Structure of the Dispase Autolysis Inducing Protein from *Streptomyces mobaraensis* and Glutamine Cross-linking Sites for Transglutaminase

David Fiebig§¶l, Stefan Schmelzǂ, Stephan Zindel§¶l, Vera Ehret§, Jan Beck§¶l, Aileen Ebenig§¶, Marina Ehret§, Sabrina Fröls║, Felicitas Pfeifer║, Harald Kolmar§, Hans-Lothar Fuchsbauer§*, Andrea Scrimaǂ*

From the ǂHelmholtz-Centre for Infection Research, Braunschweig, §the Department of Chemical Engineering and Biotechnology, University of Applied Sciences of Darmstadt and the ¶Department of Chemistry, ║Department of Biology, Technische Universität Darmstadt, Germany. l,*The authors equally contributed to the study.

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To whom correspondence should be addressed: Dr. Andrea Scrima, Helmholtz-Centre for Infection Research, Inhoffenstraße 7, 38124 Braunschweig, Telephone: +49 531 6181 7013; Fax: +49 531 6181 7099; E-mail: andrea.scrima@helmholtz-hzi.de and Prof. Hans-Lothar Fuchsbauer, Department of Chemical Engineering and Biotechnology, University of Applied Sciences of Darmstadt, Schnittspahnstraße 12, 64287 Darmstadt, Telephone: +49 6151-168203; Fax: +49 6151 168404; E-mail: hans-lothar.fuchsbauer@h-da.de

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**ABSTRACT**

Transglutaminase from *Streptomyces mobaraensis* (MTG) is an important enzyme for cross-linking and modifying proteins. An intrinsic substrate of MTG is the dispase autolysis inducing protein (DAIP). The amino acid sequence of DAIP contains five potential glutamines and ten lysines for MTG mediated cross-linking. Aim of the study was to determine the structure and glutamine cross-linking sites of the first physiological MTG substrate. A production procedure was established in *E. coli* BL21 (DE3) to obtain high yields of recombinant DAIP. DAIP variants were prepared by replacing four out of five glutamines for asparagines in various combinations via site-directed mutagenesis. Incorporation of biotin cadaverine revealed preference of MTG for the DAIP glutamines in the order of Q39>>Q298>Q345~Q65>>Q144. In the structure of DAIP the preferred glutamines do cluster at the top of the seven-bladed β-propeller. This suggests a targeted cross-linking of DAIP by MTG that may occur after self-assembly in the bacterial cell wall. Based on our biochemical and the structural data of the first physiological MTG substrate, we further provide novel insight into determinants of MTG-mediated modification, specificity and efficiency.

Microbial transglutaminase from streptomycetes (MTG, EC 2.3.2.13, P81453) is an industrial enzyme commonly used to modify food properties by protein cross-linking (1). More recently, biotechnological applications such as MTG mediated linkage of anti-cancer drugs to therapeutic antibodies or drug PEGylation are gaining in importance (2-5). Broad application of MTG can be explained by substantial differences in protein structure and specificity compared to other transglutaminases such as tissue transglutaminase (TG2) or blood coagulation factor XIII (6-8). Sequence homology...
is missing, and MTG is virtually the only transglutaminase being active in the absence of calcium ions. Unlike eukaryotic transglutaminases, the protein size is limited to the catalytic domain without any appendices such as β-sandwich and β-barrel domains. The active site of transglutaminases usually contains a catalytic triad consisting of cysteine, histidine and aspartate. In the disc-shaped MTG (PDB entry 1IU4), the distance between Cys64 and His274 is more remote than between Cys64 and Asp255 suggesting a modified proton transfer and catalysis mechanism (9). Moreover, MTG from Streptomyces mobaraensis contains an N-terminal pro-peptide, which is quite uncommon for the transglutaminase family but present in the blood coagulation factor XIII (10, 11). During culture of S. mobaraensis, pro-MTG is cleaved by at least two proteases, an activating metalloprotease of the thermolysin (M4) family and an Ala-Pro-specific tripeptidylaminopeptidase leading to the mature enzyme (12-15). Activation can also be performed by a series of other proteases such as dispase, proteinase K, bovine trypsin or chymotrypsin (11, 12, 16-18).

