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Activated transglutaminase from *Streptomyces mobaraensis* is processed by a tripeptidyl aminopeptidase in the final step

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Transglutaminase (TGase) from *Streptomyces mobaraensis* is secreted as a precursor protein which is completely activated by the endoprotease TAMEP, a member of the M4 protease family [Zotzel, J., Keller, P. & Fuchsbauer, H.-L. (2003) *Eur. J. Biochem.* **270**, 3214–3222]. In contrast with the mature enzyme, TAMEP-activated TGase exhibits an additional N-terminal tetrapeptide (Phe-Arg-Ala-Pro) suggesting truncation, at least, by a second protease. We have now isolated from the culture broth of submerged colonies a tripeptidyl aminopeptidase (SM-TAP) that is able to remove the remaining tetrapeptide. The 53-kDa peptidase was purified by ion-exchange and phenyl-Sepharose chromatography and subsequently characterized. Its proteolytic activity was highest against chromophoric tripeptides at pH 7 in the presence of 2 mM $CaCl_2$. EDTA and EGTA (10 mM) both diminished the proteolytic activity by half. Complete inhibition was only achieved with 1 mM phenylmethanesulfonyl fluoride, suggesting that SM-TAP is a serine protease. Alignment of the N-terminal sequence confirmed its close relation to the *Streptomyces* TAPs. That removal of Phe-Arg-Ala-Pro from TAMEP-activated TGase by SM-TAP occurs in a single step was confirmed by experiments using various TGase fragments and synthetic peptides. SM-TAP was also capable of generating the mature N-terminus by cleavage of RAP-TGase. However, AP-TGase remained unchanged. As SM-TAP activity against chromophoric amino acids such as Pro-pNA or Phe-pNA could not be detected, the tetrapeptide of TAMEP-activated TGase must be removed without formation of an intermediate.

Keywords: *Streptomyces mobaraensis*; transglutaminase processing; transglutaminase; tripeptidyl aminopeptidase.

Streptomyces mobaraensis belongs to a large group of Gram-positive, filamentous soil bacteria with a complex life cycle. Like other *Streptomycetes*, it has a multicellular morphology characterized by at least three distinct differentiation stages. Culture on agar plates containing glucose, yeast and malt extracts allows the organism to develop substrate and aerial mycelia culminating in the formation of spores [1]. In contrast, culture in shaking flasks containing a liquid complex medium prevents sporulation. The onset of aerial hyphae growth is closely associated with the secretion and activation of numerous hydrolases such as nucleases and proteases, the functions of which are not well understood. It would appear that they have more important roles in regulating cellular differentiation over and above the mere digestion of substrate mycelium to supply aerial hyphae with nutrients. In particular, recent results suggest that mycelium differentiation may be comparable to the events of programmed cell death in eukaryotes [2].

Transglutaminases (TGases; EC 2.3.2.13, protein glutamine:amine γ-glutamyltransferase) are multifunctional enzymes widely distributed among animals and plants [3–6]. They have also been found in some *Streptomyces* species [7–10], formerly assigned to the genus *Streptoverticillium*, and in *Bacillus subtilis* [11]. It is well known that TGases exhibit various catalytic activities, the cross-linking of proteins via N^{ε}-(γ-glutamyl)lysine bonds, the incorporation of polyamines into proteins, the deamidation of protein-bound glutamines, and the covalent attachment of proteins to lipids such as ω-hydroxyceramides [12–15]. Although much attention has been paid to the function of mammalian TGases which participate in apoptosis for example [16], less attention has been paid to the role of the bacterial enzymes and their regulation. TGase from

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Abbreviations: AP, Leu/Phe aminopeptidase; pNA, *p*-nitroanilide; SM, *Streptomyces mobaraensis*; SSI, *Streptomyces* subtilisin inhibitor; TAMEP, transglutaminase-activating metalloprotease; TAP, tripeptidyl aminopeptidase; TGase, transglutaminase.

