A Novel Transglutaminase Substrate from *Streptomyces mobaraensis* Triggers Autolysis of Neutral Metalloproteases

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**Transglutaminase (TGase) from *Streptomyces mobaraensis* is a Ca\(^{2+}\) independent enzyme that cross-links proteins to high molecular weight aggregates. A disperse autolysis inducing protein (DAIP) was identified as an intrinsic TGase substrate exhibiting accessible glutamine and lysine residues. DAIP modification during culture by TGase resulted in deamidation of reactive cysteine and lysine residues, and failure of cross-linking. The reactivity of modified DAIP can be restored to some extent by Na-lauroylamido-3-N,N\(^{-}\)-dimethylpropyl amine, thus exposing concertedly buried glutamines and lysines. The novel TGase substrate differs considerably from the well known autolysis inducing protein (DAIP) was identified as an intrinsic TGase substrate exhibiting accessible glutamine and lysine residues. In vitro experiments have shown that TAMEP is inhibited by the heat-resistant TGase substrate SSTI, which belongs to the big family of Streptomyces subtilisin inhibitors (SSI). SSTI appears to be a multi-functional protein, most likely regulating TAMEP and, indirectly, TGase activity, and protecting proteins against proteolytic degradation and heat inactivation. Accordingly, SSTI might be an important factor maintaining integrity of aerial hyphae.

Key words: dispase autolysis inducing protein; lipopolyamine; *Streptomyces mobaraensis*; transglutaminase substrate; transglutaminase substrate modulator

Streptomyces are multi-cellular soil bacteria that exhibit a complex life cycle. When spores sprout and grow into a moist substrate, a multi-branched mycelium with regular septation or distinctive cell division is formed, resulting in a cotton-like network of filamentous hyphae. Development of an aerial mycelium from the surface of the vegetative body means leaving an "environment that protects against osmotic stress, dehydration, nutrient competitors, and predators (by antibiotic compounds released into the soil). An outer barrier, preventing loss of water and essential compounds from the aerial cell wall, is thought to be composed of hydrophobic proteins, such as SapB, chaplins and rodlins. By what means the protein coat is defended against proteolytic degradation remains unclear. Spores, arising from aerial hyphae by regular septation and cell-wall thickening, run greater risk being digested without a shelter in the outer protein envelope.

*Streptomyces mobaraensis* is known as the first organism to export a Ca\(^{2+}\) independent transglutaminase (TGase, EC 2.3.2.13). Two proteases, the activating metalloprotease TAMEP and a Ca\(^{2+}\) stimulated tripeptidylaminopeptidase, TAP, consecutively dissect the precursor protein to generate the mature enzyme. In vitro experiments have shown that TAMEP is inhibited by the heat-resistant TGase substrate SSTI, which belongs to the big family of Streptomyces subtilisin inhibitors (SSI). SSTI appears to be a multi-functional protein, most likely regulating TAMEP and, indirectly, TGase activity, and protecting proteins against proteolytic degradation and heat inactivation. Accordingly, SSTI might be an important factor maintaining integrity of aerial hyphae.

TGases commonly catalyse the cross-linking of proteins to highly polymerised aggregates. Initially, an acyl-enzyme-complex is formed between the active site cysteine and the \(\gamma\)-carboxamide group of protein-bound glutamines. Subsequent reaction with an appropriate lysine donor then provides for the formation of cross-linked proteins. TGase-mediated hydrolysis of reactive SSTI glutamines during submerged culture of *S. mobaraensis* resulted in the discovery that lipoamino acids such as Na-lauroylsarcosine can function as substrate modulators by expelling cryptic carboxamide groups from dipolar and hydrophobic interactions with the protein backbone. Lipoamino acids have been reported to be formed by Streptomyces when phosphates are exhausted. Hence, phosphate deficiency after onset of aerial hyphae suggests, that lipoamino acids support protein cross-linking by TGase in the cell wall of *S. mobaraensis*.

A dispase inactivating protein (DAIP) has been characterized that can act as an additional intrinsic TGase substrate via exposed glutamine and lysine residues. Like SSTI, glutamine reactivity was lost during submerged (artificial) culture of *S. mobaraensis*, resulting in the formation of glutamic/lysine residue...
Materials and Methods

Materials. Dispase I (Bacillus polymyxa) was from Roche Diagnostics (Mannheim, Germany). All other proteases and materials were from Sigma-Aldrich (St. Louis, 110), Bachem, (Bubendorf, Switzerland), Merck (Darmstadt, Germany) and Applichem (Darmstadt, Germany).

