Microbial transglutaminase displays broad acyl-acceptor substrate specificity

ARTICLE *in* APPLIED MICROBIOLOGY AND BIOTECHNOLOGY · APRIL 2013

Impact Factor: 3.34 · DOI: 10.1007/s00253-013-4886-x · Source: PubMed

3 AUTHORS, INCLUDING:

Jeffrey Keillor
University of Ottawa
84 PUBLICATIONS **1,344** CITATIONS
SEE PROFILE

Joelle N Pelletier
Université de Montréal
77 PUBLICATIONS **1,670** CITATIONS
SEE PROFILE

Appl Microbiol Biotechnol
DOI 10.1007/s00253-013-4886-x

Microbial transglutaminase displays broad acyl-acceptor substrate specificity

Maria T. Gundersen · Jeffrey W. Keillor · Joelle N. Pelletier

Received: 22 January 2013 / Revised: 20 March 2013 / Accepted: 30 March 2013

Abstract The great importance of amide bonds in industrial synthesis has encouraged the search for efficient catalysts of amide bond formation. Microbial transglutaminase (MTG) is heavily utilized in crosslinking proteins in the food and textile industries, where the side chain of a glutamine reacts with the side chain of a lysine, forming a secondary amide bond. Long alkylamines carrying diverse chemical entities can substitute for lysine as acyl-acceptor substrates, to link molecules of interest onto peptides or proteins. Here, we explore short and chemically varied acyl-acceptor substrates, to better understand the nature of nonnatural substrates that are tolerated by MTG, with the aim of diversifying biocatalytic applications of MTG. We show, for the first time, that very short-chain alkyl-based amino acids such as glycine can serve as acceptor substrates. The esterified α-amino acids Thr, Ser, Cys, and Trp—but not Ile—also showed reactivity. Extending the search to nonnatural compounds, a ring near the amine group—particularly if aromatic—was beneficial for reactivity, although ring substituents reduced reactivity. Overall, amines attached to a less hindered carbon increased reactivity. Importantly, very small amines carrying either the electron-rich azide or the alkyne groups required for click chemistry were highly reactive as acyl-acceptor substrates, providing a robust route to minimally modified, "clickable" peptides. These results demonstrate that MTG is tolerant to a variety of chemically varied natural and nonnatural acyl-acceptor substrates, which broadens the scope for modification of Gln-containing peptides and proteins.

Keywords Amide bond formation · Microbial transformations · Biocatalysis · Peptide modification

Electronic supplementary material The online version of this article (doi:10.1007/s00253-013-4886-x) contains supplementary material, which is available to authorized users.

M. T. Gundersen · J. N. Pelletier
Département de biochimie, Université de Montréal, Montréal, Québec H3C 3J7, Canada

M. T. Gundersen · J. N. Pelletier
PROTEO, the Québec Network for Protein Function, Structure and Engineering, Québec, Canada

M. T. Gundersen · J. W. Keillor · J. N. Pelletier
CGCC, the Center in Green Chemistry and Catalysis, Montréal, Canada

J. W. Keillor
Department of Chemistry, University of Ottawa, Ottawa ON K1N 6N5, Canada

J. N. Pelletier (✉)
Département de chimie, Université de Montréal, Montréal, Québec H3C 3J7, Canada
e-mail: joelle.pelletier@umontreal.ca

Introduction

Transglutaminases (EC 2.3.2.13) catalyze the formation of peptide linkages by promoting an acyl transfer reaction between an acyl-donor substrate, the γ-carboxyamide group of glutamine, and an acyl-acceptor substrate, the ε-amino group of lysine, thus forming a new amide bond. In nature, these enzymes crosslink proteins to form insoluble protein aggregates (Pasternack et al. 1998). Microbial transglutaminase (MTG) from *Streptomyces mobaraensis* is a calcium-independent enzyme that has been used commercially since the late 1980s in the textile and the food industries, to alter the texture and appearance of products via protein crosslinking (Zhu and Tramper 2008). More recently, MTG has been utilized in novel nonnative processes such as site-specific protein PEGylation (Mero et al. 2011), antibody modification (Jeger et al. 2010; Mindt et al. 2008), formation of DNA–protein conjugates (Kitaoka et al. 2011), and the formation of hydrogels for tissue engineering and drug delivery (Zhu and Tramper 2008). It is also applied to "green" applications, such

Published online: 25 April 2013

as the biocatalytic synthesis of degradable bioplastics (Porta et al. 2011).

Biocatalysis is increasingly applied in areas spanning the preparation of bulk chemicals to high-value synthesis of chiral active pharmaceutical ingredients (APIs) (Clouthier and Pelletier 2012; Schmid et al. 2001). Amide bonds are highly represented in APIs, as well as in bulk polymers and commodity chemicals. Nonetheless, current methods of amide synthesis are generally characterized by high waste and cost, highlighting the importance of making their synthesis more sustainable. Indeed, "amide formation avoiding poor atom economy reagents" was voted the top challenge of organic chemistry in 2007 by the ACS Green Chemistry Institute (Pattabiraman and Bode 2011). To this effect, new catalytic methods for amide synthesis are called for (Pattabiraman and Bode 2011; Valeur and Bradley 2009).

A number of enzymes catalyze amide bond formation from different starting materials. Well-known examples include proteases, peptidases, transglutaminases, nitrile hydrolases, and lipoic acid ligases. Competing hydrolysis reactions frequently impede the efficiency of enzymatic amide bond formation. Transglutaminases, which natively catalyze the formation of amide bonds rather than their hydrolysis (as is the case for proteases and peptidases), offer an intrinsic advantage in this respect. The mature form of MTG is produced with high quality on a large scale, and its industrial success can be attributed to its ease of handling and stability over a broad range of pH and temperature (Umezawa et al. 2002; Yokoyama et al. 2004). Furthermore, it is active in the presence of organic cosolvents (Mero et al. 2011), thus allowing the use of poorly water-soluble compounds. Its stability and ease of application enable the use of high substrate concentrations and catalyst recycling, both important considerations in the context of biocatalyzed conversions.

In spite of the extensive industrial use of MTG, little is known about its ability to use small, non-proteogenic molecules as substrates. Ohtsuka et al. investigated a restricted range of small, non-proteogenic acyl-acceptor substrates, confirming that MTG can react with a number of primary amines, mainly natural compounds or their analogues (Ohtsuka et al. 2000b). Here, we investigate the breadth of acyl-acceptor substrate specificity of MTG in order to expand its scope and utility as a green biocatalyst for amide synthesis, by broadening the classes of compounds investigated. In particular, we revisited alkyl-based amino acids to uncover new reactivity, expanded the range of known reactive α-amino acids, and identified synthetically attractive amine substrates of non-biological origin. Our results increase the understanding of the specific characteristics of non-proteogenic acceptor substrates and broaden the scope of MTG as a green catalyst for amide synthesis.