Enzymes: transglutaminase, protein-glutamine:amine γ-glutamyltransferase from *Streptomyces mobaraensis* (EC 2.3.2.13; SwissProt entry name TGL_STRSS, accession number P81453); TAMEP, transglutaminase activating metalloprotease (SwissProt entry name TAMP_STRMB, accession number P83543); P_{14}, TAMEP inhibitory protein (SwissProt entry name SSIT_STRMB, accession number P83544); trypsin from *Bos taurus* (EC 3.4.21.4; SwissProt entry name TRY2_BOVIN, accession number Q29463); chymotrypsin from *Bos taurus* (EC 3.4.21.1; SwissProt entry name CTRA_BOVIN, accession number P00766).

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S. mobaraensis has been described as a Ca^{2+}-independent enzyme of molecular mass 38 kDa which is secreted as an inactive precursor bearing an activation peptide of 45 amino acids [7,8]. In the course of cultivation, the microbial enzyme is activated by the P1′-endoprotease TAMEP cleaving the propeptide between Ser(−5) and Phe(−4) [1]. The activity of TAMEP, a putative zinc metalloprotease, can be completely suppressed by a strong inhibitory protein of molecular mass 14 kDa (P_{14}) related to the *Streptomyces* subtilisin inhibitory (SSI) family [1]. P_{14}, one of the major extracellular proteins of submerged and surface colonies, appears to have an important role in regulating TAMEP and TGase activities.

TAMEP cleavage removes 41 amino acids from the activation peptide generating FRAP-TGase. As the intermediate already exhibits full activity, removal of the tetrapeptide by at least one additional aminopeptidase appears to be an artefact. Several monopeptidyl, dipeptidyl and tripeptidyl aminopeptidases of *Streptomyces* spp. have been identified, none with any proteolytic activity against chromophoric tetrapeptides [17–24]. Moreover, the better characterized tripeptidyl aminopeptidase (TAP) from *Streptomyces lividans* 66 obviously has inappropriate specificity (Ala-Pro-Ala↓naphthylamide) for performing the final TGase processing [17, 20]. We have now isolated a TAP from the culture broth of *S. mobaraensis* that has no sensitivity towards P_{14}. That the serine protease generates the mature N-terminus of TGase in a single step was shown by various TGase fragments and chromophoric peptides.

Materials and Methods

Materials

S. mobaraensis (strain 40847) was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen DSMZ (Braunschweig, Germany). Ala-Pro-pNA, Suc-Ala-Ala-Pro-Phe-pNA, Bz-Pro-Phe-Arg-pNA, trypsin-beaded agarose and α-chymotrypsin-beaded agarose (both from bovine pancreas) and all inhibitory compounds used were purchased from Sigma (Deisenhofen, Germany). All other synthetic peptides were from N-Zyme BioTec (Darmstadt, Germany) or Bachem (Heidelberg, Germany). Dispase I was from Roche Diagnostics (Mannheim, Germany). Additional materials were obtained in analytical grade from Merck (Darmstadt, Germany), Applichem (Darmstadt, Germany) and Sigma.

Cultivation of *S. mobaraensis*, purification of proteins (TGase, TAMEP, P_{14}) from culture broth or plate extracts, the determination of proteolytic activities and other standard procedures were performed as described previously [1,8].

Purification of the tripeptidyl aminopeptidase from *S. mobaraensis* (SM-TAP)

To a supernatant of 50-h-old cultures, obtained by centrifugation (10 000 ***g***, 15 min, 4 °C) and filtration, was added ethanol to a concentration of 70% (v/v). The precipitated proteins were dissolved in 50 mM Tris/HCl, pH 7.0, applied to a 69-mL Fractogel EMD SO_3^- column (Merck), washed with the same buffer, and eluted with 50 mM Tris/HCl containing 0.1 M NaCl followed by a linear NaCl gradient from 0.1 to 1.0 M. SM-TAP activity was found in fractions between 0.6 and 0.7 M NaCl. $(NH_4)_2SO_4$ up to 1.73 M was added to the mixture of the combined fractions, and the filtered solution was applied to a 7.5-mL phenyl-Sepharose column (Amersham-Pharmacia, Uppsala, Sweden). After a wash with 50 mM Tris/HCl, pH 7.0, containing 1.73 M $(NH_4)_2SO_4$, separation was achieved with a linear gradient from 1.73 to 0 M $(NH_4)_2SO_4$. The TAP was eluted at $(NH_4)_2SO_4$ concentrations below 0.3 M. N-Terminal sequence analysis of the purified protein was performed as described [1].