General procedures. Cultivation of S. mobaraensis DSM 40847, electrophoretic and blotting procedures, and transglutaminase and protein assays were performed as described elsewhere.5–7

Determination of protease activity and DAIP activity.

Casein degradation assay. 200 μl of alkali-soluble casein (10 mg/ml) and 200 μl of protease were incubated in 2 mM CaCl₂ and 0.1 mM Tris/HCl pH 7.5 at 37 °C for 10 min. For papain and bromelain, 0.1 mM citrate buffer, pH 6.5, was used. After the addition of 600 μl of 10% trichloroacetic acid the mixture was centrifuged, and the absorbance of the supernatant was monitored at 280 nm. One modified Anson unit (AU) was defined as the release of 1 μmol tyrosine per min. To estimate inactivation activity, protease was pre-incubated with culture supernatant or purified DAIP at an ambient temperature or 37 °C for 30 min, and the assay was continued as described above. One inactivation unit (IU) was defined as the reduction of 1 AU dispase.

Hydrolysis of Dabcyl-Ser-Phe-EDANS. The assay was performed as described previously.14 In brief, 15 μl of 1 mg/ml Dabcyl-Ser-Phe-EDANS in 475 μl of 100 mM Tris/HCl/2 mM CaCl₂ was incubated with 10 μl of 1 mg/ml dispase or thermolysin at 37 °C for 10 min. The reaction was stopped by the addition of 500 μl 20% EDTA, and emission (λex, 336 nm, λem 490 nm) was monitored using 1-cm cuvettes.

Purification of DAIP. Cultivation of S. mobaraensis was performed at 28 °C for 48–92 h, and cell aggregates were separated by suction through a Buechner funnel as rapidly as possible. The filtrate was mixed 1:1 with pre-cooled (−18 °C) ethanol at 4 °C, centrifuged at 10,000 g to remove TGase, and the ethanol of the supernatant was enhanced to 80% vol/vol. Precipitated proteins were separated at pH 4.0 using a 20-ml Fractogel EMD SO₃⁻ column (Merck), prior equilibration with 50 mM acetate buffer. After elution of non-binding proteins, chromatography was achieved by linear increasing gradient of 0–1 M NaCl. Fractions inhibiting dispase-mediated casein degradation were combined, dialyzed and lyophilized. For protein sequence analysis, tiny contaminations were removed by borate gel electrophoresis, as described elsewhere.5–7 Sequence analysis was performed by Edman degradation (Procise 494 Protein Sequencer, Applied Biosystems, Weiterstadt, Germany).

TGase mediated labelling. Labelling of DAIP was performed as described previously.8 In brief, 2.3 μM DAIP and 0.13 mM ZQGB (or 2 mM MBC) in 0.1% HEPES, pH 7.5, were incubated with 0.5 μM TGase from S. mobaraensis at 37 °C for 2 h (final volume, 30 μl). The reaction was terminated by heating to 90 °C in 30 μl of SDS buffer for 5 min. After SDS-PAGE and protein blotting, labelled proteins were visualized using avidin-alkaline phosphatase/5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium staining, as described elsewhere.3

GPC analysis. Size exclusion chromatography was performed using a 60-cm Superdex 75 column (Amersham Pharmacia, Freiburg, Germany) and 150 mM NaCl/2 mM CaCl₂ in 50 mM Tris/HCl pH 7.0 at a flow rate of 1 ml/min. Calibration proteins (S. cerevisiae alcohol dehydrogenase, 150 kDa; bovine serum albumine, 66 kDa; ovalbumine, 45 kDa; bovine carbonhydrase, 29 kDa; bovine cytochrome C, 12.4 kDa) were from Sigma.