Materials and methods

Materials

The plasmid pDJ1-3 was kindly provided by Professor M. Pietzsch (Martin-Luther-Universität, Halle-Wittenberg, Germany). pDJ1-3 encodes the proenzyme of MTG from *S. mobaraensis* inserted between the *Nde*I and *Xho*I restriction sites of the vector pET20b (Marx et al. 2007). Deionized water (18 Ω) was used for all experiments. HPLC solvents were of analytical grade, and products used for the expression and purification of MTG were of biological grade. Azides were synthesized in the laboratory of J.W.K., according to known literature procedures for the preparation of ethylamine azide (Inverarity and Hulme 2007) and propylamine azide (Park et al. 2009). Other chemicals used were purchased from the suppliers listed below. 4-Methoxybenzamide (97 % purity) and glycine ethyl ester hydrochloride were purchased from Acros Organics (Waltham, USA). Benzylamine, *m*-anisidine, N^{α}-acetyl-L-lysine methyl ester hydrochloride, β-alanine, 6-aminocaproic acid, 5-aminovaleric acid, aniline, *O*-benzylhydroxylamine hydrochloride, L-threonine methyl ester hydrochloride, L-tryptophan methyl ester hydrochloride, sarcosine, ammonium carbamate, *N*-ethylmethylamine, cyclohexane methylamine, cyclohexylamine, glycine hydrochloride, D-serine methyl ester hydrochloride, L-serine methyl ester hydrochloride, trypsin from bovine pancreas (10,000 BAEE U/mg), cadaverine, γ-aminobutyric acid, hydroxylamine, and *p*-xylenediamine were purchased from Sigma-Aldrich (St. Louis, USA). Aminoacetonitrile was purchased from Bachem (Bubendorf, Switzerland). Carboxybenzyl–glutaminyl–glycine (Z-Gln-Gly) and benzylazide were from Alfa Aesar (Ward Hill, USA). Glutathione (reduced) and thiamine were from Bioshop (Burlington, Canada). L-Cysteine ethyl ester hydrochloride and L-isoleucine methyl ester hydrochloride were purchased from Chem-Impex (Wood Dale, USA). Propargylamine was purchased from Fisher (Waltham, USA). Formic acid (98 % purity) was from Fluka Analytical (St. Louis, USA). 3-Chloro-4-fluorobenzylamine was purchased from Lancaster (Ward Hill, USA).

Expression and purification of MTG

The plasmid pDJ1-3 encodes MTG with its N-terminal prosequence and a C-terminal hexa-histidine tag. It was transformed into *Escherichia coli* BL21(DE3), using standard procedures (Sambrook et al. 1989), and maintained with 100 μg/mL ampicillin. MTG was expressed in an autoinducing medium as follows: a 5-mL culture was propagated overnight at 37 °C with agitation at 240 RPM in ZYP-0.8G medium (Studier 2005) and used to inoculate 500 mL of ZYP-505 medium (Studier 2005). The culture was incubated with shaking at 240 RPM, first at 37 °C for

2 h then overnight at 22 °C. The culture was centrifuged at 5,000 RPM (Sorvall RC 5C Plus centrifuge, SLA-3000 rotor) at 4 °C for 15 min, and the cells resuspended in 40 mL of 0.2 M Tris–HCl, pH 6.0. The cells were disrupted by sonication over ice (three cycles of 30 s pulse at 20 % intensity/1 min pause) using a Branson sonicator and further by one pass through a Constant Systems cell disruptor set at 27 kPSI and cooled to 4 °C. MTG was then activated by cleavage of the proenzyme leader sequence through incubation in a 1:9 ratio (*v*/*v*) of trypsin (1 mg/mL) to unpurified MTG for 45 min, at 30 °C. The activated MTG was purified using a 5-mL His-trap nickel-nitrilotriacetic acid (Ni-NTA) column (GE Healthcare) equilibrated in 50 mM phosphate buffer pH 8.0, with 300 mM NaCl, and eluted with an imidazole gradient (0–140 mM), on an Åkta FPLC (GE Healthcare). The purified, activated MTG was dialyzed against 0.2 M Tris–HCl buffer, pH 6.0. The average yield was 90 mg of activated MTG per liter of culture, with >85 % purity as estimated by resolution on 10 % SDS-PAGE followed by staining with Coomassie blue. Aliquots were snap frozen and stored at −80 °C in 15 % glycerol.

Determination of MTG activity

The activity of the purified, activated MTG was quantified using the hydroxamate assay, as previously described (Folk and Cole 1966). Briefly, MTG was incubated with 30 mM Z-Gln-Gly and 100 mM hydroxamate at 37 °C for 10 min. The reaction was quenched with a solution containing 2.0 M $FeCl_3 \cdot 6\ H_2O$, 0.3 M trichloroacetic acid, and 0.8 M HCl. The resulting iron complex was detected by its absorbance at 525 nm. One unit (U) of MTG produces 1 μmol of L-glutamic acid γ-monohydroxamate per min at 37 °C.

Reaction of MTG with various acceptor substrates

Amide acyl-donor substrate Z-Gln-Gly (40 mM), amine acyl-acceptor substrate (100 mM), and 10 mM glutathione were combined in 0.2 M Tris–HCl buffer in a final volume of 350 μL. The pH of each reaction was adjusted to the pK_a of the tested acceptor substrate amine, if within the range of pH 6–9. Otherwise, pH 6 or pH 9 was used, as most closely matched to the amine pK_a. For this purpose, the pK_a values were calculated online through the SPARC calculator (http://archemcalc.com/sparc; Hilal et al. 1995). The pH values of the reaction mixtures are indicated in Table S1, Supporting Information. MTG (2 U/mL final concentration) was added to the substrate mixture, and the reaction was incubated in closed 1.5-mL Eppendorf tubes at 37 °C for up to 72 h. A control reaction without MTG was run for each acyl-acceptor substrate. All experiments were performed in triplicate using MTG isolated from three independent MTG purifications (nine times total).