Partial purification of the Arg-C endoprotease

$(NH_4)_2SO_4$ (40%, w/v) was added to centrifuged and filtered supernatants of 70-h-old cultures. Precipitated proteins were removed by centrifugation (10 000 ***g***, 15 min, 4 °C) and filtration, and 2 mL of the clear solution was applied to a 1-mL phenyl-Sepharose column. After a wash with 40 mL 50 mM Tris/HCl, pH 7.0, containing 1.73 M $(NH_4)_2SO_4$, the protease was eluted with the same buffer containing 0.87, 0.43, 0.22, 0.11, 0.05 M (4 mL each) and 0 M $(NH_4)_2SO_4$ (10 mL). Fractions of 1 mL were collected and analysed using *N*-Bz-Pro-Phe-Arg-pNA.

Purification of the Leu/Phe aminopeptidase (AP)

Proteins of centrifuged and filtered culture broth were concentrated by ethanol precipitation (70%, v/v), applied to a 54-mL DEAE-Sepharose column (Amersham-Pharmacia), pre-equilibrated to pH 9 with 10 mM Tris/HCl. Active AP was found in the unbound fraction which was pumped on to a 69-mL Fractogel EMD SO_3^- column at pH 7 using a 50-mM Tris/HCl buffer and eluted with 0.2 M NaCl in the same buffer. Fractions with the highest activity only contained the TAMEP inhibitory protein P_{14} which was removed by benzamidine chromatography (Amersham-Pharmacia). Then 5 mL of the AP solution was applied to a 1-mL column equilibrated with 50 mM Tris/HCl (pH 8)/2 mM $CaCl_2$. The peptidase, eluted with 1 M NaCl, was dialysed and stored at −20 °C.

Inhibitory experiments

SM-TAP (70 μL; 37 $U \cdot mL^{-1}$) in 50 mM Tris/HCl, pH 7.0, containing 20 μL ethanol and 10 μL inhibitor (final concentration shown in Table 1) was incubated for 20 min at 28 °C before proteolytic activity was measured.

Processing of pro-TGase

Pro-TGase (2.6–4.2 nmol) in 250–400 μL 50 mM Tris/HCl, pH 7.0, was incubated at 30 °C for 30 min with 20 μL (1 pmol) TAMEP, 500 μL (20 U) immobilized chymotrypsin or 250 μL (5 U) immobilized trypsin in 50 mM Tris/HCl, pH 7.0. Immobilized proteases were removed by centrifugation before 20 μL (6 pmol) of the TAP was added. After further incubation at 30 °C for 30 min, the mixture was separated by SDS/PAGE. TGase was excised and sequenced as described [1]. In control experiments, TGase samples activated by the *endo*-proteases alone were also sequenced.

Table 1. Effect of inhibitors against SM-TAP. For residual activity monitoring, 70 μL (about 10 μg) of the enzyme was preincubated in 50 mM Tris/HCl, pH 7.0, with 10 μL inhibitor and 20 μL ethanol at room temperature for 30 min. After the addition of 0.2 mM Ala-Ala-Pro-pNA to obtain a final volume of 200 μL, residual activity was monitored at 405 nm for 20 min.

Inhibitor	Concentration (mM)	Residual activity (%)
None	–	100
EDTA	10	53
	1	81
EGTA	10	52
	1	70
Phenylmethanesulfonyl fluoride	1	0
Leupeptin	0.1	93
E-64	0.05	93
o-Phenanthroline	10	85
Pepstatin A	0.1	93
Bestatin	0.1	86
Chymostatin	0.5	90
Dithiothreitol	10	95
Iodacetamide	5	94
P_{14}	0.01	98[a]

[a] See ref [1].

