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Substrate Specificity of *Streptomyces* Transglutaminases

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Abstract

Transglutaminase (TGase) is a multifunctional enzyme vital for many physiologic processes, such as cell differentiation, tissue regeneration, and plant pathogenicity. The acyl transfer function of the enzyme can activate primary amines and, consequently, attach them onto a peptidyl glutamine, a reaction important for various in vivo and in vitro protein crosslinking and modification processes. To understand better the structure-function relationship of the enzyme and to develop it further as an industrial biocatalyst, we studied TGase secreted by several *Streptomyces* species and *Phytophthora cactorum*. We purified the enzyme from *S. lydicus*, *S. platensis*, *S. nigrescens*, *S. cinnamoneus*, and *S. hachijoensis*. The pH and temperature profiles of *S. lydicus*, *S. platensis*, and *S. nigrescens* TGases were determined. The specificity of *S. lydicus* TGase toward its acyl-accepting amine substrates was characterized. Correlation of the electronic and steric features of the substrates with their reactivity supported the mechanism previously proposed for *Streptomyces mobaraensis* TGase.

Index Entries: Transglutaminase; specificity; *Streptomyces*; *Phytophthora cactorum*; acyl-accepting amines.

Introduction

Widely distributed in bacteria, fungi, plants, and animals, transglutaminase (TGase) (EC 2.3.2.13) is a multifunctional enzyme vital for many physiologic processes, such as protein modification, tissue growth and regeneration, extracellular matrix stabilization, cell differentiation,

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apoptosis, pathogenicity, and plant defense (for recent reviews, see refs. *1–4*). The early recognized major function of TGase is its ability to catalytically link the γ-carboxamide of a peptidyl glutamine (acyl donor) and the ε-amine of a peptidyl or free lysine (acyl acceptor) into an ε-(γ-glutamyl)-lysine isopeptide. Recently, TGase was found active as a G protein (with guanosine 5'-triphosphatase– or guanosine 5'-triphosphate–binding activity), protein disulfide isomerase, or even kinase.

For its acyl transfer and transamidation function, TGase can activate various primary amines, in addition to lysine, and attach them onto a peptidyl glutamine. Physiologically, the reaction can lead to inter- or intramolecular protein crosslinkages (proteolysis resistant), modified and functionalized proteins, strengthened cellular matrices and structures, as well as other physiologic effects (e.g., blood clotting and tissue regeneration). This reaction of TGase also makes it highly desirable for various medical and industrial applications. As a medical or personal care agent, TGase may be applied in autoimmune diagnostics, wound healing, cosmetics, and other fields. As an industrial biocatalyst, TGase may be used in many food preparation fields, such as in the modification of proteins from cereal, soy, meat, milk, or egg; and for the purpose of fortification, texture or taste enhancement, reconstitution, and so on (for a recent review, see ref. *2*).

Among the various TGases, increased attention has been focused on microbial TGase, because of its potential in industrial applications. In general, a microbial TGase may be advantageous over an animal counterpart because its production, by fermentation, could be large scale, reliable, inexpensive, free of infectious viruses or prions, and environment friendly. *Streptomyces mobaraensis* TGase has been commercialized, and its lack of dependence on Ca^{2+} makes it even more suited as an industrial biocatalyst than an animal TGase (such as the well-studied guinea pig liver TGase).

To improve and expand further TGase-based biocatalysis, a better understanding of the properties and enzymology of microbial TGases is highly desirable. We carried out a comparative study of several bacterial (*Streptomyces* sp.) TGases and one fungal (*Phytophthora cactorum*) TGase and probed the specificity of *Streptomyces lydicus* TGase for its acyl-accepting amine substrates. The observed correlation between the substrates' structural property and reactivity supported the general mechanism previously proposed for the deacylation half reaction of *S. mobaraensis* TGase.

Materials and Methods

Chemicals

Chemicals used as reagents and buffers were commercial products of at least reagent grade. An Ammonia Bioanalysis Kit was purchased from R-Biopharm (#1112732). Guinea pig liver TGase was purchased from Sigma (T-5398). Novozymes SP913 and SP818, used to purify *S. mobaraensis*

and *P. cactorum* TGase, respectively, were two concentrated and formulated culture media (broths) from Novozymes. Shake-flask (SF) *Streptomyces* broths were obtained as previously reported *(5,6)*.

Streptomyces TGases were purified by multistep ultrafiltration and chromatography, similar to the procedures previously reported *(5)*. Typically, a cell-free broth was washed and concentrated on an Amicon spiral concentrator (membrane mol mass cutoff: 10 kDa) to ~2 mS conductivity and pH 7.0 before being loaded onto a Q-Sepharose column (preequilibrated with 20 m*M* sodium phosphate, pH 7.0). The eluted active fraction (in the flow-through) was adjusted to pH 5.0 and applied onto an SP-Sepharose column (preequilibrated with 50 m*M* sodium acetate, pH 5.2). A TGase-containing fraction was eluted by a 0 to 1 *M* NaCl gradient. The fraction was adjusted to 1.5 *M* $(NH_4)_2SO_4$, applied onto a Phenyl Superose column (preequilibrated with 1.5 *M* $[NH_4]_2SO_4$ and 50 m*M* sodium phosphate, pH 7.0), and eluted by a 1.5 to 0 *M* $(NH_4)_2SO_4$ gradient. Purification was monitored by a hydroxamate activity assay and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Final chromatographed TGase was concentrated and dialyzed against 50 m*M* sodium phosphate, pH 7.0.

Phytophthora TGase was purified also by multistep ultrafiltration and chromatography, similar to procedures previously reported *(6)*. Typically, a cell-free broth was washed and concentrated on an Amicon spiral concentrator (membrane mol mass cutoff: 10 kDa) to ~2 mS conductivity and pH 7.0 before being loaded onto a Q-Sepharose column (preequilibrated with 20 m*M* 3-(N-morpholino) propane-sulfonic acid hemisodium salt (MOPS), pH 7.0). The eluted active fraction (in the flow-through) was adjusted to pH 6.0 and applied onto an SP-Sepharose column (preequilibrated with 25 m*M* 2-(N-morpholino) ethane-sulfonic acid hemisodium salt (MES), pH 6.0). An elution was made with a 0 to 0.5 *M* NaCl gradient.

TGase Activity Assays

Several assays were used to measure TGase activity. For the hydroxamate assay, a substrate stock was made by mixing 50 µL of 0.1 *M* $CaCl_2$; 50 µL of 2 *M* NH_2OH; 50 µL of 20 m*M* EDTA; 23 µL of 0.2 *M* dithiothreitol (all dissolved in 1 *M* Tris-HCl, pH 6.0); 150 µL of 0.2 *M* Nα-carbobenzoxy-Gln-Gly (CBZ-Gln-Gly, dissolved in 0.1 *M* sodium borate); and 600 µL of 1 *M* Tris-HCl, pH 6.0. A color-developing stock was made by mixing equal volumes of 15% acetic acid, 5% $FeCl_3$, and 2.5 *N* HCl. To assay a TGase, 10 µL of the sample was incubated with 100 µL of the substrate stock for a selected time before being mixed with 100 µL of the color-developing stock and photometrically measured at 490 nm *(7)*. A molar absorptivity of 787 $M^{-1}cm^{-1}$ was used to calculate the activity. The background hydrolysis was measured with 26.8–268 n*M* TGase and 30 m*M* CBZ-Gln-Gly and was found negligible (under the aminolytic conditions) in comparison with the reaction when the amine substrate was present.

