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The transglutaminase activating metalloprotease inhibitor from *Streptomyces mobaraensis* is a glutamine and lysine donor substrate of the intrinsic transglutaminase

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Abstract Transglutaminase (TGase) from *Streptomyces mobaraensis* is an extra-cellular enzyme that cross-links proteins to high molecular weight aggregates. Screening for intrinsic substrates now revealed the dual *Streptomyces* subtilisin inhibitor-like inhibitor *Streptomyces* subtilisin and transglutaminase activating metalloprotease (TAMEP) inhibitor (SSTI), equally directed against subtilisin and the TGase activating metalloprotease TAMEP, is both a glutamine and a lysine donor protein. Reactivity of glutamines is lost during culture, most likely by TGase mediated deamidation, and, accordingly, cross-linking only occurred if SSTI from early cultures was used. Interestingly, release of buried *endo*-glutamines by the lipoamino acid *N*-lauroylsarcosine could restore SSTI reactivity. Formation of lipoamino acids by Streptomycetes suggests such compounds could also modulate in vivo TGase mediated SSTI cross-linking.

Keywords: *N*-Lauroylsarcosine; Protein cross-linking; Protein modulator; Subtilisin inhibitor; TAMEP inhibitor; Transglutaminase

1. Introduction

Transglutaminases (TGase, protein–glutamine: amine γ-glutamyltransferase, EC 2.3.2.13) are widely distributed in animals, plants and bacteria. They commonly catalyse the cross-linking of proteins to high molecular weight aggregates by transfer reaction between appropriate glutamine and lysine residues. While various biological functions of mammalian TGases are well established (for an overview see [1–3]), the physiological role of the extra-cellular transferase from *Streptomyces mobaraensis*, formerly termed *Streptoverticillium mobaraense*, is unknown despite its broad use in industrial processes [4]. Bacterial TGase differs from the eucaryotic counterparts in many respects. Lack of sequence homology and low molecular mass are primary features for it [5]. An activation peptide, otherwise only found in blood coagulation factor XIII, ensures a controllable processing [6]. Even the catalytic triad, composed of cysteine, histidine and aspartate, seems to be slightly modified, at least in the protein crystal [7]. The spatial arrangement suggests aspartate has taken over the role of histidine as proton acceptor, and histidine has only the function to direct the glutamine substrate to the nucleophilic cysteine. The most striking peculiarity compared with all other TGases, however, is the catalytic independence from activating Ca^{2+} making the microbial enzyme a valuable tool for technical applications [8].

We have examined the activation of microbial TGase by the metalloprotease transglutaminase activating metalloprotease (TAMEP) and a Ca^{2+} stimulated tripeptidylaminopeptidase (TAP) [9,10]. TAMEP is related to *Streptomyces griseus* metalloprotease SGMPII [11]. Previously, SGMPII or SgmA,[1] respectively, was shown to be involved in aerial mycelium formation and controlled by the transcriptional factor AdpA [12]. Transcription of *adpA* is triggered in turn by the A-factor (2-isocapryloyl-3*R*-hydroxymethyl-γ-butyrolactone), a *Streptomyces* hormone binding to the repressor protein ArpA and providing for its removal from the promoter of *adpA*. TAMEP cleaves a peptide bond within the activation peptide of the TGase precursor, thereby establishing full activity. Final removal of the remaining tetrapeptide is performed by TAP which is exported at the same time with TGase. TAMEP, and indirectly TGase as well, are inhibited by a 14 kDa protein *Streptomyces* subtilisin and TAMEP inhibitor (SSTI), a member of the large *Streptomyces* subtilisin inhibitor (SSI) family [9,13,14]. SSI are small proteins of remarkable resistance to high temperatures [15]. They possess antifungal and antiviral activities [16,17]. The TAMEP inhibitor SSTI belongs to SSI variants exhibiting binding sites for both serine and metalloproteases [18–20]. Moreover, it was recently found by two independent groups that transcription of SSI genes depends like SGMPII (SgmA) and other SSI-sensitive proteases on AdpA, also indicating the involvement of SSI in aerial mycelium formation [21,22].