Results

Discovery of the TGase substrate DAIP

S. mobaraensis took 25–30 h to produce significant inactivation activity against the neutral metalloprotease dispase from B. polymyxa. Highest activity was monitored after 3–4 d. In the best case, 30 μl of culture supernatant contained sufficient quantities to reduce the proteolytic activity of 5 μg of dispase (about 30 AU/mg) by half. Preliminary studies revealed a 37-kDa protein causing dispase to perform self-degradation. Accordingly, it was termed dispase autolysis inducing protein (DAIP). Further indications suggested that DAIP might be a TGase substrate. Investigations were performed by TGase-mediated incorporation of biotinylated compounds and avidin-alkaline phosphatase staining on NC membranes.8 Thus, mono-biotinylcadaverine (MBC, glutamine label) and 1-N-biotinyl-6-N-(carbobenzoxy-t-glutaminylglycyl)diamidohexane (ZQGB, lysine label) were allowed to react distinctively with TGase-accessible glutamines and lysines respectively. Labeling of DAIP succeeded only in a few cases, suggesting severe modification of the inactivating protein by TGase during the culture of S. mobaraensis. For that reason, DAIP was purified from supernatants of 48-h, 66-h, 73-h, and 92-h aged cultures to estimate the influence of the exported TGase and to obtain largely unmodified protein. It should be remembered that activation of TGase during submerged culture mainly occurs between 48 h and 64 h.3

Purification of DAIP

To avoid DAIP processing during purification, cell aggregates were removed as rapidly as possible by suction through a Buechner funnel. The cooled filtrate was mixed with the same volume of ethanol to precipitate TGase completely (Fig. 1). Enhancement of ethanol to 80 vol.% allowed DAIP to precipitate, and this was finally purified by Fractogel EMD SO₃⁻ chromatography at pH 4.0. DAIP was baseline separated from other contaminating proteins as the first protein at 0.35 M

![Fig. 1. Protein Patterns of DAIP Purified from 48-h Culture Supernatant of S. mobaraensis.](image-url)
NaCl. The combined fractions correspondingly showed a strong protein band at 37 kDa (Fig. 1). However, the most intriguing result was that DAIP from older cultures eluted earlier, as the increase in negative charge due to deamidation of glutamines would suggest (Fig. 2). The 66-h supernatant contained two isoforms, the smaller of which showed tiny residues of unmodified DAIP. Even the peak of the 48-h sample showed by a shoulder at the increasing flank that small amounts of DAIP were already modified (equally suggesting, that both isoforms caused the DAIP band in Fig. 1).

Under the artificial conditions in shaking flasks, *S. mobaraensis* exports large quantities, of at least 100 mg per liter DAIP, when loss during purification is not considered (Table 1). A 2.4-fold increase in DAIP activity revealed that an inhibitory factor of the culture supernatant was removed by ethanol precipitation. The final chromatography resulted in highly purified DAIP that had the capacity to diminish the dispase mediated release of 8.2 μmol of casein-bound tyrosine per min/mg (Fig. 1, Table 1).

Properties of DAIP

DAIP is a novel protein, as the N-terminal peptide ADSTSGXRAPSXTKVTGDGAVTF suggests. Alignment with known proteins failed. The apparent molecular mass of 37 kDa was equally obtained by SDS–PAGE and by GPC, signifying large differences from hitherto characterized *Streptomyces* inhibitors, such as the 14 kDa SSI and SMPI. Even the isoelectric point of 7.1–7.2 differed considerably from the SSI-like subtilisin and TAMEP inhibitory protein SSTI, with a pI of 9.0 (result not shown). SSI are heat-resistant proteins due to two cystine bridges, while the thermostability of DAIP was moderate (Fig. 3). Only short heating up to 60 °C was tolerated, and severe loss in activity occurred during prolonged incubation above 50 °C.

Labeling and cross-linking experiments

As mentioned above, DAIP from 3–4 d cultures lost as a rule the property of being labeled by TGase. Deamidation of exposed glutamines during culture of *S. mobaraensis* was the most probable event, since it was likewise observed for SSTI. To understand that processing, DAIP from 48-h to 92-h supernatants were examined for residual glutamines and lysines. As can be seen from Fig. 4A, the biotinylated glutamine marker MBC was incorporated into 48-h DAIP, while failure revealed the incapability of the other samples to form the primary acyl-enzymo-complex with TGase. MBC did not prevent completely DAIP dimerisation, as a weak band at 75 kDa displayed. It should be noticed in addition that TGase was stained as well, confirming former results. The most striking finding, however, was the vanished ability of DAIP lysines to link covalently to the biotinylated lysine marker ZQGB (Fig. 4B). The behavior of modified DAIP was in marked contrast to modified SSTI, which exhibited unimpaired lysines. Unaltered lysine labeling of modified SSTI samples gave rise to the conclusion that loss of glutamine residues must be the result of TGase mediated deamidation and not of intra-molecular cross-linking.