Product detection

Donor substrate consumption was monitored by HPLC-MS. Formic acid was added to an equal volume (0.1 mL) of reaction mixture and allowed to stand at room temperature for 5 min to quench the reaction. The volume of the quenched sample was adjusted up to 1 mL with H_2O; 0.15 mL of this solution was then combined with 0.15 mL internal standard solution (1 g/L 4-methoxybenzamide in neat DMSO), and the volume was adjusted up to 1.5 mL with H_2O. Each sample was run over a hand-packed Ni-NTA column (0.5 mL bed volume) to remove MTG and then filtered through a 0.2-μm PTFE filter to remove any particles. Samples (10 μL) were injected on a Synergi 4-μm polar-RP 80 Å, 50×2.00 mm LC column (Phenomenex), using a Waters 2545 HPLC apparatus and eluted with a 5–70 % MeOH/H_2O gradient. Masses were detected under positive ionization with a Waters 3100 single quadrupole mass detector. The consumption of donor substrate was determined by standardization with the 4-methoxybenzamide internal standard, relative to the concentration of the donor substrate in the control reaction (no MTG) for the same acceptor. The mass corresponding to the expected product was also detected. NMR analysis of the product was performed to confirm identity of the expected product for the following acceptor substrates: 5-aminovaleric acid, propargylamine, and 6-aminocaproic acid. For that purpose, the MTG-catalyzed reactions were run on a larger scale (1.4 mL). Preparative HPLC was performed with a Synergi polar-RP 80 Å, 100×21.20 mm AXIA packed column (Phenomenex) on a Waters 1525 HPLC with elution using a 5–90 % MeOH/H_2O gradient containing 0.1 % formic acid. The product was detected with a Waters 2487 dual absorbance detector. Fractions were further analyzed with direct injection mass spectrometry with a Waters 3100 single quadrupole mass detector, and those containing the mass corresponding to the expected products were pooled. Methanol was evaporated, and then, the samples were lyophilized to yield the isolated product in a powder form. ^{1}H- and ^{13}C-NMR spectra of the products were acquired on a Bruker Avance II 700 MHz spectrometer.

Click chemistry

The purified Z-N^{ε}-propargyl-Gln-Gly product served as the alkyne substrate for the azide-alkyne copper-catalyzed Huisgen cycloaddition. The reaction was performed using benzyl azide, according to the general procedure described by Himo et al. (2005). Briefly, a 0.25-M solution of the starting materials was made in 1 mL (1:1) H_2O/*t*-butanol containing 25 mM sodium ascorbate and 2.5 mM copper(II) sulfate pentahydrate (Himo et al. 2005). The reaction was monitored by LC-MS over 24 h as described above.

Results

Establishment of the reaction conditions

To gain greater insight into the reactivity of MTG with nonnative acyl-acceptor substrates, screening was performed by reacting the protected dipeptide acyl-donor substrate Z-Gln-Gly with a variety of amines as acyl-acceptor substrates. Reaction pH was adjusted in the range of pH 6–9 to aid compound dissolution while maintaining nucleophilicity of the reactive amine; MTG has been reported to exhibit >80 % activity following a 10-min incubation between pH 5 and 10 (Umezawa et al. 2002). MTG has been reported to be optimally active at 70 °C, but it loses activity over 10 min incubation at that temperature (Umezawa et al. 2002), so reactions were run at 37 °C, since MTG has been reported to maintain full activity upon 10 min incubation at 40 °C (Umezawa et al. 2002). Product formation was reported as consumption of Z-Gln-Gly donor substrate, as it could be monitored irrespective of the acceptor substrate tested.

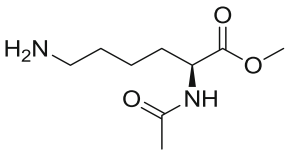
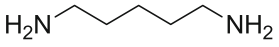
We verified MTG activity using the standard assay with hydroxylamine (1) (Table 1) as the acceptor substrate (Folk and Cole 1966), for up to 72 h, as representing a maximal acceptable time frame for most biocatalytic processes. After 72 h of incubation at pH 6 and 37 °C, 60 % of MTG activity remained, confirming its stability. Hydroxylamine (1) is chemically and sterically distinct from the native peptide- or protein-bound lysine substrates of MTG. Using hydroxylamine as a reference for small molecule acceptor substrate specificity, the donor substrate was consumed to >99 % after 24 h. Lysine, the native acceptor residue of MTG, was tested under the form of di-protected N^{α}-acetyl-L-lysine methyl ester (2), to verify reaction of the ε-amine: it reacted to >98 % after 24 h. Cadaverine (3) is an analogue of lysine, as it mimics the butylamine side chain. Substituted cadaverines have long been used as substrates of MTG (Abe et al. 2010; Jeger et al. 2010; Mindt et al. 2008; Ohtsuka et al. 2000a; Pasternack et al. 1997; Pasternack et al. 1998; Sugimura et al. 2008; Tominaga et al. 2007). Its reactivity under the conditions used herein was as high as that of lysine and hydroxylamine (>99 % after 24 h). Cadaverine (3) is a symmetric diamine and therefore has twice the amine concentration of simple amines. This may favor product formation by increasing the initial concentration of reactive amine. In addition, mono-acylated amine may serve as a substrate for a second acylation event. The formation of the diamide product was qualitatively investigated, where the donor concentration was increased to 60 mM and the acceptor concentration reduced to 30 mM. Mass spectrometry confirmed formation of the disubstituted product along with monosubstituted product (data not shown).

The low rate of MTG-mediated hydrolysis relative to mammalian transglutaminases (Umezawa et al. 2002) is one of its advantageous properties. Formation of the hydrolysis product Z-Glu-Gly was monitored during all reactions; little or no hydrolysis was observed, even after 72 h (Supporting Information, Table S1). Hydrolysis occurred almost exclusively under conditions of low acceptor substrate reactivity, particularly at pH values between 6 and 8 where MTG shows maximal activity (Umezawa et al. 2002). Under those conditions, the donor substrate concentration remained high, increasing its susceptibility to hydrolysis.

Amino acids with varying intervening chain length

We verified the reactivity of acceptor substrates with various substituents in close proximity to the reactive amine. We first determined the minimal distance allowed between the reactive amine and a negatively charged substituent.

Table 1 Reactivity of reference acyl-acceptor compounds

Acceptor compound	Structure	Donor consumed in 72 h (%)
1	H_2N-OH	>99[a]
2		>99[a]
3		>99[a]

[a] >98 % reacted in 24 h

Because of the presence of negatively charged amino acids in the vicinity of the reactive cysteine of MTG (Fig. 1), a negative charge such as a carboxylate may be detrimental to reactivity. Ohtsuka et al. previously screened amino acids with alkyl chains of one to seven carbons separating the amino group from the carboxylic acid (Ohtsuka et al. 2000b). We partly confirmed their observations, testing alkyl spacers between zero and five carbons in length (Table 2). As previously noted (Ohtsuka et al. 2000b), 6-aminocaproic acid (4) was the acceptor with the highest reactivity in that series (71 % donor consumption after 72 h). Reactivity decreased with decreasing acyl chain length (Table 2 and Fig. 2). 5-Aminovaleric acid (5), γ-aminobutyric acid (6), β-alanine (7), and glycine (8) yielded donor consumptions of 39, 12, 4.8, and 8.4 %, respectively; this is the first report of reactivity with the short-chain amino acids (6–8). The higher reactivity of glycine (8) relative to β-alanine (7) may be due to reacting glycine at pH 7.7 and β-alanine at pH 9; under the latter conditions, enzyme activity is reduced by 50 % (Umezawa et al. 2002). Carbamic acid (9) yielded no detectable product or donor consumption.

