in place of the chlorination also caused favorable effects, although the degree of the effect was significantly weaker with iodination (Figure 3).

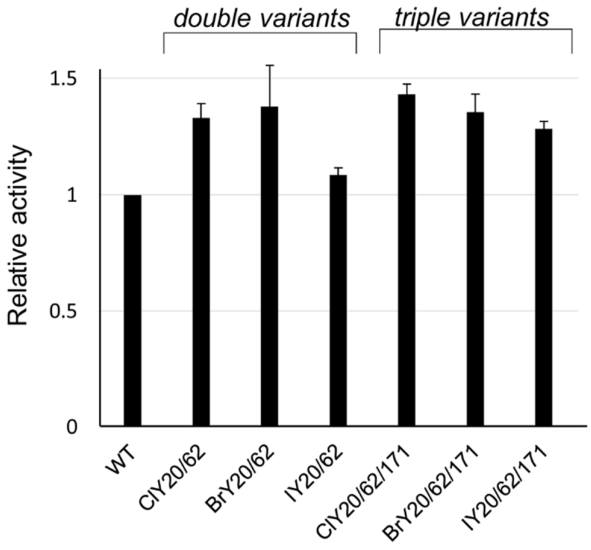

Figure 3. Basal catalytic activities of halogenated MTG variants. The activities before heating of the double and triple variants containing 3-chlorotyrosine (ClY), 3-bromotyrosine (BrY), and 3-iodotyrosine (IY) at the indicated positions are shown, relative to that of the wild-type MTG. The data were obtained from 3 experiments.

Incorporation of an α-hydroxy amino acid to achieve the autonomous release of the N-terminal inhibitory peptide from the MTG pro-enzyme. We introduced an ester bond into MTG by incorporating the α-hydroxy acid analog of N^{ε}-allyloxycarbonyl-L-lysine (AlocKOH). α-Hydroxy acids have been incorporated into proteins to modify main-chain structures,[15—18] although they had not been applied to

achieve the auto-maturation from pro-enzymes. AlocKOH was previously incorporated into proteins by using a variant of *Methanosarcina barkeri* pyrrolysyl-tRNA synthetase (PylRS), and the resulting protein hydrazides were used for peptide-protein ligation.[17] We previously developed an *M. mazei* PylRS variant (BocLyRS2) able to recognize N^{ε}-(tert-butoxycarbonyl)-L-lysine.[19] An analysis by electrophoresis on denaturing acidic polyacrylamide gels (acidic PAGE) showed that BocLyRS2 can attach AlocKOH to $tRNA^{Pyl}$, while retaining an activity towards the parent molecule AlocK (Figure 4). We then decided to isolate an *M. mazei* PylRS variant specific to AlocKOH from a library of BocLysRS2 derivatives. The isolated variant (AlocKOHRS) contained eight substitutions (R61K, M300L, A302G, L309F, C348A, Y384W, and E444G), from the wild-type PylRS, and was shown to recognize AlocKOH and not AlocK, showing its ability to distinguish between α-amino and α-hydroxy groups (Figure 4).

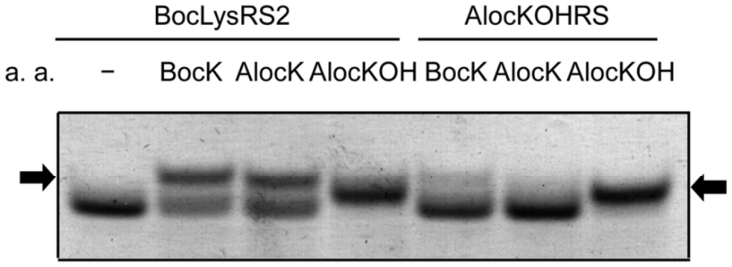

Figure 4. Activities of BocLysRS2 and AlocKOHRS toward N^{ε}-(*tert*-butoxycarbonyl)-L-lysine (BocK), AlocK, and AlocKOH. The acylation of $tRNA^{Pyl}$ with no amino acid ("–") and the indicated amino acids by the indicated PylRS variants were analyzed by acidic PAGE. Acylated $tRNA^{Pyl}$ molecules were shifted upward on the gel to the position indicated by the arrows. The same amount of tRNA (1 μg) from the reactions were applied.