At first glance, intra-molecular cross-linking of DAIP during culture appears to explain the simultaneous disappearance of accessible glutamines and lysines. However, experiments with TGase substrate modulators clearly showed that intra-molecular cross-linking of DAIP during submerged culture of *S. mobaraensis* must be excluded (Fig. 4C, D). We have found evidence for the exposure of buried SSTI glutamines by the lip-oamin acid N-lauroylsarcosine (LS). The substrate modulator likewise has the potency to expose buried glutamines of modified DAIP. However, lysines of unmodified DAIP were compromised in the presence of LS, most likely by the formation of salt bridges (both results not shown). We concluded that a positively charged substrate modulator overcomes the drawback of LS to attract and mask the basic amino acid. Accordingly, additional labeling experiments were performed using commercially available acylpolypeptide N-lauroylamido-3'-N'N'-dimethylpropylamine (LPA). LPA, like LS, has the acylamido structure to replace ω-carboxyamide groups of buried substrate glutamines (Fig. 5). Bismethylation of the second amino function was important to prevent TGase-mediated incorporation of LPA into DAIP.

As has been shown with N-lauroylsarcosine, additional glutamines of modified DAIP were exposed by 1.8 mM LPA, resulting in covalent incorporation of the biotinylated amine MBC (Fig. 4C). Equally, LPA restored at least full lysine labeling capacity. This result provides clear evidence that intra-molecular cross-linking was not the reason of DAIP modification (Fig. 4D). Intensively colored bands of DAIP and TGase suggested...
that additional lysine residues were released by the substrate modulator. Forty-eight-hours DAIP was undoubtedly the best substrate. Due to more reactive side-chains, high-molecular-weight aggregates were formed despite the presence of competitive marker molecules (Fig. 4D, line 1). Finally, it should be mentioned that 48-h DAIP was equally polymerized by TGase in the absence of a substrate modulator, and, that up to 2 mM lipopolyamine had no significant effect on inactivation activity of DAIP (not shown).

### DAIP function

DAIP from *S. mobaraensis* has more the character of an enzyme (without being a protease) than that of an inhibitor, as explained below. Molar I:P ratios of 1:20 were sufficient to diminish dispase activity by half, and complete inactivation occurred at an I:P of 1:10 under the used conditions (Fig. 6, Table 2). Only thermolysin was likewise affected, even though at considerably higher DAIP concentrations. Intrinsic TAMEP from *S. mobaraensis*, microbial collagenase, serine-, and cysteine-proteases such as trypsin, chymotrypsin, papain, and bromelain were not sensitive.

These results prompted us to study the molecular interaction of dispase and DAIP by size-exclusion chromatography. As can be seen from Fig. 7, the 35-kDa dispase needed 78 min to migrate through a 60-cm Superdex 75 column, followed by autolysis fragments after 105–140 min. Separation of dispase/DAIP mixtures then revealed the absence of any high molecular weight aggregate, as would be expected for tight enzyme-inhibitor complexes. Rather, during migration through the column, the scarcely earlier eluting DAIP caused dispase to perform autolysis continuously. Accelerated self-degradation coincided with increased amounts of DAIP, indicated by the disappearance of dispase and the amplified emergence of smaller peptides. The inactivating protein remained unimpaired, as SDS–PAGE substantiated as well (Fig. 8, lanes 1 and 2). That DAIP was not a protease was shown by each control of the casein degradation assay and the resistance of hemoglobin, myoglobin, and myosin (results not shown). Not even mutual deletion of papain or trypsin, and DAIP was observed (Fig. 8, lanes 3–6). Only thermolysin attacked DAIP successfully, and both proteins disappeared after 30 min (Fig. 8, lanes 7 and 8). A faint band at 34 kDa indicated residues of the protein network of DAIP (2.3, microbial collagenase, serine-, and cysteine-proteases such as trypsin, chymotrypsin, papain, and bromelain were not sensitive.