Protein modification mediated by transglutaminases starts via nucleophilic attack of the active site cysteine thiolate on the γ-carboxamide carbonyl carbons of the glutamine donor proteins resulting in the intermediary acyl-enzyme complex (7, 8). The enzyme may then be released in several ways (Fig. 1). If lysine donor proteins are available, cross-linking occurs by the formation of Nε-(γ-glutamyl)-L-lysine isopeptide bonds. Primary amines can replace lysine donor proteins, thus allowing the preparation of protein conjugates containing any synthetic payload. Even ω-hydroxyceramides are acyl acceptor molecules of mammalian TG1 (human TGK, P22735) and link skin proteins to the epidermal lipid envelope via ester bonds (19). Ultimately, the acyl-enzyme complex may be hydrolysed in the absence of primary amines resulting in the formation of deamidated proteins. In the case of S. mobaraensis, substrate hydrolysis or intramolecular cross-linking are the preferred reactions of MTG in submerged cultures since shearing forces most likely prevent the attachment of a lysine donor substrate to the acyl-enzyme intermediate (20).

The active site of MTG has been engineered to accelerate protein modification by improving specificity (21). Heat-resistant and heat-sensitive MTG variants were both obtained by random mutagenesis (22). A variety of approaches has been pursued to unravel binding characteristics of glutamine and lysine donors using inhibitory proteins from streptomyces (23), fluorescent derivatives of the artificial substrate Nε-benzoxycarbonyl-L-glutamylglycine (24) and diverse acyl acceptor molecules (25). The application of phage display and mRNA display techniques revealed glutamine consensus sequences for MTG that are quite different from those of the mammalian enzymes (26, 27). However, the MTG cross-linking sites in physiological substrates have not been determined, despite the fact that patterns of the tertiary structure can be more important for protein-protein interaction than the mere amino acid sequence (28).

We have purified and characterized several proteins from Streptomyces mobaraensis that are glutamine- and lysine-donor substrates of MTG (20, 29-31). The dispase autolysis inducing protein (DAIP, P84908) has a molecular mass of 37 kDa containing five glutamine and ten lysine residues (32). Substrates are metalloproteases of the thermolysin family M4 (P00800) that undergo rapid autolysis in the presence of DAIP. A mechanism to trigger self-degradation has not yet been published, but proteolytic activity was excluded, and influence of Ca²⁺ and Zn²⁺ on DAIP activity was negligibly small (29).

Aim of the present study was to determine the structure and glutamine cross-linking sites for the first physiological substrate of MTG and to contribute to more site-specific protein modification procedures. For this purpose, a production procedure for DAIP was established in E. coli. DAIP glutamines were replaced by asparagines through consecutive site-directed mutagenesis keeping one of the five available residues in place. Additionally, we succeeded in determining the seven bladed β-propeller structure of DAIP to 1.7 Å resolution and correlate the structural data with the mutagenesis data and efficiency of MTG mediated biotin cadaverine incorporation. Enzymatic linkage of biotin cadaverine to the DAIP variants preferentially occurs on glutamine cross-linking sites of the microbial transglutaminase that cluster on one side.
of the DAIP β-propeller structure, suggesting a site directed and orientation-specific cross-linking.

RESULTS AND DISCUSSION

Primary structure of the dispase autolysis inducing protein (DAIP)—DAIP was originally purified from culture broth of Streptomyces mobaraensis (29). Determination of the N-terminal amino acid sequence suggested a novel protein, since the sequence was not related to any protein characterized so far. Since attempts to identify the DAIP-encoding gene using an appropriate DNA probe failed, the entire genome sequence of S. mobaraensis was determined to obtain the full-length sequence (Fig. 2A). In parallel, the genome sequence of the same strain was published by Yang and others (32). Both genome sequences perfectly coincided and depicted the DAIP encoding gene without any deviation (not shown).