Results

Proteases of liquid cultures

S. mobaraensis was cultured in a glucose/starch medium that always enabled the production of large quantities of TGase [8]. Numerous attempts failed to demonstrate TAMEP activity with pro-TGase or the P1′ substrates shown in Table 2. Screening for other proteases was then restricted to those that may be relevant in TGase processing,

Table 2. Peptidase activities in liquid cultures of *S. mobaraensis*. FA, furylacryloyl; ND, not detectable.

Protease	Substrate	Activity ($nmol \cdot min^{-1} \cdot ml^{-1}$)
TAMEP (N-Phe)	FA-Ala-Phe-NH_2[a]	ND
	FA-Gly-Leu-NH_2[a]	ND
Chymotrypsin-like (Phe-C)	Suc-Ala-Ala-Pro-Phe-pNA[b]	< 0.1
Trypsin-like (Arg-C)	Bz-Pro-Phe-Arg-pNA[b]	1.4
SM-TAP	Ala-Pro-pNA[b]	16.3
	Cbz-Gly-Pro-pNA[b]	0.2
AP	Leu-pNA[b]	5.5
	Phe-pNA[b]	8.2

[a] 30 μL culture supernatant in 160 μL 50 mM Tris/HCl, pH 8.0, containing 2 mM $CaCl_2$ was incubated with 10 μL 10 mM furylacryloyl peptide at room temperature. ΔA_{340} was recorded for 20 min. [b] 50 μL culture supernatant in 50 μL 50 mM Tris/HCl, pH 7.0, containing 2 mM $CaCl_2$, was incubated with 100 μL 0.4 mM *p*-nitroanilide at room temperature. ΔA_{405} was recorded for 20 min.

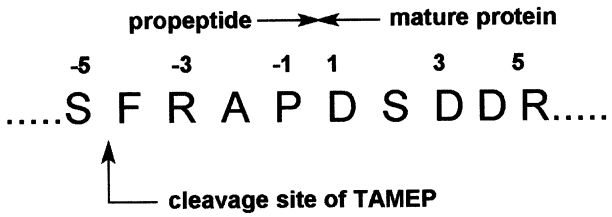

Fig. 1. Amino acids at the cleavage site of TGase from *S. mobaraensis*. The peptide bond between the activation peptide and the mature enzyme as well as the cleavage site of TAMEP are indicated by arrows.

and commercially available peptides were chosen corresponding to the amino acids at the TGase cleavage site (Fig. 1, Table 2). Two aminopeptidases and a trypsin-like (Arg-C) endoprotease were identified despite P_{14} being present in all culture supernatants (Table 2). Low proteolysis of Suc-Ala-Pro-pNA was a side reaction of SM-TAP as shown below.

The Arg-C endoprotease was partially purified in order to study its proteolytic potency against TGase. All attempts to activate TGase failed. Similarly, purified AP was unable to remove phenylalanine from the TAMEP product FRAP-TGase (Table 3). We therefore abandoned the characterization of the properties of both enzymes. In contrast, TAP, first detected with Gly-Pro-pNA and Ala-Pro-pNA, was obviously the enzyme required to complete TGase processing. Preliminary experiments showed its potency to cleave the tetrapeptide from TAMEP-activated TGase. In addition, the appearance of SM-TAP in the culture broth correlated with the increase in TGase (Fig. 2).

Purification of SM-TAP

SM-TAP was purified by ethanol precipitation and ion-exchange and phenyl-Sepharose chromatography (Table 4). Solvent precipitation was associated with considerable loss of activity, but more than 90% of other proteins were eliminated. Chromatography on Fractogel EMD SO_3^- generally produced high yields. Fractions with the highest activities only exhibited a few proteins with a molecular mass of 50 kDa or above; SM-TAP gave the main electrophoresis band at ≈50 kDa (Table 4, pool A; Fig. 3, lane 3). No proteolytic activity, apart from

Table 3. N-Terminal sequences of TGase from *S. mobaraensis* after proteolytic truncation by proteases.