α-Amino acid acceptors

We probed the reactivity of MTG toward chemically diverse α-amino acids (Table 3). Because a negatively charged carboxylate near the reactive amine was unfavorable for reactivity (Table 2), esterified amino acids were tested, as previously demonstrated (Ohtsuka et al. 2000b). Esterification effectively increased reactivity, as shown by the faster reaction of glycine ethyl ester (10) (Table 3; >99 % in 4 h) relative to its parent glycine (8) (Table 2; 8.4 % in 72 h). The beneficial effect of esterification is also evident on comparison of unreactive L-threonine (11) with L-threonine ethyl ester (12), which yielded 4.1 % product in 72 h. The lower reactivity of L-threonine ethyl ester (12) relative to glycine ethyl ester (10) suggests that the steric hindrance due to β-branching is incompatible with high reactivity, as observed with the unreactive valine ethyl ester (Ohtsuka et al. 2000b).

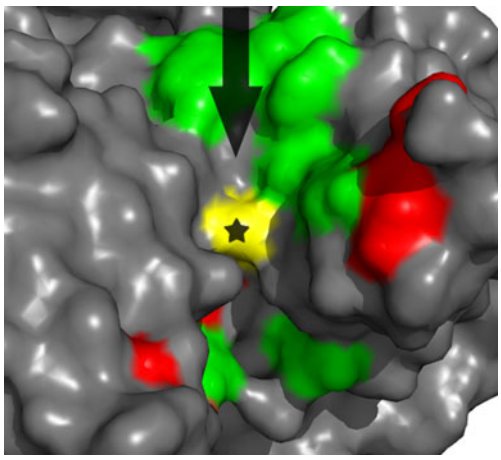


Fig. 1 The active site of MTG (1IU4) (Kashiwagi et al. 2002) *in gray* surface rendering, with the reactive cysteine indicated in *yellow with a black asterisk*, and aromatic and acidic residues surrounding the active site indicated in *green* and *red*, respectively. The active site cleft is indicated by the *arrow*

The reactivity of the unhindered L-serine methyl ester (13), D-serine methyl ester (14), and L-cysteine ethyl ester (15) over 72 h was 5.8, 3.0, and 2.7 %, respectively. These similar values are much lower than the >99 % in 4 h observed for glycine ethyl ester. The bulky tryptophan methyl ester (16) also yielded a reactivity of 4.5 % over 72 h, similar to the unhindered, polar amino acids. However, the nonpolar, β-branched L-isoleucine methyl ester (17) did not form any detectable product. This result indicates that the bulky bicyclic indole, three carbons away from the reactive amine, is less detrimental to acceptor reactivity than is β-branching, two carbons away from the amine.

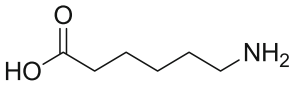
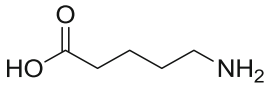
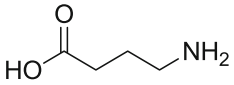
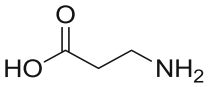
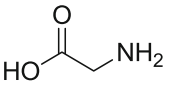
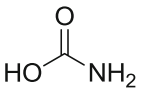
Aromatic acceptors

In addition to negatively charged residues, the active site of MTG also contains a number of nearby aromatic residues (Fig. 1) (Kashiwagi et al. 2002). Hypothesizing that they may contribute to substrate binding, we tested simple aromatic amines. Benzylamine (18) and aniline (19) showed reactivity of 94 and 19 % respective substrate consumption over 72 h (Table 4). In contrast, the nonaromatic cyclohexylmethylamine (20) and cyclohexylamine (21) were approximately 50 % as reactive as their respective aromatic analogues and were of reactivity comparable to other compounds containing an amino group attached to a secondary carbon (Tables 2 and 4).

Conjugated substituents on the aromatic ring yielded a reduction in reactivity. *m*-Anisidine (22) reacted slowly, with 11 % conversion, a reduction of approximately 40 % relative to its unsubstituted analogue aniline. Similarly, the dihalogenated 3-chloro-4-fluorobenzylamine (23) showed 23 % reactivity, a 75 % reduction relative to its unsubstituted benzylamine analogue. Despite having a doubled amine concentration like cadaverine (3) (Table 1), the diamine *p*-xylenediamine (24) yielded a reactivity of 63 %, which is a 30 % reduction compared to the monosubstituted benzylamine (18). As for the reaction of cadaverine, both amines of *p*-xylenediamine (24) were reactive, yielding both mono- and diamide products (data not shown).

We attempted to combine the beneficial effect of the benzyl ring with that of the highly reactive hydroxylamine (1) by testing the reactivity of *O*-benzylhydroxylamine (25). The longer linker between the benzene and the amine combined with the reactivity of the hydroxylamine was expected to increase the reactivity of MTG relative to benzylamine

Table 2 Reactivity of amino acids with varying chain length as acyl-acceptor substrates

Acceptor compound	Structure	Donor consumed in 72 h (%)
4		70.6 ± 6.4
5		38.5 ± 3.2
6		11.8 ± 6.7
7		4.8 ± 1.3
8		8.4 ± 2.4
9		nd

nd not detected (<2 % product)

(18) and aniline (19). Surprisingly, *O*-benzylhydroxylamine (25) yielded only 2.6 % conversion. This may be due to the reduced polarity of the hydroxylamine group in *O*-benzylhydroxylamine, though it appears more likely that the bulk of *O*-benzylhydroxylamine relative to hydroxylamine (1)—the smallest compound tested—may be the cause of reduced reactivity.