Figure 1

DAIP consists of 348 amino acids preceded by a 26mer signal peptide (Fig. 2A). Ala-Gln-Ala is most likely the binding site of the putative signal peptidase, which is in accordance with the experimentally determined N-terminal peptide sequence. The mature protein contains five glutamines and ten lysines. Consensus sequences for microbial transglutaminase (MTG) were not detectable in the potential glutamine and lysine cross-linking sites (Table 1). Moreover, amino acids, predicted by phage display and mRNA display libraries (26, 27), were not found at the proposed positions referring to the central glutamines Q39, Q65, Q144, Q298, and Q345. The DAIP motifs contain neither aromatic amino acids (26) nor arginines in position -3 (27). Proline (27) and arginine (26) are not preferred amino acids for position +1, and even double-Q pairs that have been found in random peptides as preferred substrate sites are missing (27). It thus seems plausible that the interaction of MTG and its physiological substrate DAIP is governed by yet unidentified properties directing efficient modification by MTG at the glutamine cross-linking sites.

Table 1

Production of ΔQ deficient DAIP variants—C-terminally His6-tagged rDAIP turned out to be unsuitable for the characterization of glutamine binding sites, since modification of rDAIP with a C-terminal His6-tag resulted in partial reduction in dispase-autolysis activity. We thus used untagged DAIP derivatives in order to avoid any impact of the tag on activity and MTG-mediated modification efficiency. Tag-free rDAIP was produced in E. coli using the optimized gene as described in the experimental section. The described procedure resulted in high yields of purified wt-rDAIP (Fig. 2B) with similar activity compared to DAIP from S. mobaraensis (Sm-Q5), suggesting that the overall conformation was not altered (Table 2).

Figure 2 and Table 2

Next, four out of the five DAIP glutamines were substituted by asparagines via site directed mutagenesis to yield Q1 variants containing only a single glutamine residue. The ΔQ deficient proteins are denoted as Q39, Q65, Q144, Q298, and Q345. Besides wt-rDAIP, still possessing five glutamines (Q5), a null-Q variant (Q0) was generated as control. All productions resulted in considerably lower but sufficient amounts of modified rDAIP to perform the experiments described below (Table 2).

Functional conformation of the ΔQ deficient proteins was verified by DAIP activity. All tag-free variants destroyed dispase as fast as wt-DAIP (Table 2).

Alkylation of microbial transglutaminase—Access of microbial transglutaminase to the still available glutamines of modified rDAIP was examined by labeling and blotting procedures (20, 29). However, MTG and DAIP are comparable in size, and the enzyme MTG is capable of autocatalytic incorporation of lysine equivalents such as (mono)biotin cadaverine (MBC). To distinguish unambiguously between biotinylated glutamine-deficient DAIP variants and the modifying enzyme, masking of MTG glutamines was essential. Carboxamide transamidation was the most appropriate method since transglutaminases are thought not to react with alkylated glutamines (8). The MTG mediated self-modification was
studied by incorporation of several alkyl amines into the accessible glutamines. Amphiphilic N-lauroylsarcosine (LS) was added to enhance efficiency of MTG alkylation (20). After incubation for several hours and dialysis against water to remove excess of alkyl amines and LS, MTG activity was comparable to non-alkylated MTG (Fig. S2A). In contrast, self-biotinylation of alkylated MTG was considerably impaired as indicated by significantly weakened bands (Fig. S2B). Since impact on self-biotinylation activity was comparable between the different alkylated MTG, methylated MTG (hereafter referred to as MTGmet) was used for subsequent labeling reactions.

**MTG specific glutamine cross-linking sites**—Sorting of DAIP glutamines was carried out by MTGmet mediated incorporation of biotin cadaverine (MBC) into tag-free ΔQ deficient variants. Labeling mixtures were separated by electrophoresis, blotted, if necessary, and stained by a fluorescent streptavidin conjugate, Coomassie and polyclonal antibodies as described in the experimental section.

The Q39, Q298 and Q345 variants showed intensively stained biotinylated proteins, identifying these glutamine residues as preferred cross-linking sites of MTG (Fig. 2C). Moreover, it became obvious that MTG has access to Q65 and Q144 as well, at least under the conditions used. In contrast, the null-Q variant could not be detected by the fluorescent probe, excluding unspecific incorporation of MBC.