For the enzymatic NH_3-detection assay, a substrate stock was made by mixing 120 μL of 0.1 *M* CBZ-Gln-Gly; 40 μL of 0.1 *M* glutathione; an appropriate amount of an acyl-accepting amine (all made in 0.2 *M* Tris-HCl, pH 6.0); and 0.2 *M* Tris-HCl, pH 6.0, to a final volume of 0.4 mL. The stock was mixed with 0.39 mL 0.2 *M* Tris-HCl of pH 6, preincubated at 25°C for 5 min, added to 10 μL of a TGase stock, and incubated at 25°C for 10 min. The reaction was stopped by heating at 95°C for 1 min. After a brief centrifugation, 0.3 mL of supernatant was mixed in a cuvet with equal volumes of H_2O and solution #2 of the Ammonia Bioanalysis Kit from R-Biopharm. After 5 min, 6 μL of the solution #1 from the kit was added. After 20 min at 25°C, the absorption was read at 340 nm, and a molar absorptivity of 6.3 $mM^{-1}cm^{-1}$ was used to calculate activity. The assay was used to determine TGase specificity toward acyl-accepting amines. Used as control, reaction solutions without TGase were assayed. No significant ammonia was produced, and the baseline reading was subtracted from the assay results obtained in the presence of TGase. TGase-omitted solutions were also spiked with ammonia, and they were compared to water spiked with ammonia by the assay. No assay interference from the substrates was observed.

Casein gelation assay was carried out on α-casein. Typically, 300 μL of 9% α-casein (dissolved in 0.1 *M* Tris-HCl, pH 7.9) was incubated with 0.03–0.25 mg/mL of TGase at 37°C. The fluidity of the solution was periodically visualized.

Egg-white gelation assay was carried out as follows: First an egg-white sample was mixed with four parts of water. Because the white of a large hen egg (~1.25 oz) generally has 2 mg of calcium, 3 g of protein, and ~104 mg of other major elements (P, Na, and K), the 20% egg-white solution should contain ~3 m*M* Ca^{2+}. After adjusting the pH from ~9.3 to 8.0 with 1 *M* Tris-HCl, pH 6.0, 0.4 mL egg-white solution was mixed with 25 μL of 3 mg/mL TGase. Another TGase dosing, 50 μL of 3 mg/mL, was also tested. The mixture was incubated at 37°C and visually examined periodically.

For all of the assays, solutions without TGase served as control.

Other Assays

The pH dependence of TGase activity was measured by hydroxamate assay with Britton & Robinson buffer, pH 3.0–11.0 (made by mixing 0.1 *M* phosphoric acid, 0.1 *M* acetic acid, and 0.1 *M* boric acid and adjusting the with 0.5 *M* NaOH) at 25°C. The reaction pH was measured by a pH meter before adding TGase.

To study the pH-activity profile, we assumed that the substrates were unprotonated inside the TGase-active site, and that the following fast equilibriums (relative to the catalysis) occur for the acidic and alkaline pH–induced activity loss, respectively:

$$\text{TGase-H}^+ \text{ (inactivated)} \leftrightarrow \text{TGase} + \text{H}^+, \text{ apparent acid dissociation constant: } K_1$$

$$\text{TGase} \leftrightarrow \text{TGase}^{-}\text{ (inactivated)} + H^{+}\text{, apparent acid dissociation constant: } K_2$$

Subsequently the Michaelis-Menten equation could be modified to

$$v = V_{max}[S]/(K_m + [S])/(1 + [H^+]/K_1 + K_2/[H^+])$$

or

$$\text{Relative rate} = 1/(1 + [H^+]/K_1 + K_2/[H^+])$$

in which $[H^+] = 10^{-pH}$.

Fitting the relative rate equation to the pH-activity profile (by nonlinear regression, using GraphPad Prism software) would allow the extraction of K_1 and K_2. When the alkaline pH–induced activity loss was not considered, the relative rate equation became

$$\text{Relative rate} = 1/(1 + [H^+]/K_1)$$

When the observed pH profile could not be fitted satisfactorily, K_1 or K_2 was estimated from the pH corresponding to ~50% activity loss.

The temperature dependence of TGase activity was also measured by the hydroxamate assay. Typically, 50 µL of the substrate stock was mixed with 5 µL of TGase in 0.7-mL centrifuge tubes for 30 min at a selected temperature. After brief centrifugation, the supernatant was mixed with 50 µL of the color-developing stock.

The portion of the temperature profile at which the activity increased as temperature increased was fitted (by nonlinear regression) to the Arrhenius equation

$$\text{Relative rate} = \{E_a/[8.314(T + 273)]\}$$

in which T is in degrees centigrade, to extract the apparent activation energy, E_a (J/mol).

Protein concentration was determined using a Pierce BCA Kit.

Potential protease side activity was detected with FTC-casein (Pierce). Typically, 10 µL of sample was mixed with 20 µL of 0.25 *M* MOPS, pH 7.0, and 20 µL of 5% FTC-casein. After 1 h of incubation at 37°C, 150 µL of 5% trichloroacetic acid was added and the mix was kept on ice for 20 min. After centrifugation, 20 µL of the supernatant was mixed with 180 µL of 0.5 *M* sodium borate, pH 9.0, and measured for fluorescence at 538 nm (excitation at 485 nm).

N-terminal amino acid sequencing was carried out in an Applied Biosystems 476A Protein Sequencer. Ultraviolet (UV)-visible absorption spectroscopy was performed on either a Shimadzu UV160U or a Hitachi U-2001 spectrophotometer, as well as a Molecular Devices Thermomax microplate reader.

Specific Activity

Various amines were reacted with CBZ-Gln-Gly under the catalysis of *S. lydicus* TGase at pH 6.0. Typical reaction solutions contained 0.2–5 × Km acyl-accepting amine, 30 m*M* CBZ-Gln-Gly, and 2.7–540 n*M S. lydicus* TGase in 0.2 *M* Tris-HCl, pH 6.0, buffer containing 10 m*M* glutathione. The reac-

tion was monitored by the enzymatic NH_3 detection method. Observed data were fitted to the Michaelis-Menten kinetic equation to extract the apparent K_m and k_{cat}.

The specificity toward the acyl donor was measured on 5–67 m*M* CBZ-Gln-Gly with either 11.1 m*M* hydroxylamine or 2 m*M* 2-bromoethylamine as the acyl-accepting amine substrate.