Next, MTG variants with an in-frame UAG at the end of the pro-peptide were

expressed in a conventional *E. coli* strain, together with AlocKOHRS and $tRNA^{Pyl}$. The UAG codon was translated by these molecules to AlocKOH. The pro-peptide originally had a Phe-Arg-Ala-Arg (FRAR) sequence at its C-terminal end, and this sequence was changed to four different sequences (FAPF, FRAx, FAPx, and FRAPx), three of which included AlocKOH at the positions indicated by "x". The resulting variants of the MTG pro-enzyme were incubated at pH 11.0, and revealed various efficiencies of auto-cleavage; the pro-peptide with an FRAx sequence was much more efficiently removed from the pro-enzyme than those with the other sequences either at 4°C or room temperature (Figure 5). This sequence produced a minor amount of the mature enzyme even before an exposure to the alkaline conditions (Figure 5, lane: NT-FRAx). These findings indicated the influence of the sequence context around the ester-bond position on the efficiency of cleavage, and the context might be varied to control the rate of the auto-maturation of enzymes.

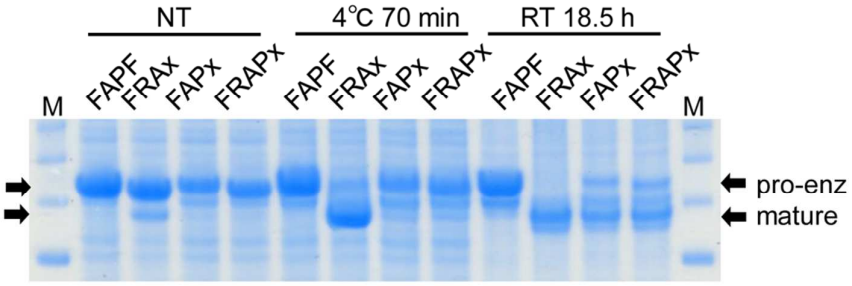

Figure 5. Autonomous cleavage of the N-terminal inhibitory peptides with various terminal sequences. The pro-enzymes with the indicated terminal sequences were roughly purified and subjected to alkaline treatment [4°C at 70 min or room-temperature (RT) for 18.5 h], and then separated from their products (mature MTG) on the polyacrylamide gel. The positions of the pro-enzyme and mature MTG are indicated by the arrows labeled with "pro-enz" and "mature", respectively. "NT"

represents “no alkaline treatment”. “M” indicates marker lanes.

Engineering of a thermostabilized auto-maturing MTG variant. The incorporation of an α-hydroxy acid together with 3-chlorotyrosine required a second codon to specify the non-amino-acid component, when UAG is assigned to the tyrosine derivative. We previously engineered the *E. coli* AGG-27.3/Har strain, a derivative of B-95.ΔA.[20] The AGG arginine codon is reassigned to L-homoarginine in this strain, and the supplementation of this arginine derivative in a growth medium is necessary for cell growth. AGG is the sense codon occurring in the *E. coli* genome with the least frequency, and this low usage minimized the impact of the reassignment of AGG. Homoarginine is thus incorporated in a codon-specific manner, and does not replace arginine at any of the five remaining arginine codons. Since the AGG-27.3/Har cells lack both the endogenous molecules recognizing UAG and AGG, these codons are simultaneously available for the incorporation of non-natural components. Nevertheless, there is an important difference in the availability between these codons. UAG has no specific assignment and can be reassigned to any amino acid in a sustainable manner, whereas AGG already has an assignment (homoarginine), and is reassigned to other molecules when homoarginine is removed from growth media.

We introduced two heterologous pairs of tRNA and aminoacyl-tRNA synthetase into AGG-27.3/Har, to create B-95.ΔA/ClY/OH. One pair was the aforementioned pair for translating UAG to 3-chlorotyrosine, and another was the archaeal pair of AlocKOHRS and $tRNA^{Pyl}$ with a CCU anticodon complementary to AGG. The B-95.ΔA/ClY/OH cells harboring MTG variant genes were first grown in a rich medium supplemented with homoarginine, and then transferred to another rich medium containing AlocKOH

instead of homoarginine. The expression of MTG was induced after this medium change. The cells proliferated vigorously in the first medium, and their wet weight reached about 0.5 g from a 200-ml culture at the log phase. Although the cells did not continue growing in the second medium, the yield of the AlocKOH-containing pro-enzyme of MTG(ClY20/62/171) was 5 mg. The mass spectrum of this variant revealed the major peak corresponding to the calculated mass (Figure S2E), and no peaks indicating the incorporation of arginine or homoarginine in place of AloKOH. The signal with a smaller mass (38,178 Da) corresponds to the product of the spontaneous maturation.