Finally, to explore the extent to which aromaticity compensates for steric hindrance, we tested the reactivity of

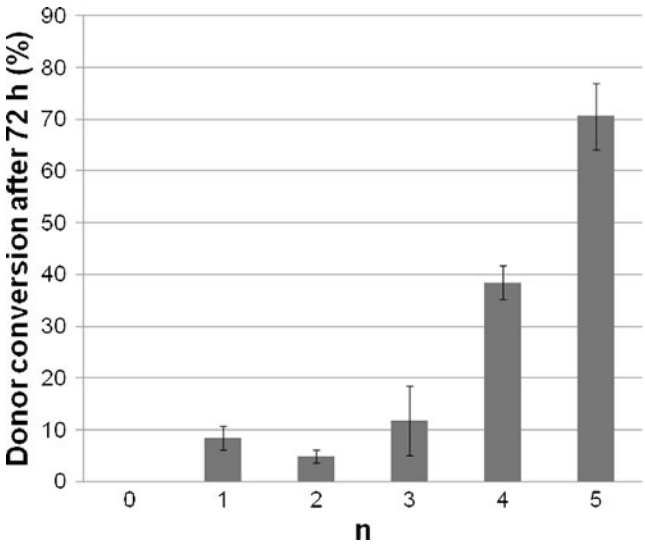


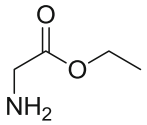
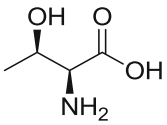
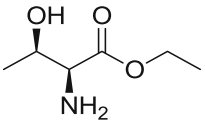
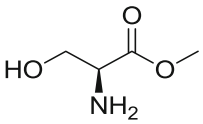
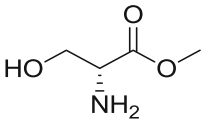
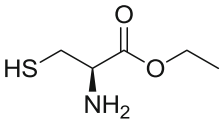
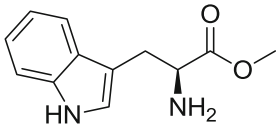
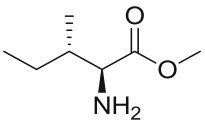
Fig. 2 Reactivity of alkyl-based amino acids as acyl-acceptor substrates over 72 h. Reactivity for amino acids having the structure $H_2N–(CH_2)_n–CO_2H$ with varying spacer length, $n=0$ to 5. *Error bars* indicate standard deviation from the mean from three experiments performed in triplicate

thiamine (26), also known as vitamin B_1. Its primary amine belongs to an aminopyrimidine ring, which is further attached to a thiazole ring. Thiamine was unreactive; its greater bulk or higher polarity than benzylamine (18) may hinder binding to the active site.

Electron-rich amines

Halogenated moieties provide a starting point for synthetic modification. Furthermore, halogens are increasingly found in pharmaceutical compounds to increase lipophilicity and thus improve drug uptake (Hernandes et al. 2010). Above, we showed that 3-chloro-4-fluorobenzylamine (23) was reactive as an acyl-acceptor substrate (Table 4), illustrating the utility of MTG for installing modifiable moieties on the donor peptide. The smaller 2-bromoethylamine (27) did not yield detectable product (Table 5), potentially due to the electron density of bromine, or its bulk, near the amine. While Ohtsuka et al. (2000b) previously reported the MTG-catalyzed addition of pentylamine or hexylamine-linked sugar moieties to proteins, glucosamine (28) did not yield any product. This may result from the amine being adjacent to a secondary carbon and/or to the hydrophilicity in the immediate vicinity of the amine. Nitriles are a further functional group of high value in synthesis of APIs, as their high permeability and bioavailability, combined with low metabolite rates in the cell, make them a favorable substituent (Fichert et al. 2003; Fleming et al. 2010). In light of the above results, we reasoned that its lack of bulky substituents or negative charge should make aminoacetonitrile (29) compatible with reactivity. Indeed, it reacted to completion

Table 3 Reactivity of α-amino acids as acyl-acceptor substrates

Acceptor compounds	Structure	Donor consumed in 72 h (%)
10		>99[a]
11		nd[b]
12		4.1 ± 9.5
13		5.8 ± 1.4
14		3.0 ± 1.9
15		2.7 ± 1.7
16		4.5 ± 1.1
17		nd

nd not detected (<2 % product)

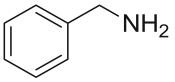
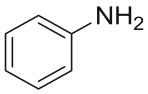
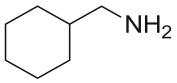
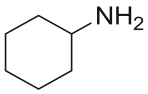
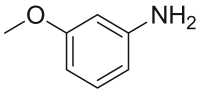
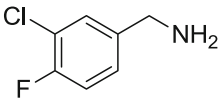
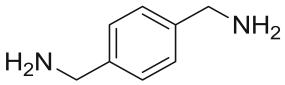
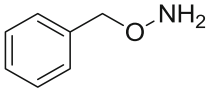
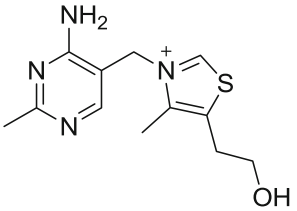
[a] >99 % reacted in 4 h

within 24 h (Table 5), which makes it one of the most reactive compounds identified, as previously reported for tissue transglutaminase (Leblanc et al. 2001). Having demonstrated the reactivity of MTG toward aminoacetonitrile, we probed its reactivity toward the similarly small, π electron-rich propargylamine (30), which yielded 90 % reaction after 72 h (Table 5). This is consistent with the high reactivity observed for propargylamine with tissue transglutaminase (Gnaccarini et al. 2012). The resulting reaction product, Z-N^{ε}-propargyl-Gln-Gly, can subsequently serve as the alkyne in a click reaction with a suitable azido-substituted compound (Gnaccarini et al. 2012). To increase the flexibility imparted by MTG in preparing modified peptides for click reactions, we tested its reactivity toward two simple amino azides, propylamine azide (31) and ethylamine azide (32), which reacted to an extent of 99 and 98 %, respectively, over 72 h. These results demonstrate that MTG tolerates the presence of both alkynes and azides in close proximity to the reactive amine of the acceptor substrate.

Acceptors other than primary amines

We investigated limits of MTG's acyl-acceptor substrate specificity by testing compounds other than primary amines as potential acyl-acceptor substrates (Supporting Information, Table S2). Neither of the secondary amines *N*-ethylmethylamine (33) or sarcosine (34) yielded product. Non-primary amines are poorer nucleophiles than primary amines; their increased steric hindrance and the carboxylate of sarcosine (34) may be a further deterrent to reactivity. We also probed the reactivity of an unhindered alcohol and a

Table 4 Reactivity of aromatic amino acids as acyl-acceptor substrates

Acceptor compound	Structure	Donor consumed in 72 h (%)
18		93.6 ± 1.3
19		18.9 ± 4.4
20		46.3 ± 1.6
21		3.1 ± 1.6
22		11.3 ± 1.4
23		23.1 ± 5.7
24		62.7 ± 2.4
25		2.6 ± 5.6
26		nd

nd not detected (<2 % product)

thiol as alternative nucleophiles: neither butanol (35) nor butanethiol (36) yielded product. These results indicate that weaker nucleophiles than amines cannot serve as acceptor substrates for MTG and that an unhindered primary amine is required as the reactive species.