Incubation mixture	N-Terminal sequence
pro-TGase [8]	DNGAG...
mature TGase [26]	DSDDR...
pro-TGase + SM-TAP	DNGAG...
pro-TGase + TAMEP [1]	FRAP-DSDDR...
pro-TGase + chymotrypsin	RAP-DSDDR...
pro-TGase + trypsin [8]	AP-DSDDR...
FRAP-TGase + Leu/Phe-AP	FRAP-DSDDR...
FRAP-TGase + SM-TAP	DSDDR...
RAP-TGase + SM-TAP	DSDDR...
AP-TGase + SM-TAP	AP-DSDDR...

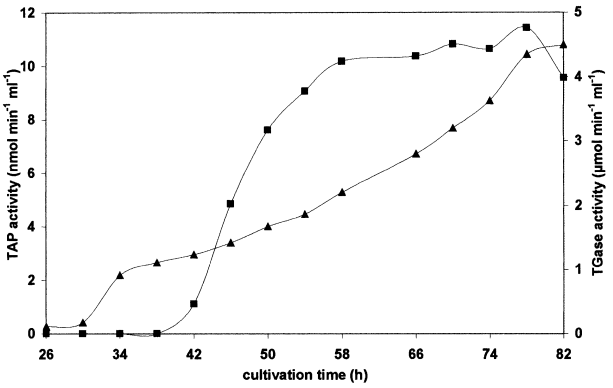

Fig. 2. Activity of TGase (▲) and SM-TAP (■) of submerged *S. mobaraensis* cultures. Enzyme activity was measured by the incorporation of hydroxylamine into Cbz-Gln-Gly (TGase) and by the release of pNA from Gly-Pro-pNA (SM-TAP) as described [1].

SM-TAP activity and that relevant to TGase processing, could be detected.

Purification of SM-TAP was continued by hydrophobic interaction chromatography to remove proteins of higher molecular mass. This procedure only moderately enhanced the specific activity, mainly to the detriment of the yield (Table 4; Fig. 3, lane 4). Such high activity loss on filter membranes used for desalting or concentrating suggested that the binding forces between SM-TAP and phenyl-Sepharose were so strong that only small amounts of the enzyme could be released at low salt concentrations.

Properties of SM-TAP

According to SDS/PAGE, SM-TAP has an apparent molecular mass of 53 kDa. The optimum pH, determined in Tris/acetate buffer, was 7.0–7.5. Activity could be further enhanced by the addition of small amounts of $CaCl_2$. For instance, Ala-Pro-pNA was hydrolysed in the presence of 50 µM Ca^{2+} at double the normal rate. Further increasing the Ca^{2+} concentration had only a small effect (less than 10%), indicating moderate stimulation of SM-TAP activity by the bivalent ion. Correspondingly, EDTA and EGTA at concentrations up to 10 mM were both unable to inhibit SM-TAP completely. Catalytic activity was reduced at most by half in the presence of the chelating agents (Table 1).

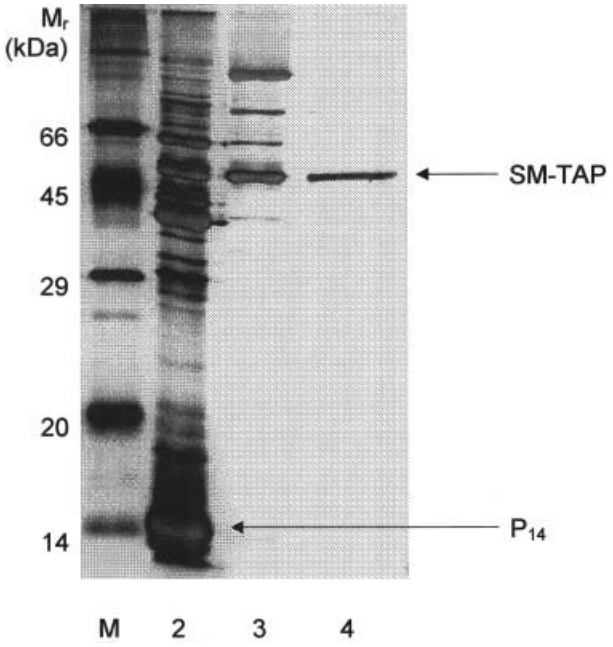

Fig. 3. Results of SM-TAP purification indicated by silver-staining and SDS/PAGE. Lane M, molecular mass markers; lane 2, ethanol precipitate; lane 3, pool A of Fractogel EMD SO_3^- chromatography; lane 4, pool A of phenyl-Sepharose chromatography.