Results

Purification

Fermentation broths from nine *Streptomyces* species were subjected to multistep chromatographic purification. For *S. nitrosporus*, *S. fradiae*, and *S. thermovulgaris*, the initial broths showed on SDS-PAGE a distinct protein band (approximately one-fifth of total protein) of ~45–50 kDa, a molecular mass range similar to that of the TGase purified from other *Streptomyces* species. However, the initial activity and the putative protein band quickly diminished after two rounds of chromatography, likely owing to proteolysis, resulting in unsuccessful attempts at purification.

For *S. mobaraensis*, *S. lydicus*, *S. platensis*, *S. cinnamoneus*, *S. nigrescens*, *S. hachijoensis*, and *P. cactorum*, TGases of a molecular mass of ~45 to 50 kDa were purified (Table 1). The purified TGases were found to be free of detectable protease side activity. Preparation of *S. mobaraensis* and *S. lydicus* TGase showed the correct N-terminal sequences *(8)*. Because of their abundance, only *S. mobaraensis*, *S. lydicus*, *S. platensis*, and *S. nigrescens* TGases were characterized in terms of pH and temperature profiles, and only *S. lydicus* TGase was characterized for its substrate specificity.

TGase Activity

Table 2 presents the specific activity of the purified TGases on CBZ-Gln-Gly and NH_2OH, measured by the hydroxamate assay. Up to an ~20-fold difference was observed among the *Streptomyces* TGases.

Figure 1A presents the pH profiles of several *Streptomyces* TGases, as measured on CBZ-Gln-Gly and NH_2OH. While *S. lydicus* TGase had significant activity at pH 5.0–9.0, *S. platensis* TGase's activity was optimal from pH 6.0–9.0 under our conditions. The *Streptomyces* TGases lost activity at acidic pH. Assuming that NH_2OH remained unprotonated inside the active site and that there was a fast equilibrium between TGase and its protonated inactivated form, an apparent pK_a (equal to $-\log K_1$) of 4.4 and 5.5 was found for *S. lydicus* and *S. platensis* TGase, respectively (Fig. 1A). For *S. nigrescens*, a pK_a of ~5 to 6 might be estimated from the pH corresponding to ~50% relative activity.

The *Streptomyces* TGases also lost activity at alkaline pH. Assuming another fast (relative to the catalysis) equilibrium between TGase and its deprotonated inactivated form, an apparent pK_a ($-\log K_2$) of 10.1 was found for *S. platensis* TGase (Fig. 1A). For *S. lydicus* and *S. nigrescens*, a pK_a of ~10

Table 1
Purification of Various Microbial TGases

TGase	Source	Yield[a]	Purification fold[b]	Purity (%)[c]
S. mobaraensis	SP913	76	7.3	>95
S. lydicus	SF broth	5	30	>95
S. platensis	SF broth	28	60	>95
S. cinnamoneus	SF broth	26	30	>75
S. nigrescens	SF broth	63	17	>85
S. hachijoensis	SF broth	5	92	>90
S. nitrosporus[d]	SF broth	—	—	—
S. fradiae[d]	SF broth	—	—	—
S. thermovulgaris[d]	SF broth	—	—	—
P. cactorum[d]	SP818	—	—	—

[a]Based on activity toward CBZ-Gln-Gly and NH_2OH.
[b]Based on specific activity.
[c]Based on major band on SDS-PAGE.
[d]Purification was unsuccessful for *S. nitrosporus*, *S. fradiae*, *S. thermovulgaris*, and *P. cactorum* TGase because of rapid loss of the target protein during chromatography.

Table 2
Molecular and Enzymatic Properties of Various Purified TGases

TGase	Mol mass (kDa)	Activity (IU/mg)[a]	T_{opt} (°C)	pH_{opt}
S. mobaraensis	37	3.9	37	8
S. lydicus	37	2.2	37	6
S. platensis	38	1.5	37	7–8
S. cinnamoneus	39	0.18	—	—
S. nigrescens	36	0.59	37	8
S. hachijoensis	35	1.9	—	—
P. cactorum	52	~0	—	—
Guinea pig liver	75	0.4	40[b]	6[b]

[a]Activity on CBZ-Gln-Gly and NH_2OH, measured by the hydroxamate assay.
[b]The values for T_{opt} and pH_{opt} were from refs. *19* and *20*.

and 8 might be estimated, respectively, from the alkaline pH corresponding to ~50% relative activity.

Figure 1B presents the temperature profiles of several *Streptomyces* TGases, as measured on CBZ-Gln-Gly and NH_2OH. *S. mobaraensis*, *S. lydicus*, *S. platensis*, and *S. nigrescens* TGases showed very similar thermal activity profiles, with an optimal temperature (T_{opt}) of ~37°C and a sudden inactivation at $T > 40$°C. For the range of 4–37°C, an apparent E_a of ~36 kJ/mol was observed for all four TGases.

When incubated at pH 6.0 and 20, 40, 45, 50, or 55°C, *S. lydicus* TGase retained 100, 107, 87, 45, or 7% initial activity, respectively. The abrupt activity loss was similar to that previously reported *(8)*. The acyl donor

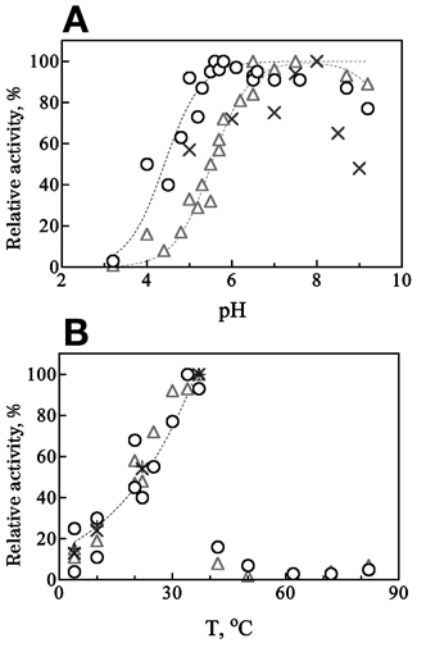

Fig. 1. Initial activity dependence on **(A)** pH and **(B)** temperature. **(A)** Fitting with Relative rate = $1/(1 + [H^+]/K_1)$: For *S. lydicus* TGase (- - -), $-\log K_1 = 4.4 \pm 0.1$; for *S. platensis* TGase (curve not shown), $-\log K_1 = 5.5 \pm 0.1$; for *S. nigrescens*, no satisfactory fit was obtained. Fitting with Relative rate = $1/(1 + [H^+]/K_1 + K_2/[H^+])$: For *S. platensis* (- - -), $-\log K_1 = 5.5 \pm 0.1$ and $-\log K_2 = 10.1 \pm 0.3$; for either *S. lydicus* or *S. nigrescens*, no satisfactory fit was obtained. **(B)** Fitting with Relative rate = $A \times \exp\{E_a/[8.314(T + 27)]\}$ was made for the temperature range of 4–37°C. Estimated activation energy, E_a (kJ/mol): *S. mobaraensis*, 36 ± 3; *S. lydicus*, 37 ± 6; *S. platensis*, 36 ± 4; *S. nigrescens*, 37 ± 4. For clarity, only the fitting curve for *S. lydicus* is shown (- - -). (○) *S. lydicus*; (△) *S. platensis*; (×) *S. nigrescens*; (+) *S. mobaraensis*.