The MTG(ClY20) and MTG(ClY20/62/171) pro-enzymes each with AlocKOH were incubated at pH 11.0 at 25°C for 30 min, to be activated (Figure 6A). A trace of the pro-enzyme was detected on the SDS-gel for each of the MTG molecules. Then, heating was done at 60°C for 10 min in a buffer different from that used for assaying the enzymatically activated MTG variants. The residual activities of the auto-matured MTG(ClY20) and MTG(ClY20/62/171) were 75% and 81%, respectively (Figure 6B). The residual activity for the auto-matured wild-type MTG was 37%. The half-life extension was calculated to be 4.7-fold, a similar value to that (5.1-fold) with the enzymatically activated counterpart, which showed the compatibility of the thermostabilization by halogenation with the auto-maturation mechanism.

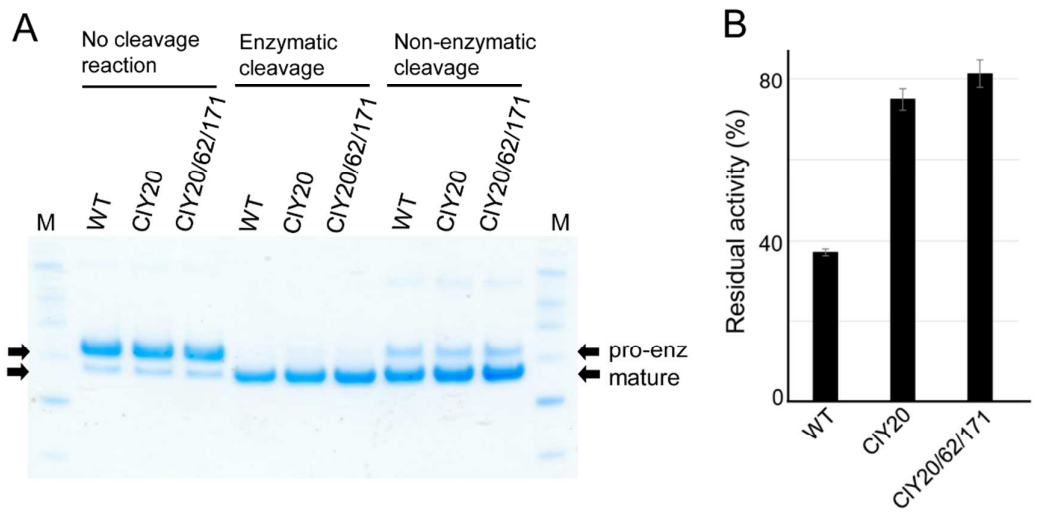

Figure 6. The auto-maturing MTG variants with enhanced thermostability. (A) Cleavage of the N-terminal peptide of MTG and halogenated variants. The AloKOH-containing pro-enzymes of MTG and its chlorinated variants were separated from their products (mature MTG) of the indicated cleavage reactions on the polyacrylamide gel. The positions of the pro-enzyme and mature enzyme are indicated by the arrows labeled with "pro-enz" and "mature", respectively. (B) Heat resistance of the thermostabilized MTG variants matured non-enzymatically. The residual activities of the wild-type MTG and the single and triple chlorinated variants are shown. These molecules were matured by alkaline treatment, and subsequently treated at 60°C for 10 min. The data were obtained from 3 experiments.

DISCUSSION

In the present study, we wanted to explore the impact of a synthetic genetic code on protein engineering, and conducted a two-step modification to MTG. In the typical case of stabilizing a protein, favorable amino acid substitutions are searched for by the large-scale screening of variants. This conventional approach was taken in the two

previous studies on the thermostabilization of MTG; 5,500 and 1,500 variants were sifted to achieve 2.7- and 12-fold, respectively, longer half-lives at 60°C.[5,6] By contrast, we achieved a comparable degree of thermostability by examining only 22 variants. A major factor in this drastic reduction in screening size was the preferential substitution of tyrosine residues, which is justified by an important role of this amino acid in the network of interresidue interactions.[7, 21] Another factor was the additive nature of the stabilizing effects of halogenation; a stabilizing position can be combined with another one or a neutral position to strengthen the effect, and it was unnecessary to sift through all possible combinations between tyrosine positions.