Other inhibitors were tested in order to assign SM-TAP to a protease family (Table 1). Only phenylmethanesulfonyl fluoride at a concentration of 1 mM completely inhibited proteolytic activity, suggesting that a serine residue may be located in the active site. P_{14}, which is related to the serine protease inhibitory family SSI and present in the culture broth (Fig. 3, lane 2), did not have any effect on the peptidase, at least at the concentration used (10 µM).

N-Terminal sequence analysis performed by automated Edman degradation revealed a 35-amino acid segment of high homology to putative TAPs deduced from DNA of *Streptomyces coelicolor* and *S. lividans* ([25], C. Binnie, M.J. Butler, J.S. Aphale, M.A. DiZonno, P. Krygsman, E. Walczyk, & L.T. Malek, unpublished observation) (Fig. 4). Their molecular masses calculated from the putative mature proteins correspond closely to the experimental data for SM-TAP.

Table 4. Purification protocol for SM-TAP. One unit is defined as the release of 1.0 nmol *p*-nitroaniline per min using Ala-Pro-pNA in the assay.

Purification step	Volume (ml)	Activity (U)	Protein (mg)	Specific activity ($U \cdot mL^{-1}$)	Specific activity ($U \cdot mg^{-1}$)	Purification factor (%)	Yield
Culture supernatant	320	4960	2560	15.5	1.94	1	100
Ethanol precipitate	80	2424	131	30.3	18.5	10	49
Fractogel EMD SO_3^-							
Pool A	65	1482	7.9	22.8	188	97	30
Pool B	65	813	6.9	12.5	117	60	16
Phenyl-Sepharose							
Pool A	10	216	0.99	21.6	218	112	4
Pool B	15	211	1.41	14.1	150	77	4

Table 5. Substrate specificity of SM-TAP. SM-TAP (100 μL; ≈ 50 μg) was incubated with 100 μL 0.4 mM amino acid or peptide in 50 mm Tris/HCl (pH 7.0)/ 2 mm $CaCl_2$ for 30 min at 28 °C. Amino acids with the same position at cleavage sites of TGase are printed bold.

Substrates	Activity ($nmol \cdot min^{-1} \cdot ml^{-1}$)	Relative activity (%)
Pro-pNA	< 1	< 0.05
Phe-pNA	< 1	< 0.05
Leu-pNA	< 1	< 0.05
Ala-pNA	< 1	< 0.05
Ala-Pro-pNA	153	3.6
Suc-**Ala-Pro**-pNA	5	0.1
Gly-**Pro**-pNA	15	0.3
Cbz-Gly-**Pro**-pNA	6	0.1
Ala-Ala-pNA	2	0.05
Ala-Phe-pNA	< 1	< 0.05
Gly-Glu-pNA	< 1	< 0.05
Gly-Arg-pNA	< 1	< 0.05
Ala-**Ala-Pro**-pNA	4304	100
Ala-Phe-**Pro**-pNA	3258	76
Ala-**Ala**-Ala-pNA	2080	48
Pro-Leu-Gly-pNA	199	4.6
Ala-**Ala**-Phe-pNA	86	2.0
Suc-Ala-**Ala**-Phe-pNA	< 1	< 0.05
Val-Leu-Lys-pNA	5	0.1
Cbz-Pro-Phe-Arg-pNA	< 1	< 0.05
Ala-Ala-Val-Ala-pNA	46	1.1
Ala-Ala-Pro-Leu-pNA	< 1	< 0.05
Suc-Ala-Ala-Pro-Phe-pNA	< 1	< 0.05