CBZ-Gln-Gly showed a K_m of 23 ± 2, similar to a previously reported value *(8)*, and a k_{cat} of 13 ± 1 s^{-1} when 11.1 m*M* NH_2OH served as the amine donor. With 2 m*M* 2-bromoethylamine as the amine donor, CBZ-Gln-Gly showed a K_m of 26 ± 6 m*M* and a k_{cat} of 8 ± 1 s^{-1}. When the concentration of NH_2OH was varied from 0.1 to 4 m*M* in the presence of 30 m*M* CBZ-Gln-Gly, the activity remained relatively constant, giving an estimated $K_m \leq 0.1$ m*M* for NH_2OH. Given the detection limits of the assay, it was not possible to test below this value. When $[NH_2OH] > 10$ m*M*, the activity appeared to drop, possibly indicating substrate inhibition or inactivation. The ability of TGase to crosslink protein was tested on α-casein. Under our conditions, the apparent time for 0.03 mg/mL of *S. mobaraensis* and *S. lydicus* TGases to convert 9% fluidic α-casein solution into immovable gel was 210 and 315 min, respectively. For 0.08 mg/mL of *S. mobaraensis*, *S. platensis*, and *S. nigrescens* TGases, the apparent time was 120, 240, and 280 min, respectively. For 0.17 mg/mL of *S. platensis* and *S. nigrescens* TGases, the apparent time was

Table 3
Properties of Various Amines as *S. lydicus* TGase Substrates[a]

Amine	pK_a	σ*	K_m (m*M*)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
Aminoacetonitrile	5.5	1.30	1.2 ± 0.2	7.7 ± 0.5	$6.4\ 10^3$
Hydroxlyamine	6.0	1.34	<0.1	9.8	$>9.8\ 10^4$
Glycine ethyl ester	7.4	0.82	0.13 ± 0.04	10.2 ± 0.5	$7.8\ 10^4$
3-Aminopropionitrile	7.7	0.87	1.9 ± 0.4	9.3 ± 0.7	$4.9\ 10^3$
2-Bromoethylamine	8.5	0.49	0.26 ± 0.05	4.7 ± 0.2	$1.8\ 10^4$
Benzylamine	9.3	0.24	3.5 ± 0.4	12.1 ± 0.5	$3.5\ 10^3$
Ethanolamine	9.6	0.21	9.6 ± 0.9	8.1 ± 0.3	$8.4\ 10^2$
Histamine	9.8	–0.10[c]	0.38 ± 0.05	3.8 ± 0.2	$1.0\ 10^4$
Propylamine	10.7	–0.10	2.4 ± 0.4	6.6 ± 0.5	$2.8\ 10^3$
Isopropylamine	10.5	–0.19	—	<0.2	—
1-Ethylpropylamine	10.4	–0.23	—	<0.2	—
Glucosamine	10.7[b]	–0.06[d]	—	<0.2	—
Aniline	4.6	0.75	—	<0.2	—
1-Adamantanamine	10.7[b]	–0.18[b]	—	<0.2	—
Cyclohexylamine	10.7	–0.18	—	<0.2	—

[a]pK_a: Values for X-NH_3^+ *(16)*; σ*: Taft constant for substituent X in X-NH_2 *(15)*.
[b]Value of cyclohexylamine.
[c]Value of propylamine.
[d]Value of 2-hydroxypropylamine.

145 and 190 min, respectively. For 0.25 mg/mL of *S. platensis* and *S. nigrescens* TGases, the apparent time was 120 and 145 min, respectively.

Specificity Toward Acyl-Accepting Amine

Fifteen amines with different stereoelectronic properties were assayed as the acyl-accepting substrate for S. lydicus TGase (Table 3). The amines with a cyclic substituent at the Cα site showed minimal activity. By contrast, the substituted but not Cα-branched amines showed significant activity, which allowed derivation of the apparent Michaelis kinetic parameters K_m and k_{cat}. Figure 2 shows the dependence of the kinetic parameters on the pK_a (or basicity) of the amines or the inductive Taft constant σ* of the substituents. Between pK_a and $\log(k_{cat}/K_m)$ or $\log k_{cat}$, a small negative correlation was observed, whereas between σ* and $\log(k_{cat}/K_m)$ or $\log k_{cat}$, a small positive correlation was observed.

Over the range of 0.1–4 m*M*, isopropylamine, 1-ethylpropylamine, aniline, and glucosamine led to rates close to those of the TGase's CBZ-Gln-Gly hydrolysis (deamidation, H_2O as the acyl acceptor) reaction (~0.14 s^{-1}). Over the range of 0.1–40 m*M*, cyclohexylamine and 1-adamantanamine also led to rates close to those of the hydrolysis reaction.

Over the range of 2.5–150 m*M*, lysine showed Michaelis-like kinetics with a K_m of 6 ± 1 m*M* and a k_{cat} of 3.3 ± 0.2 s^{-1}. Over the range of 0.1–12 m*M*, Gly-His-Lys acetate showed a linear rate-concentration dependence, indicating a K_m of >12 m*M*. Over the range of 0.1–2 m*M*, physalaemin (D-Glu-