The previous studies pointed to an important role of the N-terminal domain in the stabilization of MTG, based on the distribution of the stabilizing amino-acid substitutions in the tertiary structure.[5,6] Consistently, the stabilizing positions 20 and 62, identified in the present study, are both located in this domain, and the halogenation on tyrosine at position 20 caused a predominant effect. The crystal structure of MTG[13] indicates that the phenolic ring of this tyrosine is deeply buried in the core of the N-terminal domain, and there is a space about the size of a chlorine atom in the proximity of position 3 of the phenolic ring. Thus, halogenation filled this space, and probably enhanced the structural stability of the N-terminal domain, which led to the overall stabilization of the enzyme.

A thermostable variant of MTG can be applied for preventing the shrink of wool textiles and maintaining their strength by crosslinking constituent proteins.[22] This modification to the textile is better performed at high temperature, as the proteins are partly denatured and expose potential reaction sites to MTG. In addition, this process benefits from the acceleration of the reaction at higher temperatures. Note that the

stabilization of MTG was achieved without reducing the catalytic activity, and some of the chlorinated variants even exhibited better activities than the wild-type enzyme. Detailed analyses on the kinetics of the desirable variants at high temperature will be required for industrial application.

We previously succeeded in reassigning two codons (UAG and AGG) in the genetic code of *E. coli*.[20] The availability for the incorporation of non-natural amino acids is different between these two codons. UAG has no specific meaning and allows the reassignment to any amino acid in a sustainable manner, whereas AGG is normally translated into L-homoarginine, and the change in its assignment required the removal of this essential non-natural amino acid from growth media. Since the AGG position was contaminated by neither arginine nor L-homoarginine in the auto-maturing MTG variants, non-natural amino acids could be incorporated at multiple AGG positions, together with a second one being incorporated at multiple UAG sites. The present study was the first demonstration of the synthesis of a purposeful target, not a mere model protein, using a synthetic code with two codon reassignments. The cells grew vigorously until the removal of the essential homoarginine from the medium, to support the synthesis of recombinant proteins with non-natural components. The representation of various synthetic molecules in one genetic code would help to advance protein science and engineering.

METHODS

Non-natural amino acids. N^{ε}-allyloxycarbonyl-L-lysine (AlocK), N^{ε}-(*tert*-butoxycarbonyl)-L-lysine (BocK), and L-homoarginine were purchased from Bachem.

The α-hydroxy acid derivative of AlocK (AlocKOH) was from Shinsei Chemical Co. Ltd.

Biosynthesis of halogenated MTG. The sequence of the N-terminal end of the MTG pro-enzyme was: MAHHHHHHDN GAGEETKSYAETYRLTADDVANINALNESAPAASSAGPSFRARDS…, where the pro-peptide was underlined. The numbering of the residues of the mature enzyme starts with the first Asp (position 1). Arg at position −1 was specified by the AGG codon. The MTG genes were terminated with TAA. The plasmid pACET-26A TG/IYoN3 was a derivative of pET-26b(+) (Novagen), which contained the replication origin of pACYC184 in place of the original one and the ampicillin resistance gene. The genes coding for MTG, iodoTyrRS-*mj*, and UAG-reading tRNA were also carried on pACET-26A TG/IYoN3, with MTG being expressed from the T7 promoter. The IYoN3 part of the plasmid was derived from the IYN3 unit[9] expressing iodoTyrRS-*mj* and UAG-reading tRNA gene, with the modification of synonymously replacing the AGA and AGG codons and the codon optimization in the iodoTyrRS-*mj* gene. B-95.ΔA was transformed with pACET-26A TG/IYoN3, and incubated at 28°C for 20 h in the terrific broth (TB) medium supplemented with 3-chlorotyrosine (0.2g/l). The auto-induced expression and purification of MTG was performed according to the method described previously for GST.[7]

Biosynthesis of MTG with two different synthetic components. For incorporating both 3-chlorotyrosine and AlocKOH, AGG-27.3/Har, which already harbored the previously described plasmid pHar,[20] was transformed with both pACET-26A TG/IYoN3 and pCDF-1b AlocLysOHRS/pylT(CCU)×3, to create B-95.ΔA/ClY/OH. The latter plasmid was a derivative of the previously described plasmid for expressing PylRS and $tRNA^{Pyl}$.[23] The enzyme was replaced in pCDF-1b AlocLysOHRS/pylT(CCU)×3 with AlocKOHRS, together with the triplicated gene coding for $tRNA^{Pyl}$ with a CCU anticodon. The resulting B-95.ΔA/ClY/OH