### Table 1. Purification Protocol of DAIP from 48-h Culture Supernatant of *S. mobaraensis*

<table>
<thead>
<tr>
<th>Culture supernatant</th>
<th>Volume (ml)</th>
<th>Protein (mg/ml)</th>
<th>Total protein (mg)</th>
<th>Specific activitya (IU/ml)</th>
<th>Total activitya (IU)</th>
<th>Yield (%)</th>
<th>Purification factor</th>
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<tbody>
<tr>
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<td>4.63</td>
<td>431</td>
<td>0.45</td>
<td>0.098</td>
<td>42</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>20</td>
<td>3.39</td>
<td>68</td>
<td>5.05</td>
<td>1.49</td>
<td>101</td>
<td>240</td>
<td>15</td>
</tr>
<tr>
<td>Fractogel EMD SO4−</td>
<td>12</td>
<td>0.74</td>
<td>6.07</td>
<td>8.2</td>
<td>73</td>
<td>174</td>
<td>84</td>
</tr>
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aOne inactivation unit (IU) was defined as the amount of DAIP reducing the dispase release capacity by 1 μmol tyrosine per min at pH 7.5 at 37 °C on casein degradation assay.

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**Fig. 3.** Thermostability of Purified DAIP.

DAIP (30 μl, 0.3 mg/ml) and 155 μl of 2 mM CaCl₂ in 0.1 M Tris/ HCl pH 7.5 were heated to 20–90 °C for 5 min (○), 20 min (●), and 60 min (△). After further incubation with added 15 μl of dispase (0.3 mg/ml) for 30 min at an ambient temperature, residual protease activity was measured using the casein degradation assay, as described in “Materials and Methods.”

**Fig. 4.** TGase-Mediated Biotinylation of DAIP from *S. mobaraensis*.

DAIP (2.3 μM) from variously aged cultures was allowed to react in the presence of 0.5 μM TGase from *S. mobaraensis* with biotinylated lysine and glutamine equivalents MBC (A, C) and ZQGB (B, D) at 37 °C for 2 h as described in “Materials and Methods.” Mixtures of (C) and (D) contained 1.8 mM N-laurylamido-3-N,N′-dimethylpropylamine in addition. Proteins were separated by SDS–PAGE, blotted onto NC membranes, and visualised by avidin-alkaline phosphatase BCIP-NBT staining: Lanes 1–4, DAIP from 48-h, 66-h, 73-h and 92-h cultures; TG, used transglutaminase.

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**Fig. 5.** TGase-Mediated Biotinylation of DAIP from *S. mobaraensis*. Molar I:P ratios of 1:20 were sufficient to diminish dispase activity by half, and complete inactivation occurred at an I:P of 1:10 under the used conditions (Fig. 6, Table 2). Only thermolysin was likewise affected, even though at considerably higher DAIP concentrations. Intrinsic TAMEP from *S. mobaraensis*, microbial collagenase, serine-, and cysteine-proteases such as trypsin, chymotrypsin, papain, and bromelain were not sensitive.

These results prompted us to study the molecular interaction of dispase and DAIP by size-exclusion chromatography. As can be seen from Fig. 7, the 35-kDa dispase needed 78 min to migrate through a 60-cm Superdex 75 column, followed by autolysis fragments after 105–140 min. Separation of dispase/DAIP mixtures then revealed the absence of any high molecular weight aggregate, as would be expected for tight enzyme-inhibitor complexes. Rather, during migration through the column, the scarcely earlier eluting DAIP caused dispase to perform autolysis continuously. Accelerated self-degradation coincided with increased amounts of DAIP, indicated by the disappearance of dispase and the amplified emergence of smaller peptides. The inactivating protein remained unimpaired, as SDS–PAGE substantiated as well (Fig. 8, lanes 1 and 2). That DAIP was not a protease was shown by each control of the casein degradation assay and the resistance of hemoglobin, myoglobin, and myosin (results not shown). Not even mutual deletion of papain or trypsin, and DAIP was observed (Fig. 8, lanes 3–6). Only thermolysin attacked DAIP successfully, and both proteins disappeared after 30 min (Fig. 8, lanes 7 and 8). A faint band at 34 kDa indicated residues of the protein network of DAIP (2.3 μM) from *S. mobaraensis*, is objective of further studies.
Metalloproteases such as dispase and thermolysin can be inactivated by removal of essential metal ions. For instance, EDTA is known to trigger the autolysis of thermolysin.\textsuperscript{15) }EDTA inactivation of dispase was likewise revealed by GPC, supposing that DAIP is a metal binding protein. However, two observations were in marked contrast to any metal transfer: (i) metalloproteases, such as TAMEP and collagenase, maintained full activity in the presence of DAIP, and (ii) EDTA concentrations had to surmount DAIP by at least 4 orders of magnitude to achieve similar inactivation of dispase (results not shown). In order to obtain further evidence, DAIP-mediated dispase inactivation was studied in the presence of 0–10 mM Ca\textsuperscript{2+} (stabilizing thermolysin structure, see ref. \textsuperscript{15}) and 0–500 mM Zn\textsuperscript{2+} (essential for catalysis). For this purpose, DAIP was carefully purified from contaminating metals using 1,10-phenanthroline by the method of Holmquist and Vallee.\textsuperscript{16) }