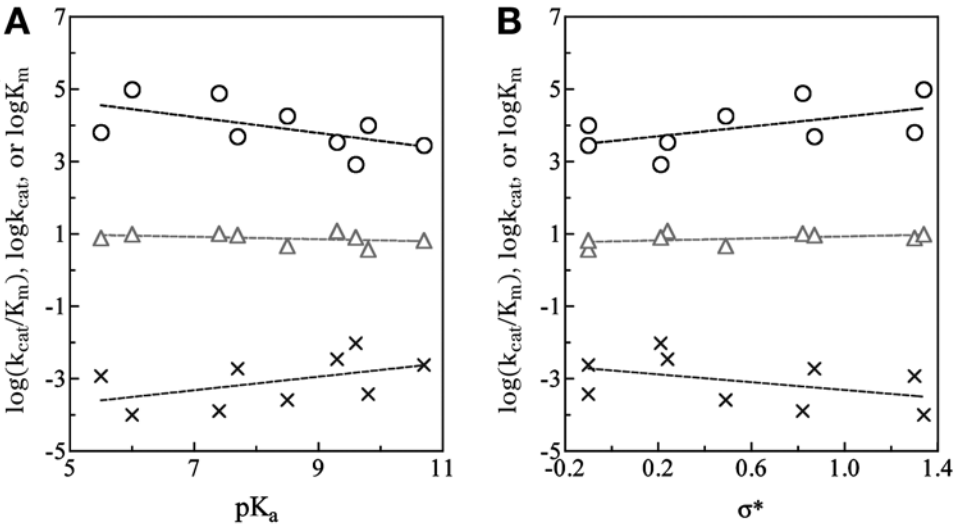

Fig. 2. Correlation between enzymatic and chemical parameters for acyl-accepting amines. **(A)** Correlation with pK_a: (○) log(k_{cat}/K_m) = (–0.2 ± 0.1)pK_a + (6 ± 1); (△) log(k_{cat}) = (–0.03 ± 0.03)pK_a + (1.2 ± 0.3); (×) log(K_m) = (0.2 ± 0.1)pK_a + (–5 ± 1). **(B)** Correlation with Taft σ*: (○) log(k_{cat}/K_m) = (0.7 ± 0.4)σ* + (3.6 ± 0.3); (△) log(k_{cat}) = (0.1 ± 0.1)σ* + (0.8 ± 0.1); (×) log(K_m) = (–0.5 ± 0.4)σ* + (–2.8 ± 0.3). Units: k_{cat}/K_m ($M^{-1}s^{-1}$); k_{cat} (s^{-1}); K_m (M^{-1}).

Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met) also showed a linear rate-concentration dependence, indicating a K_m of >2 m*M*. Over the range of 0.1–0.75 m*M*, D-Ala-β-(2-naphthyl)-D-Ala-Trp-D-Phe-Lys amide seemed to cause an inhibition at >0.5 m*M* on *S. lydicus* TGase. Over the range of 0.1–2 m*M*, hydrapeptide fragment 7-11 (Lys-Val-Ile-Leu-Phe) was sparingly active.

Difference Between Streptomyces and Phytophthora TGases

Under the specified conditions, *P. cactorum* TGase showed negligible, if any, activity to crosslink CBZ-Gln-Gly with NH_2OH and other simple, primary amines. However, *P. cactorum* TGase was able to gel diluted egg white under our conditions, although the time needed (~16 h) was longer than that of the *S. mobaraensis* TGase (~4 h). The identity of the modified egg-white protein(s) was not analyzed, although the most abundant ovalbumin was likely targeted. The fungal TGase has previously been shown to incorporate 5-(biotinamido)pentylamine into *N,N*-dimethyl casein, implant 1,4-^{14}C-putrescine onto α-casein, as well as polymerize α-casein *(6)*.

Discussion

Streptomyces TGases

S. mobaraensis, *S. lydicus*, *S. platensis*, and *S. cinnamoneus* TGases have high homology (~80%) in terms of protein sequence *(5,8)*. The suggested

catalytic amino acid residues, a Cys, an Asp, and a His, for *S. mobaraensis* TGase are conserved among the enzymes (Fig. 3). For *S. mobaraensis* TGase, the N-terminus, the loop between the β4 and β5 strands, and the loop between the β6 and β7 strands make up the entrance and wall of the active-site cleft, while the loop between the α2 and α3 helices, the β5 and β6 strands, and the α11 helix make up the bottom of the active-site cleft *(9)*. The sequences of the *S. lydicus*, *S. platensis*, and *S. cinnamoneus* TGases corresponding to these *S. mobaraensis* segments are highly conserved, although there are a few substitutions such as a Ser replacement by Lys on the loop between the β4 and β5 strands in *S. platensis*. Thus, the main characteristics of the *Streptomyces* TGases would be expected to be similar, as observed by this and other studies *(8)*.

There were some minor differences between our pH and temperature profiles and those previously reported for *S. mobaraensis* or *S. lydicus* TGase *(5,8)*, most likely owing to the different assays, buffers, pH values, or reaction times used in these studies. The apparent inhibition or inactivation of TGase by NH_2OH has not been reported before, and this observation may call the need for reexamination of previous kinetic data obtained with this widely used substrate.

Substituted Amine Substrates: Steric Effect

Structural and mechanistic studies have established that vertebrate TGase employs an active Cys-His-Asp triad, similar to cysteine proteases, to catalyze an isopeptide formation reaction, which can be regarded as a "reversed" proteolytic reaction *(7)*. For *S. mobaraense* TGase, the Cys from the putative active site is proposed to carry out acyl transfer and isopeptide formation, while the Asp from the putative active site is proposed to serve as the general acid/base *(9)*. In its "ping-pong" mechanism, the catalytic acylation half reaction is hypothesized to involve a nucleophilic attack of the Cys thiolate on the Cγ of a glutamyl (acyl-donating) substrate to form a tetrahedral intermediate, followed by release of an NH_3 (after an H^+ transfer from the general acid Asp) and formation of (Cys-)S-acyl. The catalytic deacylation half reaction is hypothesized to involve a nucleophilic attack of an (acyl-accepting) amine substrate on the Cγ of the S-acylated glutamyl moiety (helped by an amine H^+ transfer to the deprotonated general base Asp) to form another tetrahedral intermediate, followed by release of the isopeptide product (containing a γ-amidated glutamyl moiety) and regeneration of the active Cys thiolate (Fig. 4).

With the same acyl-donating substrate CBZ-Gln-Gly (although not saturating under our conditions), the initial rate of reaction depended on the acyl-accepting amine substrate, indicating that the deacylation half reaction was rate limiting, similar to the observation previously made on vertebrate TGases *(7,10,11)*. We attempted to decipher further which of the steps postulated for the half reaction control the deacylation reaction, by examining a series of acyl-accepting amines with different electronic and steric properties.