Discussion

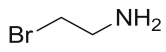
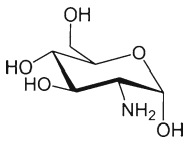
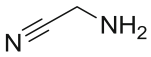
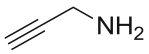
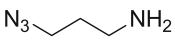
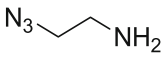
The reaction conditions investigated indicate that MTG is a good biocatalyst candidate for running lengthy reactions (72 h). To gain insight into the tolerance of MTG to chemically and sterically varied acceptor substrates, we tested its reactivity using acceptor substrates with substituents proximal to the reactive amine, using the donor substrate Z-Gln-Gly. Donor substrate binding is the first event in this ordered reaction, and the bound-donor substrate is therefore an integral part of the active site with respect to acceptor binding.

Very short-chain amino acids can serve as acceptor substrates

In investigating the minimal distance allowed between the reactive amine and a negatively charged substituent (compounds 4–9), we detected conversion not only for the long chain amino acids 4 and 5 but, contrary to the report of Ohtsuka et al. (2000b), also for the amino acids 6–8 having only one to three intervening carbons. This may be due to the higher enzyme concentrations and increased reaction time used here. Thus, even a single intervening carbon between the carboxylate and amino groups is sufficient to allow some reactivity with MTG as an acceptor substrate. While the reactivity with amino acids 4–8 was modest, its optimization would be great utility for general synthetic biocatalytic applications.

Decreasing reactivity with decreasing spacer length (Fig. 2) is consistent with the reactivity of MTG with other negatively charged acceptor substrates such as amino alkyl

Table 5 Reactivity of halogenated amines and labeling agents as acyl-acceptor substrates

Acceptor compound	Structure	Donor consumed in 72 h (%)
27		nd
28		nd
29		>99[a]
30		90.1 ± 0.9
31		98.5 ± 0.5
32		97.7 ± 0.5

nd not detected (<2 % product)

[a] >99 % reacted in 24 h

sulfonates and phosphates (Ohtsuka et al. 2000b; Tanaka et al. 2004, 2005) and likely results from electrostatic repulsion with negatively charged residues in the active site (Fig. 1) (Kashiwagi et al. 2002). Repulsion may be augmented by the bound donor substrate Z-Gln-Gly, which harbors a negatively charged carboxylate. However, in the absence of structural confirmation of the mode of substrate binding to MTG, this remains speculative.

Side-chain volume reduces reactivity of α-amino acid acceptor substrates

α-Amino acids and their derivatives are heavily used in the synthesis of high-value APIs because they provide a readily accessible pool of chemically varied chiral starting materials. The comparison of glycine and L-threonine with their respective esters illustrates that esterification effectively increased reactivity, confirming previous observations (Ohtsuka et al. 2000b). The reaction rate with glycine ethyl ester (10) (Table 3; >99 % in 4 h) even surpassed that of N^{α}-acetyl-L-lysine methyl ester (2) (Table 1; 80 % in 4 h), which served as a mimic of the natural, protein-bound lysine substrate, consistent with previous reports (Ohtsuka et al. 2000b). This observation is consistent with use of an N-terminal Gly or a poly-Gly tag as an acceptor substrate in protein–protein conjugations (Tanaka et al. 2005) and raises the question as to whether N-terminal Gly may also be a native acceptor substrate of MTG. While MTG natively catalyzes protein side-chain crosslinking, a potential high-value application is the biocatalysis of peptide bonds between the α-amino and α-carboxyl groups of natural or nonnatural amino acids, as we have demonstrated using mutants of tissue transglutaminase (Keillor et al. 2008). Despite esterification, the reactivity of the α-amino group of a set of chemically diverse α-amino acids was at least 18-fold lower than that of glycine ethyl ester. Thus, amines linked to a primary carbon are more reactive than amines linked to secondary carbons. Considering that alanine ethyl ester showed only a threefold decrease in reactivity (Ohtsuka et al. 2000b), this illustrates the negative impact of increasing side-chain volume. Nonetheless, the reactivity of the bulky tryptophan methyl ester (16) was comparable to that of the serine (13, 14) and cysteine (15) ethyl esters. Indeed, a bulky substituent farther removed from the reactive amine was better tolerated than β-branching two carbons away from the amine, as illustrated by the unreactive L-isoleucine methyl ester (17). Nonetheless, the reactivity of the β-branched L-threonine ethyl ester (12) (4.1 % in 72 h) indicates that β-branching can be tolerated to some extent. Overall, its robustness and reactivity toward a number of amino acids make MTG a good candidate for further development as a peptide bond catalyst, although the stringent acyl-donor substrate specificity of MTG currently precludes reaction ofan amino acid α-carboxyl group. We note that no significant stereoselectivity was observed upon reaction of the L or D isomers of serine ethyl ester (13 and 14); MTG may require more voluminous substituents for stereoselectivity to be observed.

Unsubstituted aromatic amines are more reactive acyl-acceptor substrates

In addition to a number of negatively charged residues in and around the active site, the MTG active site also holds a

number of aromatic residues; several of which have been shown to be important for donor substrate binding (Fig. 1) (Tagami et al. 2009; Kashiwagi et al. 2002). We hypothesized that they may also aid in binding of acceptor substrates, via hydrophobic interactions and/or π stacking. The higher reactivity of benzylamine (18) than aniline (19) (Table 4) is in agreement with the trends we observed above, where an amino group attached to a primary carbon yielded higher reactivity than an amino group attached to a more highly substituted carbon—a secondary carbon in the case of the α-amino acids investigated and an aromatic ring carbon in the case of aniline (19). It is also consistent with the higher nucleophilicity of benzylamine (18) relative to aniline (19). The higher reactivity of aniline relative to α-amino acids, and of benzylamine and aniline relative to their nonaromatic analogues cyclohexylmethylamine (20) and cyclohexylamine (21), appears to be related to aromaticity rather than to the cyclic structure. Increased reactivity may thus result from the formation of favorable π–π interactions between the acceptor substrate and MTG and/or the carboxybenzyl group of the MTG-bound donor substrate, from the planar geometry of the benzyl ring, as opposed to the puckered cyclohexane ring, or to a combination of those two factors. Ring substituents on substrates 22 to 24 reduced reactivity, again indicating the detrimental effect of increased steric bulk. Despite reduced reactivity of *p*-xylenediamine (24) relative to benzylamine (18), we demonstrated the feasibility of using MTG for homodisubstitution of the acyl-acceptor substrates *p*-xylenediamine (24) and cadaverine (3), where the resulting diamine has either a rigid (24) or a flexible (3) linker.