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          1              11             21             31
S. mob.   QADIK DRILK IPGMK FVEEK PYQGY RYLVM TYRQP
           •||| ||•|  ||||   •||| || || |   |    | ||
S. coe.   AVDIK DRLLS IPGMS LIEEK PYTGY RFFVL NYTQP
          || |||| ||||| ||||| ||||| ||||| |||| || ||
S. liv.   AVDIK DRLLS IPGMS LIEEK PYTGY RFFVL NYAQP
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Fig. 4. N-Terminal sequence of SM-TAP. Corresponding segments of putative TAPs from *S. coelicolor* (line 2) and *S. lividans* (line 3) are also shown ([25], C. Binnie, M.J. Butler, J.S. Aphale, M.A. DiZonno, P. Krygsman, E. Walczyk, & L.T. Malek, unpublished observation). Identical residues are in bold and linked by a vertical line.

Processing of TGase fragments by SM-TAP

Purified pro-TGase that remained unchanged by SM-TAP treatment was digested with trypsin, chymotrypsin and TAMEP from *S. mobaraensis* to produce the active fragments AP-TGase, RAP-TGase and FRAP-TGase, respectively (Table 3).

First, it was shown that purified AP could not release phenylalanine or any other amino acid of FRAP-TGase, excluding its participation in the final TGase processing (Table 3). In further experiments, mixtures of SM-TAP and a TGase fragment were incubated for 30 min and separated by SDS/PAGE. N-Terminal sequence analysis of TGase clearly showed that SM-TAP removes Arg-Ala-Pro and Phe-Arg-Ala-Pro from the chymotrypsin-activated and TAMEP-activated intermediate, respectively. However, the trypsin fragment (AP-TGase) remained resistant to proteolytic attack, suggesting that SM-TAP generates mature TGase in a single step (Table 3). To our knowledge, a peptidase able to shorten proteins by removal of tetrapeptides has not yet been described in the literature. Further studies using chromogenic amino acids and peptides were therefore necessary to substantiate the unusual specificity of SM-TAP.

Activity of SM-TAP against chromogenic peptides

All the amino acids and peptides used exhibited a pNA residue on the C-side. The already slight yellowing of the solution indicated SM-TAP activity against the compound in the incubation mixture.

SM-TAP has a clear preference for tripeptides as can be seen from Table 5. The highest activity was found for Ala-Ala-Pro-pNA, which includes two amino acids identical with FRAP-TGase. Substitution of alanine with phenylalanine or proline with alanine reduced the rate of hydrolysis comparably moderately (up to 50%). However, if the tripeptide pattern differed considerably from the TGase appendage, release of pNA declined by an order of magnitude. The affinity of Pro-Leu-Gly-pNA or Ala-Ala-Phe-pNA for SM-TAP corresponded to that of Ala-Ala-Val-Ala-pNA or Ala-Pro-pNA, exhibiting precisely the sequence of FRAP-TGase.

Yellowing of the Ala-Ala-Val-Ala-pNA solution must be the result of direct cleavage of the anilide bond. Ala-pNA and Ala-Ala-pNA were not substrates (or only extremely poor ones) of SM-TAP.

The high specificity of SM-TAP was also underlined by other dipeptides and tetrapeptides. Any modification of the Ala-Pro motif resulted in a dramatic loss of SM-TAP activity. Furthermore, a second commercially available tetrapeptide investigated here, Ala-Ala-Pro-Leu-pNA, had a structure that did not fit into the SM-TAP active site.

It was also interesting to find that SM-TAP displayed weak activity against Suc-AP-pNA and Cbz-GP-pNA, which was not observed for other N-protected peptides. It is possible that these peptides are accepted by SM-TAP like poor tripeptides.

Finally, SM-TAP activity against chromogenic amino acids was studied. None of the anilides used, even Phe-pNA and Pro-pNA, was cleaved by the peptidase. As Gly-Arg-pNA and AP-TGase (see above) were also not substrates, it appears that SM-TAP removes the tetrapeptide from FRAP-TGase in a single step.