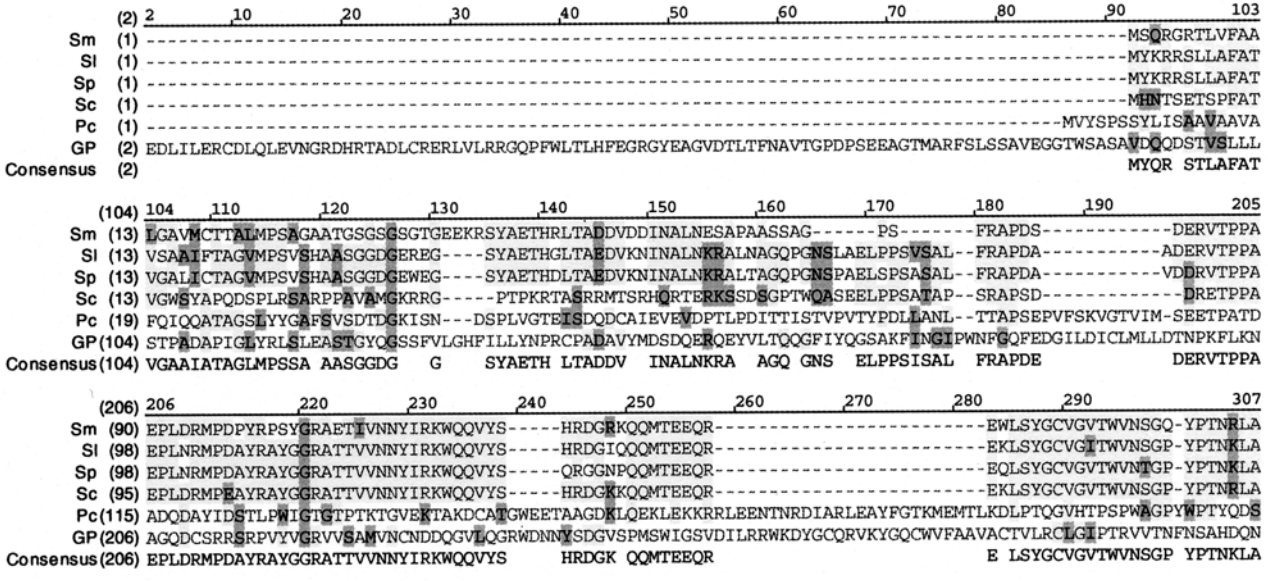
(2) 2 10 20 30 40 50 60 70 80 90 103
Sm (1) ----------MSQRGRTLVFAA
Sl (1) ----------MYKRRSLLAFAT
Sp (1) ----------MYKRRSLLAFAT
Sc (1) ----------MHNTSETSPFAT
Pc (1) ----------MVYSPSSYLISAAVAAVA
GP (2) EDLILERCDLQLEVNGRDHRTADLCRERLVLRRGQPFWLTLHFEGRGYEAGVDTLTFNAVTGPDPSEEAGTMARFSLSSAVEGGTWSASAVDQQDSTVSLLL
Consensus (2) MYQR STLAFAT
(104) 104 110 120 130 140 150 160 170 180 190 205
Sm (13) LGAVMCTTALMPSAGAATGSGSGSGTGEEKRSYAETHRLTADDVDDINALNESAPAASSAG------PS-------FRAPDS-----------DERVTPPA
Sl (13) VSAAIFTAGVMPSVSHAASGGDGEREG----SYAETHGLTAEDVKNINALNKRALNAGQPGNSLAELPPSVSAL--FRAPDA-----------ADERVTPPA
Sp (13) VGALICTAGVMPSVSHAASGGDGEWEG----SYAETHDLTAEDVKNINALNKRALTAGQPGNSPAELSPSASAL--FRAPDA-----------VDDRVTPPA
Sc (13) VGWSYAPQDSPLRSARPPAVAMGKRRG-----PTPKRTASRRMTSRHQRTERKSSDSGPTWQASEELPPSATAP--SRAPSD------------DRETPPA
Pc (19) FQIQQATAGSLYYGAFSVSDTDGKISN---DSPLVGTEISDQDCAIEVEVDPTLPDITTISTVPVTYPDLLANL--TTAPSEPVFSKVGTVIM-SEETPATD
GP (104) STPADAPIGLYRLSLEASTGYQGSSFVLGHFILLYNPRCPADAVYMDSDQERQEYVLTQQGFIYQGSAKFINGIPWNFGQFEDGILDICLMLLDTNPKFLKN
Consensus (104) VGAAIATAGLMPSSA AASGGDG G SYAETH LTADDV INALNKRA AGQ GNS ELPPSISAL FRAPDE DERVTPPA
(206) 206 220 230 240 250 260 270 280 290 307
Sm (90) EPLDRMPDPYRPSYGRAETIVNNYIRKWQQVYS-----HRDGRKQQMTEEQR----------EWLSYGCVGVTWVNSGQ-YPTNRLA
Sl (98) EPLNRMPDAYRAYGGRATTVVNNYIRKWQQVYS-----HRDGIQQQMTEEQR----------EKLSYGCVGITWVNSGP-YPTNKLA
Sp (98) EPLNRMPDAYRAYGGRATTVVNNYIRKWQQVYS-----QRGGNPQQMTEEQR----------EQLSYGCVGVTWVNTGP-YPTNKLA
Sc (95) EPLDRMPEAYRAYGGRATTVVNNYIRKWQQVYS-----HRDGKKQQMTEEQR----------EKLSYGCVGVTWVNSGP-YPTNRLA
Pc (115) ADQDAYIDSTLPWIGTGTPTKTGVEKTAKDCATGWEETAAGDKLQEKLEKKRRLEENTNRDIARLEAYFGTKMEMTLKDLPTQGVHTPSPWAGPYWPTYQDS
GP (206) AGQDCSRRSRPVYVGRVVSAMVNCNDDQGVLQGRWDNNYSDGVSPMSWIGSVDILRRWKDYGCQRVKYGQCWVFAAVACTVLRCLGIPTRVVTNFNSAHDQN
Consensus (206) EPLDRMPDAYRAYGGRATTVVNNYIRKWQQVYS HRDGK QQMTEEQR E LSYGCVGVTWVNSGP YPTNKLA