MTG is reactive toward small, π electron-rich amines

We have observed reactivity with acyl-acceptor compounds that provide avenues for further synthesis. High reactivity was confirmed for the small, unbranched, π electron-rich amines aminoacetonitrile (29), propargylamine (30), as well as for propylamine azide (31) and ethylamine azide (32) (Table 5), opening the door to click chemistry. The Cu(I)-catalyzed azide/alkyne [3+2]-cycloaddition reactions are established techniques for specific labeling of proteins in vivo. These selective reactions rapidly proceed with high accuracy under mild conditions (Kolb et al. 2001) and have been applied to specific labeling of proteins with fluorescent labels and sugars, as we demonstrated with tissue transglutaminase (Gnaccarini et al. 2012). Performing click chemistry with proteins generally requires noncanonical amino acids containing either an azido or alkyne moiety (Best 2009), making the method costly and poorly accessible. The specific addition of the azido or alkyne moiety to the target protein with a transglutaminase can constitute a significant advantage (Gnaccarini et al. 2012).

The reactivity of MTG with small amino azides is of particular interest because many commercially available labels are alkyne substituted. We note that, following 4 h incubation of MTG with 0.1 M of either azide (31) or (32), no further conversion was observed (data not shown), suggesting rapid initial reaction followed by inactivation of the enzyme. Indeed, azides can serve as inhibitors of cysteine proteases (Le et al. 2006). To demonstrate the utility of an MTG-mediated alkyne-modified peptide in a copper-catalyzed cycloaddition reaction, we reacted the purified Z-N^{ϵ}-propargyl-Gln-Gly with benzylazide under previously reported conditions (Himo et al. 2005). Product formation was confirmed by MS; no degradation of the starting materials was observed. MTG thus demonstrates flexibility towards substitution of peptides for further modification by click chemistry. Furthermore, we demonstrate that MTG tolerates the π electron density of the nitrile, alkyne, and azido groups in proximity to the reactive amine.

In conclusion, this work significantly increases our knowledge of the acyl-acceptor specificity of MTG. Our results expand the range of acyl-acceptor substrates known to be accepted—or not accepted—by MTG, for formation of a secondary amide bond with a peptide-bound glutamine. We have shown, for the first time, that even very short-chain alkyl-based amino acids such as glycine can serve as acceptor substrates, with reactivity increasing with the chain length separating the amine and the carboxyl groups. We observed reactivity with the α-amino group of chemically diverse α-amino acids. Additional steric hindrance in the immediate vicinity of the amine was detrimental to reactivity. Nonetheless, the bulky, aromatic Trp—but not Ile—showed reactivity; this suggests that steric bulk farther removed from the reactive amine is better tolerated. Extending the search to nonnatural compounds, an aromatic ring was beneficial for reactivity of the acceptor substrate. Ring substitution reduced reactivity, apparently as a result of steric hindrance. Finally, only primary amines were reactive, and bonding of the amine to a less hindered carbon increased reactivity.

While specific molecules were shown to be reactive, they likely indicate a trend where other similar compounds will exhibit comparable reactivity. Importantly, very small amines carrying either the electron-rich nitrile, azide, or the alkyne groups required for click chemistry were highly reactive as acceptor substrates, facilitating the synthesis of minimally modified, "clickable" peptides. These results demonstrate that MTG is tolerant to a variety of chemically varied natural and nonnatural acceptor substrates. We expect that the reactivity of the acyl-acceptor substrates observed here with the Z-Gln-Gly dipeptide as the acyl-donor substrate is predictive of their relative reactivity toward reactive, protein-bound glutamines, as previously demonstrated with mammalian transglutaminase (Gnaccarini et al. 2012)

and MTG (Lee et al. 2013). These results broaden the scope for modification of Gln-containing peptides and the use of MTG as a biocatalyst.

Acknowledgments This work was supported by funding from the Natural Sciences and Engineering Research Council of Canada and CGCC, the Center in Green Chemistry and Catalysis. We thank Fabrice Galaud and Pierre Lavallée, in the Combinatorial Chemistry Laboratory at the Université de Montréal, for their generous technical help, Alexandra Furtos in the Regional Mass Spectrometry Center at Université de Montréal for her assistance, and Maria Stoïca and Dr. Christophe Pardin for the synthesis of azide compounds and acknowledge Klavdja Annabel Fignolé for assisting in experimental work and Natalie Rachel for commenting and revising the manuscript.

References

Abe H, Goto M, Kamiya N (2010) Enzymatic single-step preparation of multifunctional proteins. Chem Commun 46:7160–7162

Best MD (2009) Click chemistry and bioorthogonal reactions: unprecedented selectivity in the labeling of biological molecules. Biochemistry 48:6571–6584

Clouthier CM, Pelletier JN (2012) Expanding the organic toolbox: a guide to integrating biocatalysis in synthesis. Chem Soc Rev 41:1585–1605

Fichert T, Yazdanian M, Proudfoot JR (2003) A structure–permeability study of small drug-like molecules. Bioorg Med Chem Lett 13:719–722

Fleming FF, Yao L, Ravikumar PC, Funk L, Shook BC (2010) Nitrile-containing pharmaceuticals: efficacious roles of the nitrile pharmacophore. J Med Chem 53:7902–7917

Folk JE, Cole PW (1966) Transglutaminase: mechanistic features of the active site as determined by kinetic and inhibitor studies. Biochim Biophys Acta Enzymol Biol Oxid 122:244–264

Gnaccarini C, Ben-Tahar W, Mulani A, Roy I, Lubell WD, Pelletier JN, Keillor JW (2012) Site-specific protein propargylation using tissue transglutaminase. Org Biomol Chem 10:5258–5265

Hernandes MZ, Cavalcanti SMT, Moreira DRM, De Azevedo Junior WF, Leite ACL (2010) Halogen atoms in the modern medicinal chemistry: hints for the drug design. Current Drug Targets 11:303–314

Hilal SH, Karickhoff SW, Carreira LA (1995) A rigorous test for SPARC's chemical reactivity models: estimation of more than 4300 ionization pKa's. Quant Struc Act Rel 14:348–355