cells were incubated at 37°C for 4 h in a TB medium (200 ml) containing homoarginine (5 mM), gentamycin (7 mg/l), spectinomycin (100 mg/l), carbenicillin (100 mg/l), and glucose (1% w/v). When the optimal density at 600 nm reached 0.6—0.9 in the logarithmic phase, the cells were harvested and suspended in 25 ml of phosphate-buffered saline (PBS), followed by another round of centrifugation to collect cells. The washed cells were inoculated in 100 ml of the TB medium containing AlocLysOH (10 mM), 3-chlorotyrosine (0.2g/l), IPTG (1 mM), spectinomycin (100 mg/l), and carbenicillin (100 mg/l), followed by an incubation at 28°C for 15 h. The wet cells, finally harvested, weighed 0.42—0.53 g from the initial culture volume (200 ml). MTG with AlocLysOH was then purified in a similar manner to that used for the halogenated MTG.

Mass spectrometry. ESI-TOF-MS analyses were commercially performed by Mass Spectrometry Service, Research Resources Division, RIKEN Center for Brain Science (Wako, Japan).

Engineering and analysis of AlocKOHRS. AlocKOHRS was isolated from a library of the variants of BocLysRS2, using a similar screening method to that used for isolating BocLysRS2.[19] For incorporating AlocKOHRS into MTG, we used a synthetic gene for expressing AlocKOHRS in which the AGG codons were replaced synonymously. The purification of BocLysRS2, AlocLysOHRS, the *in vitro* preparation of $tRNA^{Pyl}$ and the *in vitro* aminoacylation assays were performed in a manner similar to the methods described previously.[20, 24—26] The N-terminally tagged BocLysRS2 and AlocLysOHRS were synthesized in BL21(DE3). The aminoacylation reaction was performed in a 100 mM sodium-HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer (pH 7.2) containing either BocLysRS2 or AlocLysOHRS (3.6 μM), the T7 transcript of $tRNA^{Pyl}$ (4.6 μM), 10 mM $MgCl_2$, 2 mM ATP, 4 mM dithiothreitol (DTT), and one of BocK, AlocK, and AlocKOH (1 mM). The

reaction mixture was incubated at 37 °C for 1 h, and the products were analyzed by acidic urea polyacrylamide gel electrophoresis [10% (w/v) polyacrylamide, 8M urea gel (pH 5.0)] run for 16—20 h at 4 °C with an electrical current of 25—30 mA.

Alkaline cleavage of the N-terminal peptide from the MTG pro-enzyme. The concentration of MTG was adjusted to A_{280}=1.0, and an aliquot was mixed with the equal volume of 0.5M CAPS buffer (pH 11.0), and then incubated at 25°C for 30 min. Alkaline cleavage was terminated by mixing the reaction with the equal volume of 1M MES buffer (pH 6.0). The MTG activity was measured by the colorimetric hydroxamate procedure using *N*-carbobenzoxy-L-glutaminylglycine (Z-Gln-Gly).[27] After heat treatment, 12 µl of the mixture was mixed with 100 µl of Solution A [0.03 M Z-Gln-Gly, 0.1 M NH_2OH, 0.01 M glutathione (reduced form) and 50 mM MES (pH 6.0)], followed by an incubation at 37°C for 10 min. The reaction was stopped by mixing with 100 µl of Solution B [1 N HCl, 4% TCA and 5% $FeCl_3$-$6H_2O$]. The optimal density at 525 nm was determined. For enzymatically activating MTG, Alcalase 2.4L (Sigma) was mixed at a final concentration of 0.00002% (v/v), with an MTG solution adjusted to an optical density of 1.0 at 280 nm, and the mixture was then incubated at 30°C for 18 h.

ASSOCIATED CONTENTS

Supporting Information

Figure S1: Mapping the tyrosine residues in the crystal structure of MTG, and the chemical structures of halogenated tyrosine derivatives; Figure S2: Mass spectrometric data; Figure S3: PAGE data; Table S1: List of MTG variants; Table S2: Yields of MTG variants.

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Author contributions

K. O., K. H., M. D. K. Y. and K. S. designed the experiments. K. S. wrote the manuscript. K. O. and M. T. did the MTG experiments. T. M. and F. I. developed and analyzed the hydroxy-acid-specific PylRS.

Notes

The authors declare no competing financial interest.

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Engineering an auto-maturing transglutaminase with enhanced thermostability by genetic code expansion with two codon reassignments

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