As can be expected from activity measurements using 2 mM Ca\textsuperscript{2+} in buffer, the effect of Ca\textsuperscript{2+} on DAIP activity was negligible. The highest dispase activity was monitored at 2 mM Ca\textsuperscript{2+}, and autolysis of the enzyme in the

![Proposed Mechanism of N-Lauroylamido-3-N,N-dimethylpropylamine (LPA) Mediated Exposure of Protein-Bound Glutamines.](image1)

![Effect of Purified DAIP on Dispase Activity.](image2)

![Effects of DAIP on Metalloproteases](image3)

![Effects of DAIP on Various Endoproteases.](image4)

![Autolysis of Dispase in the Presence of DAIP.](image5)

![Effects of DAIP on Various Endoproteases.](image6)

![Effects of DAIP on Various Endoproteases.](image7)

![Effects of DAIP on Various Endoproteases.](image8)
presence of DAIP could not be encountered decisively by the stabilizing factor (not shown).

Next, DAIP was studied using 2 mM Ca\(^{2+}\) and Zn\(^{2+}\) in ranges of 100–500 \(\mu\)M, 20–100 \(\mu\)M, and 0–10 \(\mu\)M. While 500 \(\mu\)M Zn\(^{2+}\) diminished dispase activity by half, a continuous decrease in proteolytic activity was still observable in a range of 20–100 \(\mu\)M Zn\(^{2+}\). Only at 0–5 \(\mu\)M Zn\(^{2+}\), the inhibitory effect of the metal was absent or negligibly small (results not shown). Under these conditions, DAIP activity was clearly affected by small amounts of Zn\(^{2+}\), but only when low concentrations (1 \(\mu\)M < 0.1) were used in the assay (Fig. 9). In the experiment, Zn\(^{2+}\) and Ca\(^{2+}\) surmounted DAIP by 2 and 6 orders of magnitude respectively, and Zn\(^{2+}\) (at the most effective concentrations) dispase by a factor of 3–10. Accordingly, it is reasonable to assume that the metal binding sites of DAIP (if any) are saturated, and that Zn\(^{2+}\) stabilizes dispase by diminishing the apoenzyme concentration. Thus, reduction of apoenzyme most likely inhibits the DAIP-mediated formation of unpitigious, autolysis-susceptible conformations.

Partially unfolded, autolysis-prone protein structures have been reported as the beginning of thermolysin inactivation at elevated temperatures.\(^{[3]}\) The activation energy for thermolysin and dispase autolysis varies considerably, as a T50 shift of 27°C to lower temperatures indicates (Fig. 10). When DAIP was added to dispase in a molar ratio of 1:186, T50 was further reduced by 10°C, and initial proteolytic activity was not further achieved at 37°C. All indications suggest that DAIP diminishes, like an enzyme, the transition energy of dispase refolding, and supports by this means self-degradation of the enzyme.

**Discussion**

Streptomyces are multi-cellular soil bacteria comprising a large number of genetically and morphologically diverse variants. *S. coelicolor* A3(2) are commonly used to study the development of bacterial life, while other strains are more the focus of industry. *S. mobaraensis* is known to export a Ca\(^{2+}\) independent transglutaminase (TGase) that differs greatly from the eucaryotic counterparts. Considerable amounts in the culture broth of *S. mobaraensis* (>100 mg/l) suggest an important role of TGase during bacterial life. However, even 20 years after discovery,\(^{[4]}\) and despite broad industrial applications,\(^{[5]}\) the biological function of the enzyme has remained unclear.