Fig. 3

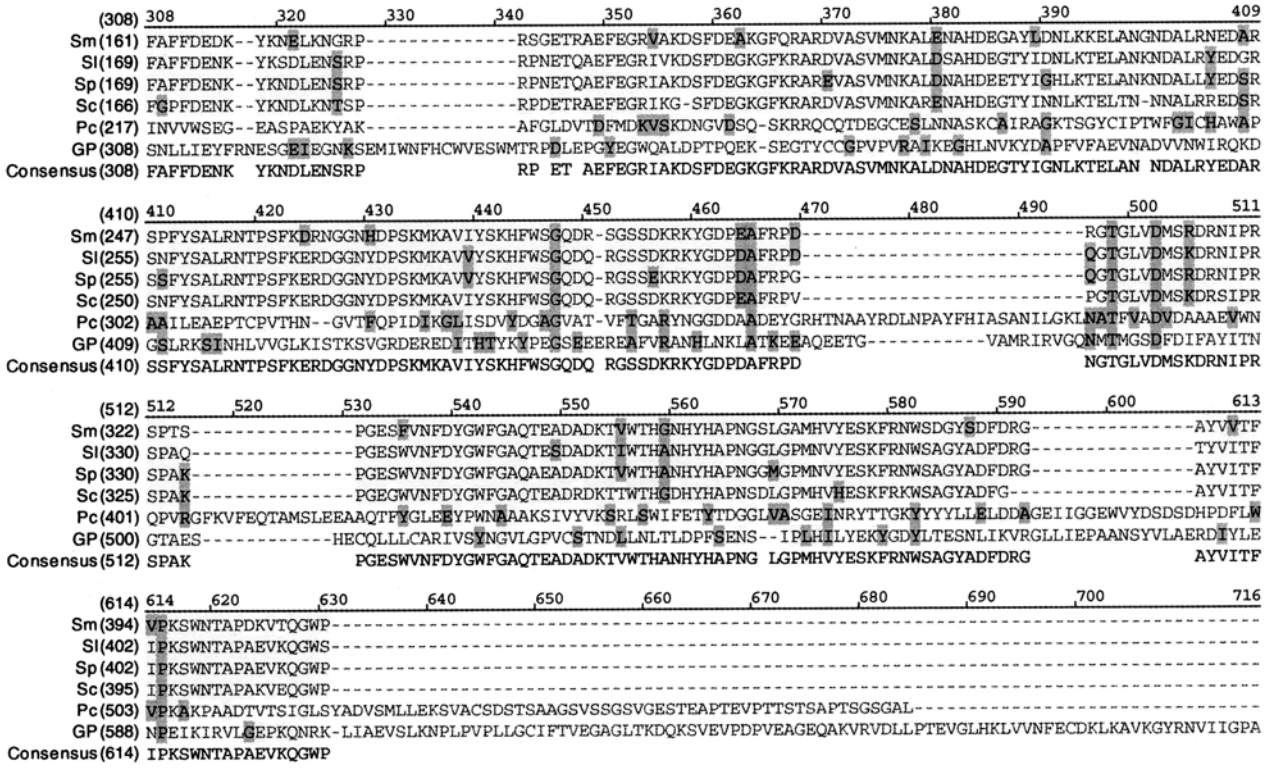

Fig. 3. Amino acid sequences of various TGases. Source: Sm, *S. mobaraensis* (EMBL AY241675); Sl, *S. lydicus (5,8)*; Sp, *S. platensis* (EMBL AY555726); Sc, *S. cinnamoneus* (TREMBL Q9RIS1); Pc, *P. cactorum (5,8)*; GP: guinea pig liver (Swiss-Prot P08587). The sequences of *S. lydicus*, *S. platensis*, *S. cinnamoneus*, and *P. cactorum* TGase had 84, 84, 79, and 27% homology to that of *S. mobaraensis* TGase, but only 10, 9, 9, and 7% homology to that of guinea pig liver TGase, respectively. The putative catalytic amino acid residues in *S. mobaraensis* were Cys143, Asp344, and His363 *(9)*. The inner wall of the putative active site in *S. mobaraensis* comprised segments Asp77-Ala86 (N-terminus), Asn115-Asn129 (loop between β4 and α5 sheets), and Asn352-Met364 (loop between β6 and β7 sheets), and the bottom comprised Ser137-Val141 (loop between β2 and β3 helices), Phe130-Gly136 (β5 sheet), Val347-Gly351 (β6 sheet), and Phe371-Glu376 (α11 helix).

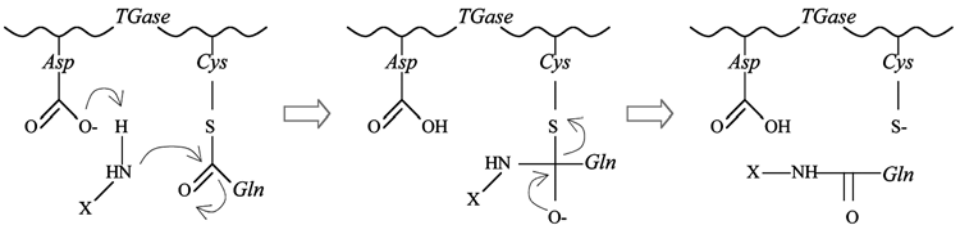

Fig. 4. Putative mechanism for deacylation half reaction of *S. mobaraensis* TGase catalysis *(9)*. The nucleophilic amine (X-NH_2) attacks the γ-C of the putative Cys-S-acylated Gln, assisted by the H^+ transfer from S-C(O)¨NH_2-X to the putative general acid/base Asp. The resulting oxyanion rearranges, leading to deacylation from the putative active Cys, release of the isopeptide or γ-amidated Gln product, and regeneration of the putative active Cys thiolate.

Six of the amines had branched Cα, four of them with their $–NH_2$ attached to bulky hexa-ring structures. Under our conditions, none of the branched amines showed an acyl-accepting activity stronger than that of the solvent H_2O. Consistent with previously reported observations *(7,10–12)*, the Cα-substitution (even by a methyl) severely limited the activity of the acyl-accepting amines, likely owing to unfavorable steric hindrance inside the active-site cleft.

Substituted Amine Substrates: Basicity Effect

Eight of the amines had unbranched Cα or equivalent structure and showed significant acyl-accepting activity. Compared to NH_3, these amines had substituents (to the $–NH_2$ group) of different electronic-inductive property, capable of affecting the electron density on the N and leading to different basicity and nucleophilicity of the $–NH_2$.

Chemical aminolysis of thioesters involves $>NH^+$-$C(O^–)$S- tetrahedral intermediate. For the nonenzymatic reaction, amines having higher basicity in general possess higher reaction rates. The Brønsted plot, log(rate) vs pK_a, has a positive slope, β (Brønsted coefficient). When the formation or breakdown of the tetrahedral intermediate is rate limiting, β 0.1 or 0.8 is commonly observed, respectively *(13,14)*. The positive β implies the presence of net positive charge (or electron density deficiency) on N at the key transition state *(7,13,14)*.

Figure 2A presents the Brønsted plots of *S. lydicus* TGase–catalyzed aminolysis of the enzyme-linked thioester. For k_{cat}/K_m (the apparent quasi-first-order rate) and k_{cat}, β –0.2 and –0.03 was observed, respectively, quite different from the chemical aminolysis reactions. A negative β has also been reported for guinea pig liver TGase *(7)*. The negative sign and relatively small magnitude of the β indicated that at the key transition state there would be a small net electron density "surplus" on the N of the amine substrate. Thus, it seemed that the neutral X-NH_2, rather than X-NH_3^+, served as the nucleophile for the catalytic deacylation. An H^+ transfer from

the N site in S-C(O)¨NH_2-X to the putative general acid/base Asp (which would create a transient, net negative charge on the N) participated in the formation of the key transition intermediate (as postulated in Fig. 4).

According to their pK_a (Table 3), a significant portion of our amines would be protonated (as X-NH_3^+) under our conditions (pH 6.0). Prior to the nucleophilic attack on the TGase S-acyl, a protonated amine would need to be deprotonated, likely by a carboxyl group at or near the active-site cleft. Smaller pK_a should facilitate deprotonation, making, e.g., NH_2OH (pK_a = 6.0) more active than propylamine (pK_a = 10.7) in this respect. The Brønsted plot for k_{cat}/K_m in Fig. 2A seems to support this. The relatively small magnitude of the β, however, indicates that the effect might not be involved at the key step of the catalytic deacylation reaction.

Substituted Amine Substrates: Inductive Effect

The schematic mechanism of Fig. 4 for the deacylation half reaction of TGase is supported by several structural and kinetic observations. When the amine nucleophilically attacks the S-acyl and the resulting tetrahedral intermediate decomposes, N-substituents may impact both steps via inductive effect, which can be quantified by the Taft parameter σ* *(15,16)*. A substituent with larger (more positive) or smaller (more negative) σ* would tend to withdraw or donate electrons from or to the N, respectively.

With an electron-withdrawing substituent, the amine would become less nucleophilic, unfavorable for the formation of the tetrahedral intermediate. The electron-withdrawing substituent might also divert the electron migration from the O^- to the S (Fig. 4), potentially slowing down the deacylation. However, an electron-withdrawing substituent could facilitate the H^+ transfer from S-C(O)¨NH_2-X to the putative general acid/base Asp, favorable for the formation of the tetrahedral intermediate.