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Abbreviations: LS, *N*-lauroylsarcosine; MBC, monobiotinylcadaverine; MDC, monodansylcadaverine; SIL, subtilisin inhibitor-like proteins; SSI, *Streptomyces* subtilisin inhibitor; SSTI, *Streptomyces* subtilisin and TAMEP inhibitor; TAMEP, transglutaminase activating metalloprotease; TAP, tripeptidylaminopeptidase; TGase, transglutaminase; ZQGB, 1-*N*-biotinyl-6-*N*′-(carbobenzoxy-L-glutaminylglycyl) hexane diamine; ZQGD, 1-*N*-(carbobenzoxy-L-glutaminylglycyl)-5-*N*′-(5′-*N*″,*N*″-dimethylaminonaphthalenesulfonyl)pentane diamine

[1]Despite divergent entry names in the SwissProt database, the sequences of both SGMPII (Q9R5Q0_STRGR) and SgmA (Q83WH1_STRGR) are identical.

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During our studies to address a physiological role of bacterial TGase, SSTI turned out to be both a glutamine and lysine donor substrate of the cross-linking enzyme. Such a result was unexpected since it has already been reported various SSI or subtilisin inhibitor-like proteins (SIL), among them SIL-V6 from a strain of *S. mobaraensis*, cannot be cross-linked by bacterial TGase [23]. Only formation of SIL-V6 β-casein conjugates, intensified by 10 mM DTT, was observed, not allowing the discrimination between both TGase accessible side-chains. Since sequence information is only available for SSTI [9], its identity with SIL-V6 remains in question as well. Our crucial discovery was the early modification of SSTI by TGase during submerged culture of *S. mobaraensis* resulted in an entire loss of accessible glutamines and the capability to be cross-linked. Partial compensation of disappeared reactive sites by *N*-lauroylsarcosine (LS) mediated exposure of buried glutamines gives rise to the assumption that lipoamino acids, structurally related to carboxamide side-chains, could act as TGase substrate modulators in vitro and in vivo.

2. Materials and methods

2.1. Materials

S. mobaraensis (strain 40847) was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen DSMZ (Braunschweig, Germany). Subtilisin Carlsberg from *Bacillus licheniformis* (EC 3.4.21.62), monodansylcadaverine (MDC) and anti-rabbit IgG-alkaline phosphatase antibodies were from Sigma (Deisenhofen, Germany), LS sodium from Merck (Darmstadt, Germany), avidin–alkaline phosphatase and monobiotinylcadaverine (MBC) from Pierce (Rockford, USA), and 1-*N*-biotinyl-6-*N*′-(carbobenzoxy-L-glutaminylglycyl)hexane diamine (ZQGB) from Zedira (Darmstadt, Germany). 1-*N*-(Carbobenzoxy-L-glutaminylglycyl)-5-*N*′-(5′-*N*″,*N*″-dimethylaminonaphthalene sulfonyl)pentane diamine (ZQGD) and transglutaminase from *S. mobaraensis* were prepared as described [9,24,25]. All other biochemicals were from Applichem (Darmstadt, Germany), Merck, Sigma or Bachem, respectively.

2.2. General procedures

Determination of protein content and TGase activity, SDS–PAGE and Western blotting using polyclonal antibodies raised against SSTI and proTGase were performed as described elsewhere [6].

2.3. Purification of SSTI

S. mobaraensis was cultured in a starch mineral salt medium at 28 °C for 43–93 h as described [6,9], and formed cell aggregates were removed by suction through a Buechner funnel. The filtrate was cooled immediately to 4 °C, mixed with 50 vol% pre-cooled ethanol (to precipitate transglutaminase as rapid as possible) and centrifuged. SSTI was then precipitated by increasing ethanol to 80% and separated by Fractogel EMD SO_3^- chromatography (18 ml bed volume, 1 ml/min flow rate) at pH 4.0 using 50 mM acetate buffer. Elution was performed by linear increasing NaCl of 0–1 M, and fractions, containing inhibitory activity against subtilisin, were combined, desalted and stored at −18 °C.