Recently, we obtained the first indication that TGase is part of a defense system of *S. mobaraensis*.\(^{[6]}\) The inhibitory protein against subtilisin from *Bacillus* spp. and the intrinsic TGase activating metalloprotease TAMEP (SSTI) was found to be provided with sufficiently exposed glutamine and lysine residues, allowing SSTI cross-linking by TGase. Accordingly, TGase mediated incorporation into outer protein layers of bacterial hyphae can be regarded as an effective weapon against proteolytic degradation by predatory serine and metalloproteases.

A decisive breakthrough was the discovery that SSTI is deamidated in an early phase of submerged culture, just after the onset of TGase activation, and despite available lysine residues, enzymatic polymerisation of the modified protein failed. The lipoamino acid N-lauroylsarcosine (LS) restored SSTI cross-linking to some extent by exposure of additional, buried glutamines.

Such insights resulted in the discovery of the novel TGase substrate DAIP, which is much more suited to defend bacterial protein envelopes against potentially harmful metalloproteases, such as dispase and thermolysin, from *Bacillus polymyxa* and *thermoproteolyticus* rokko. The high affinity of TGase for DAIP can be deduced from the rapid hydrolysis of glutamine carboxamides during culture. The exposure of buried side-
chains by the lipoamino acid LS is in line with the behavior of the inhibitory substrate SSTI. In contrast, the formed glutamates of modified DAIP compromise strongly the reactivity of lysines by electrostatic attraction. That intra-molecular cross-linking can be excluded was shown by TGase mediated labelling and cross-linking in the presence of the novel substrate modulator N-laurolamido-3-N,N'-dimethylpropylamine (LPA). LPA releases buried glutamines and lysines of DAIP more effectively than LS.

DAIP has the unique biological function of inducing autolysis of neutral metalloproteases. Since the intrinsic TGase activating metalloprotease TAMEP, a member of the thermolysin (M4) family, is not affected, it is likely that the targets of DAIP are mainly of external origin. Inactivating activity is accompanied by remarkable resistance against various endoproteases. Only thermolysin was found to have access to vulnerable peptide bonds. DAIP is neither a protease (as the integrity of casein, hemoglobin, myoglobin, myosin, and most of the proteases used showed) nor an inhibitory protein like SSTI. GPC experiments failed to identify a DAIP dispase complex. Rather, total degradation of dispase occurred at 1 order of magnitude lower DAIP concentrations, and even in the presence of dispase stabilizing metals such as Ca$^{2+}$ and Zn$^{2+}$. The benefit of 1–5 μM Zn$^{2+}$ on dispase integrity is thought to be more an effect of the reduced apoenzyme concentration than of DAIP metal binding site saturation. Most likely, DAIP supports the formation of autolysis-prone conformations, which are postulated to be the beginning of thermolysin inactivation. 17)

DAIP is an antibiotic protein, as can be shown by the inhibited growth of moulds (not shown). The existence of reactive glutamines and lysines for specific TGase-mediated incorporation into protein layers gives rise to the assumption that the inactivating protein is, along with SSTI, part of the outer defense system of *S. mobaraensis*. Antibiotic substrate modulators, exhibiting the structural features of lipoamino acids and lipopolymamines such as LS and LPA, might assist in the construction of resistant, non-leaking composites by releasing additional side-chains from backbone attachments and sealing the protein layer. Lipoamino acids from Streptomycetes emerge when phosphates are exhausted, as mentioned above, 10,11 and various lipopolymamines have been determined to be antibiotic. 19,20 Moreover, a recently characterized penicillin-V-acylase from *S. mobaraensis* has the capability to catalyze the synthesis of lipoamino acids and lipopeptides. 21 Accordingly, it is highly probable that *S. mobaraensis* has developed a unique antimicrobial system consisting of transglutaminase, anti-proteolytic substrates and acylamido compounds to build up a tight protein coat protecting against degradation, leakage of essential substrates, dehydration, and thermal denaturation.

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**References**