A third inductive effect would negate the first and second effects. The observed Taft plots for the k_{cat}/K_m and k_{cat} of *S. lydicus* TGase–catalyzed reactions showed a slope (Taft coefficient ρ) of 0.7 and 0.1, respectively (Fig. 2B), suggesting that the inductive effect on the H^+ transfer from S-C(O)¨NH_2-X to the putative general acid/base Asp might be slightly dominating.

It should be pointed out that molecular and electronic effects other than the σ-bond polarization might contribute to the kinetics as well. Among the active amine substrates tested, glycine ethyl ester and benzylamine had a branched Cβ, histamine had a branched Cγ, and the rest had an unbranched structure. The branching, as well as the phenyl and imidazole substituents, might cause steric hindrance for a substrate. The hydroxyl, ester, and imidazole substituents might also interact with TGase via H bond. Such interactions might contribute to the substrate binding to TGase, causing the K_m scattering in Fig. 2.

Mechanism of Catalytic Deacylation Half Reaction

In reviewing the discussion on the effects from the basicity (pK_a), nucleophilicity, and inductivity (σ*), it seems that the deprotonation of the

amine substrate (at the neutral form), by the putative general acid/base Asp, plays a key role in the overall TGase catalysis, as previously hypothesized based on other experiments including isotope effect *(7)*. Likely the deprotonation by the general base and the nucleophilic attack on the S-acyl were concerted, rather than stepwise.

Streptomyces TGases lost activity at acidic pH, possibly associated with a protonation with an apparent pK_a of 4.4, 5.5, and ~5 to 6 for *S. lydicus*, *S. platensis*, and *S. nigrescens* TGase, respectively (Fig. 1A). A similar activity-reducing protonation with an apparent pK_a of ~4.5 has been previously reported for *S. mobaraensis* TGase *(8,17)*. The inactivation might be caused by the protonation of the postulated carboxylate needed for deprotonating a protonated amine substrate at the beginning of the catalytic deacylation half reaction. When fully solvated in water, the carboxylate of an Asp or Glu should have a pK_a of ~4. Increased pK_a might indicate an intramolecular environment favorable to the stabilization of the deprotonated state. Among the amino acid changes in the sequence segments that corresponded to those constituting the inner surface of the putative active site in *S. mobaraensis (9)*, only *S. platensis* TGase had a change accompanied by a net change of charge: it had a Lys333 at the position of the Ser325 in *S. mobaraensis* (Fig. 3). The extra positive charge might stabilize the postulated carboxylate needed for deprotonating a protonated amine substrate, resulting in a shift in pH profile. Other pH-inducible events, particularly amine substrate protonation and TGase conformational change, might also contribute to the observed pH dependence.

Streptomyces TGases remained active in neutral-alkaline pH (Fig. 1A), indicating that the putative general acid/base Asp, which should have a pK_a of ~4 when fully solvated in H_2O, could keep its initial protonation form even at pH > 6.0, a result likely caused by the local environment of the active-site cleft. *S. lydicus* and *S. platensis* TGases had a slightly more alkaline optimal pH than that of *S. mobaraensis* TGase, probably because of the minor sequence difference at the active-site cleft among these TGases.

The mechanism shown in Fig. 4 also invoked a deprotonated active Cys thiolate, which should have a pK_a of ~10 when fully solvated in H_2O. In cysteine proteases and vertebrate TGases (such as human coagulation factor XIIIa), the Cys thiolate is believed to be created via an ionic pair with the active His (in histidinium form) *(18)*. How *Streptomyces* TGases generated their active Cys thiolates, which would likely be exposed to solvent *(9)*, remained unclear.

The *Streptomyces* TGases showed a T_{opt} (~37°C) and an E_a (~37 kJ/mol) similar to those of guinea pig liver TGase (~40°C, ~45 kJ/mol) *(19,20)*. The thermal stability of the TGases was also similar.

Difference Between Bacterial and Fungal TGases

Streptomyces TGases may be quite different from human TGases in terms of sequence homology (~10–40%), crystallographic structure (as exemplified by *S. mobaraensis* TGase and human coagulation factor XIIIa),

mechanism (catalytic triad), and kinetics (as exemplified by their Ca^{2+} dependence) *(2,9,18)*. As a microbial TGase, *P. cactorum* TGase seemed quite different from its bacterial *Streptomyces* counterpart.

In terms of protein sequence, *P. cactorum* TGase was only ~10% homologous to *Streptomyces* TGases, and ~10–30% homologous to human TGases. A ClustalW alignment failed to identify a catalytic triad in *P. cactorum* TGase that would correspond to that from either *S. mobaraensis* TGase or human coagulation factor XIIIa (Fig. 3). Unlike *Streptomyces* TGases, *P. cactorum* TGase was Ca^{2+} dependent for its activity, similar to vertebrate TGases *(6,21)*. Similar to previous reports *(6,21)*, we observed *P. cactorum* TGase activity on crosslinking linear, small primary amines with native or modified α-casein, most likely onto its Gln via γ-glutamyl amide or isopeptide bonds. In contrast to both *Streptomyces* and vertebrate TGases, however, *P. cactorum* TGase seemed unable to activate CBZ-Gln-Gly and other small acyl-donating substrates.

A bovine α-s1-casein molecule had ~16 Gln. They were linked to four charged, six polar, and six hydrophobic amino acids at the N-terminal side, and five charged, two polar, and nine hydrophobic amino acids at the C-terminal side *(22)*. It was not clear whether *P. cactorum* TGase was specific to only part of the Gln sites. It seemed likely that the TGase was specific for the Gln-flanking moieties and might have an active acyl-donating substrate pocket with multiple subsites, whose interaction with the Gln-flanking moieties (either directly or via Ca^{2+}) could play an important role in the catalytic acylation half reaction. This speculation needs to be verified by future studies such as a comparative study employing different acyl donors *(11,23–25)*.

Conclusion

TGase is a multifunctional enzyme. The acyl transfer and isopeptide-forming function of the enzyme is important for not only physiologic but also biocatalytic reactions *(1–4,26,27)*. Among the various TGases, microbial TGase is increasingly attracting attention for its potential in industrial applications, because it may be produced by large-scale, inexpensive fermentation from hosts free of toxin, virus, or other infectious agents. Although *S. mobaraensis* TGase has been successfully commercialized for the past two decades for various food and specialty chemicals processes, developing new TGases with higher or extended stability and reactivity, as well as lower cost, is vital for the successful application of the enzyme to a wider range of industrial, personal care, medical, and other biocatalytic uses *(28)*.

We carried out a comparative study of several *Streptomyces* TGases and provided insight into the mechanistic specificity of *S. lydicus* TGase. The study, although still preliminary and in need of follow-ups (particularly characterization on the effect of calcium and the mechanism of the thermal inactivation), may help us to understand better the structure-function relationship of TGase and to develop it further as a versatile biocatalyst.

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