Himo F, Lovell T, Hilgraf R, Rostovtsev VV, Noodleman L, Sharpless KB, Fokin VV (2005) Copper(I)-catalyzed synthesis of azoles. DFT study predicts unprecedented reactivity and intermediates. J Am Chem Soc 127:210–216

Inverarity IA, Hulme AN (2007) Marked small molecule libraries: a truncated approach to molecular probe design. Org Biomol Chem 5:636–643

Jeger S, Zimmermann K, Blanc A, Grunberg J, Honer M, Hunziker P, Struthers H, Schibli R (2010) Site specific and stoichiometric modification of antibodies by bacterial transglutaminase. Angew Chem Int Ed 49:1–4

Kashiwagi T, Yokoyama K-I, Ishikawa K, Ono K, Ejima D, Matsui H, Suzuki E (2002) Crystal structure of microbial transglutaminase from *Streptoverticillium mobaraense*. J Biol Chem 277:44252–44260

Keillor JW, Chica RA, Chabot N, Vinci V, Pardin C, Fortin E, Gillet SMFG, Nakano Y, Kaartinen MT, Pelletier JN, Lubell WD (2008) The bioorganic chemistry of transglutaminase—from mechanism to inhibition and engineering. Can J Chem 86:271–276

Kitaoka M, Tsuruda Y, Tanaka Y, Goto M, Mitsumori M, Hayashi K, Hiraishi Y, Miyawaki K, Noji S, Kamiya N (2011) Transglutaminase-mediated synthesis of a DNA-(enzyme)n probe for highly sensitive DNA detection. Chemistry 17:5387–5392

Kolb HC, Finn MG, Sharpless KB (2001) Click chemistry: diverse chemical function from a few good reactions. Angew Chem Int Ed 40:2004–2021

Le GT, Abbenante G, Madala PK, Hoang HN, Fairlie DP (2006) Organic azide inhibitors of cysteine proteases. J Am Chem Soc 128:12396–12397

Leblanc A, Gravel C, Labelle J, Keillor JW (2001) Kinetic studies of guinea pig liver transglutaminase reveal a general-base-catalyzed deacylation mechanism. Biochemistry 40:8335–8342

Lee J-H, Song C, Kim D-H, Park I-H, Lee S-G, Lee Y-S, Kim B-G (2013) Glutamine (Q)-peptide screening for transglutaminase reaction using mRNA display. Biotechnol Bioeng 110:353–362

Marx CK, Hertel TC, Pietzsch M (2007) Soluble expression of a pro-transglutaminase from *Streptomyces mobaraensis* in *Escherichia coli*. Enzyme Microb Technol 40:1543–1550

Mero A, Schiavon M, Veronese FM, Pasut G (2011) A new method to increase selectivity of transglutaminase mediated PEGylation of salmon calcitonin and human growth hormone. J Controlled Release 154:27–34

Mindt TL, Jungi V, Wyss S, Friedli A, Pla G, Novak-Hofer I, Grünberg J, Schibli R (2008) Modification of different IgG1 antibodies via glutamine and lysine using bacterial and human tissue transglutaminase. Bioconjugate Chem 19:271–278

Ohtsuka T, Ota M, Nio N, Motoki M (2000a) Comparison of substrate specificities of transglutaminases using synthetic peptides as acyl donors. Biosci Biotechnol Biochem 64:2608–2613

Ohtsuka T, Sawa A, Kawabata R, Nio N, Motoki M (2000b) Substrate specificities of microbial transglutaminase for primary amines. J Agric Food Chem 48:6230–6233

Park KD, Morieux P, Salomé C, Cotten SW, Reamtong O, Eyers C, Gaskell SJ, Stables JP, Liu R, Kohn H (2009) Lacosamide isothiocyanate-based agents: novel agents to target and identify lacosamide receptors. J Med Chem 52:6897–6911

Pasternack R, Dorsch S, Otterbach JT, Robenek IR, Wolf S, Fuchsbauer HL (1998) Bacterial pro-transglutaminase from *Streptoverticillium mobaraense*—purification, characterisation and sequence of the zymogen. Eur J Biochem/FEBS 257:570–576

Pasternack R, Laurent HP, Rüth T, Kaiser A, Schön N, Fuchsbauer HL (1997) A fluorescent substrate of transglutaminase for detection and characterization of glutamine acceptor compounds. Anal Biochem 249:54–60

Pattabiraman VR, Bode JW (2011) Rethinking amide bond synthesis. Nature 480:471–479

Porta R, Di Pierro P, Sorrentino A, Mariniello L (2011) Promising perspectives for transglutaminase in "bioplastics" production. J Biotechnol Biomaterial 01:1–4

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor

Schmid A, Dordick JS, Hauer B, Kiener A, Wubbolts M, Witholt B (2001) Industrial biocatalysis today and tomorrow. Nature 409:258–268

Studier FW (2005) Protein production by auto-induction in high-density shaking cultures. Protein Expression Purif 41:207–234

Sugimura Y, Yokoyama K, Nio N, Maki M, Hitomi K (2008) Identification of preferred substrate sequences of microbial transglutaminase from *Streptomyces mobaraensis* using a phage-displayed peptide library. Arch Biochem Biophys 477:379–383

Tagami U, Shimba N, Nakamura M, Yokoyama K-I, Suzuki E-I, Hirokawa T (2009) Substrate specificity of microbial transglutaminase as

revealed by three-dimensional docking simulation and mutagenesis. Protein Eng Des Sel 22:747–752

Tanaka T, Kamiya N, Nagamune T (2004) Peptidyl linkers for protein heterodimerization catalyzed by microbial transglutaminase. Bioconjugate Chem 15:491–497

Tanaka T, Kamiya N, Nagamune T (2005) N-terminal glycine-specific protein conjugation catalyzed by microbial transglutaminase. FEBS Lett 579:2092–2096

Tominaga J, Kemori Y, Tanaka Y, Maruyama T, Kamiya N, Goto M (2007) An enzymatic method for site-specific labeling of recombinant proteins with oligonucleotides. Chem Commun 401–3

Umezawa Y, Ohtsuka T, Yokoyama K (2002) Comparison of enzymatic properties of microbial transglutaminase from *Streptomyces* sp. Food Sci Technol Res 8:113–118

Valeur E, Bradley M (2009) Amide bond formation: beyond the myth of coupling reagents. Chem Soc Rev 38:606–631

Yokoyama K, Nio N, Kikuchi Y (2004) Properties and applications of microbial transglutaminase. Appl Microbiol Biotechnol 64:447–454

Zhu Y, Tramper J (2008) Novel applications for microbial transglutaminase beyond food processing. Trends Biotechnol 26:559–565