2.4. TGase mediated labelling

Four to five micrograms of SSTI, 0.13 mM ZQGB (2 mM MBC, 2 mM ZQGD, or 8 mM MDC, respectively) in 0.1 M HEPES, pH 7.5 were incubated with 0.9 μg bacterial TGase at 37 °C for 2 h (final volume of 30 μl). The reaction mixture was combined with 30 μl SDS application buffer and heated to 90 °C for 5 min. After SDS–PAGE (10 μl), labelled proteins were visualised by illumination at 365 nm (dansylated samples) and silver staining or semi-dry blotted (biotinylated samples) onto nitrocellulose membranes. Washing and blocking of unspecific binding sites using non-fat milk were performed as described [6]. The saturated membrane was then incubated over night with an avidin–alkaline phosphatase conjugate (50 ng/ml) at 4 °C. After three-fold washing using 10 mM Tris–HCl/150 mM NaCl/0.05% Tween20, pH 8.0 and 1 min equilibrating to pH 9.5 using 0.1 M Tris–HCl/0.1 M NaCl/5 mM $MgCl_2$, colour development was achieved using 51 μl 5-bromo-4-chloro-3-indolyl phosphate (50 mg/ml in DMF) and 66 μl nitro blue tetrazolium (75 mg/ml in 70% DMF) in 15 ml equilibration buffer. Gels and blots were photographed using a Biometra BioDocAnalyzer.

2.5. TGase mediated protein crosslinking

Cross-linking and labelling procedure were the same with the exception of absence or presence of labelling compounds. In brief, 3–4 μg SSTI in 0.1 M HEPES, pH 7.5 were allowed to react in the presence of 0.9 μg TGase at 37 °C. After separation by SDS–PAGE, the gel was silver-stained, or blotted and immuno-stained using SSTI antiserum, respectively, and photographed.

2.6. Measurement of protease activity

Two milligrams casein and 10 μg commercial subtilisin in 0.1 M Tris–HCl/2 mM $CaCl_2$, pH 7.5 were incubated at 37 °C for 10 min (final volume of 400 μl). If SSTI activity was measured, appropriate subtilisin inhibitor mixtures were pre-incubated in the same buffer for 30 min at ambient temperature. The reaction was terminated by addition of 600 μl trichloroacetic acid, and absorbance of the centrifuged supernatant was measured at 280 nm. One protease unit is defined as the release of 1 μmol tyrosine/min using the extinction coefficient of 1.219 ml μmol^{-1} cm^{-1}.

2.7. Isoelectric focusing

125 mm × 125 mm Servalyt precotes gels (Serva, Heidelberg, Germany) were used to determine SSTI pI. After equilibration using ampholyte mixtures pH 7–9 and pH 9–11, separation occurred for 3.5–4 h up to a final voltage of 2000 V (6 W maximum). Gels were silver-stained according to the manufacturer's protocol.

3. Results and discussion

3.1. SSTI from S. mobaraensis is a transglutaminase substrate

TGase mediated cross-linking of proteins occurs by acyl transfer of exposed glutamine residues onto ε-amino groups of appropriate *endo*-lysines resulting in the formation of N^{ε}-(γ-glutamyl)lysine isopeptide bonds. Glutamine donor proteins are usually determined by the incorporation of primary amines provided with a radioactive label, a fluorescent dye or biotin, respectively (Fig. 1A). The determination of reactive lysines requires labelled glutamine peptides consisting of two or more amino acids according to TGase specificity. The dipeptide Cbz-glutaminylglycin (ZQG) is well suited to attach to microbial TGase. Affinity is still enhanced by linkage of a label to the glycine carboxyl group (Fig. 1B) [24]. It should be remarked in addition that the glutamine and lysine probes act as both label and inhibitor of the enzymatic cross-linking reaction.

In this study, we used monodansylated and monobiotinylated cadaverine (MDC, MBC) as well as dansylated and biotinylated ZQG (ZQGD, ZQGB) to discover physiological substrates of TGase from *S. mobaraensis*. All attempts failed to label proteins of submerged cultures, even when cadaverine (1,5-diaminopentane) was present in the culture medium. Cadaverine is an inhibitor of protein cross-linking, and incorporation into available glutamine donor proteins by the exported TGase should enhance the number of primary amino groups in parallel. In an early phase of culture, the *endo*-protease inhibitor SSTI is secreted belonging to the *Streptomyces* subtilisin inhibitory (SSI) family. The subtilisin inhibitor, one of the most prominent proteins in culture broth, was determined to be directed against