Conclusions

We recently reported the activation of TGase from *S. mobaraensis* by the P1′-metalloprotease TAMEP which cleaves a peptide bond between Ser(−5) and Phe(−4) [1]. Protease activity and, correspondingly, the extracellular cross-linking activities of the microbe seem to be strictly regulated by a strong inhibitory 14-kDa protein (P_{14}) related to the *Streptomyces* subtilisin inhibitor (Fig. 5). The intermediate FRAP-TGase formed has the full activity of the mature enzyme, suggesting that the final processing step is only an artefact of an aminopeptidase coincidentally secreted with TGase.

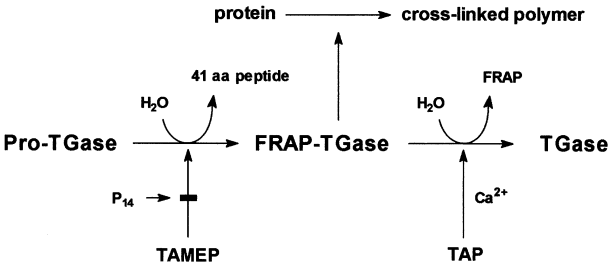

Fig. 5. Scheme of TGase processing. Pro-TGase is cleaved by TAMEP releasing a peptide of 41 amino acids. P_{14} may inhibit the reaction by attaching to TAMEP in equimolar concentrations [1]. FRAP-TGase exhibits the full activity of the mature enzyme [1]. Final processing is achieved by SM-TAP in a single step which removes the tetrapeptide without forming any intermediate. The last reaction is promoted by low concentrations of Ca^{2+} ions.

We have now purified a TAP from *S. mobaraensis* that produces mature TGase. The enzyme belongs to the serine protease family, as shown by inhibitory experiments and sequence alignment. Nevertheless, unlike other serine proteases, no sensitivity to P_{14} could be detected. However, SM-TAP has a very high specificity. The Ala-Pro motif is a crucial building block which FRAP-TGase can attach to SM-TAP even if AP-TGase is not processed (probably, in this case, the additional, positively charged arginine is needed to keep the hydrophobic dipeptide in the aqueous environment). Experiments using synthetic dipeptides and tripeptides clearly indicated that any substitution of alanine or proline was associated with a decrease in proteolytic activity. Our study also revealed the strong preference of SM-TAP for tripeptides. Designation of the enzyme as a tripeptidyl aminopeptidase is therefore logical. However, a side reaction with the tetrapeptide Ala-Ala-Val-Ala-pNA was revealed. The inability of the peptidase to hydrolyse Ala-Ala-pNA and Ala-pNA (or other chromogenic amino acids) at reasonable rates clearly indicates exclusive cleavage of the anilide bond of Ala-Ala-Val-Ala-pNA. Our results also provide convincing evidence that FRAP-TGase is processed by SM-TAP without passing through an intermediate. Phenylalanine cannot be removed, as shown by the Phe-pNA experiment. Cleavage of the peptide bond between Arg(−3) and Ala(−2) implies formation of AP-TGase which is resistant to SM-TAP proteolysis. Ultimately, truncation of the tripeptide Phe-Arg-Ala would yield P-TGase as a final product, as Pro-pNA is also not a substrate of the peptidase. Processing of TGase from *S. mobaraensis* apparently proceeds as shown in Fig. 5. Whether the stimulation of SM-TAP activity by small amounts of Ca^{2+} is of physiological importance remains in question.

The unusually high specificity of SM-TAP towards the appendage of TAMEP-activated TGase suggests that the function of the tetrapeptide may be to regulate already activated TGase by retaining the partially processed enzyme in the murein layer. Ionic interactions may occur between negatively charged cell wall components and the positively charged tetrapeptidyl arginine, only allowing movement of TGase by SM-TAP processing or high salt concentrations. Our finding that active TGase is formed by surface colonies but cannot be extracted from the agar medium at low salt concentration would be consistent with such a model. Formation of TGase isoforms at distinct differentiation stages is being investigated.

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