**Labeling kinetics**—The tag-free DAIP variants were also studied to determine semi-quantitatively the rate of MBC incorporation by MTG at pH 8.0. Highly purified DAIP from *S. mobaraensis* (Sm-Q5), the null-Q variant (Q0), and a β-glucosidase (βGlc) from almonds were used as controls. Reaction mixtures contained 8 µM DAIP and 0.2 mM MBC. The reaction was started by the addition of 0.5 µM MTGmet, and various samples were taken in the first hour and after incubation overnight (Fig. 2D). Constantly increasing fluorescent bands, already indicated after three minutes, showed that Q39 was biotinylated almost as fast as the wildtype protein Sm-Q5. In contrast, significantly stained protein bands of Q298 and Q345 only emerged when the incubation mixtures were allowed to react for at least 30 minutes. MTG mediated linkage of the biotinylated amine to Q65 and Q144 obviously occurred with even lower velocity, and the controls Q0 and βGlc clearly revealed absence of any cross-linking site for bacterial transglutaminase. The latter enzyme was not labeled by MTG despite of 14 glutamines, of which at least 10 are solvent accessible in a predicted structure model based on the structure of the related β-glucosidase from *Oryza sativa* (Fig. 3A,B). Weak bands of the control proteins became only visible after incubation overnight.

**Figure 3**

There was a linear increase in the degree of labeling over time as the emission intensity of the fluorescent streptavidin conjugate suggested (Fig. 2E). The highest biotinylation rate was monitored for DAIP from *S. mobaraensis* (Sm-Q5), corresponding with an increase in fluorescence of 278 rfu/min after protein blotting and streptavidin conjugate treatment. Enzymatic linkage of MBC to the DAIP variants was slower but Q39 was the best cross-linking site by far. The labeling rate of Q39 was equivalent with a fluorescence increase of 159 rfu/min and reached about 57 percent of that of Sm-Q5. Assuming similar protein blotting and biotin orientation, preference of MTG for the DAIP glutamines was in the order of Q39 (159 rfu/min, 57.2 %) >> Q298 (48 rfu/min, 17.5 %) > Q345 (33 rfu/min, 12.0 %) ~ Q65 (33 rfu/min, 11.8 %) >> Q144 (5 rfu/min, 1.8 %).

**Molecular structure of DAIP**—To gain more insights into the overall structure, the location of glutamines and lysines in DAIP, as well as to identify potential structural properties governing efficient modification by MTG, we determined the crystal structure of DAIP to 1.7 Å resolution by X-ray crystallography (Fig. 4 and Table S2). The structure was solved by Yb-SAD phasing and comprises two molecules in the asymmetric unit with residues 2 to 346 built from a total of 348 residues, with a cis-peptide bond between Ser40 and Val41. The structure of DAIP shows the classical seven bladed β-propeller (Fig. 4A). In contrast to the blades I-VI, which contain four sequential β-strands A-D, the last blade VII is composed of two β-strands derived from the C-terminus (A-B, aa 326-340) and one from the N-
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terminus (C, aa 20-25). These two elements from both the N- and C-terminus interlock and form blade VII, thus closing and stabilizing the ring-shaped propeller structure. The DAIP structure is further stabilized by two cysteines, Cys12 and Cys72, that form a single disulfide bridge covalently linking the N-terminal tail at position 12 to the β-turn between C1 and D1 (aa 68-74) of the first propeller blade. Four elongated loops (DIV-AV, BCV, BCVI and CVII-AI) are found on the top of the propeller (Fig. 4B), two of which, namely CVII-AI and BCVI, contain Q39 and Q298, respectively.

The structure provides first insights into the orientation of the microbial transglutaminase (MTG) binding sites. Interestingly, four out of the five glutamines are located on the same side in close proximity (Fig. 4A and 4E). The ten lysines of DAIP are instead scattered over the whole surface of the propeller without showing a preferred surface location. Glutamines are in general well resolved in the 2Fo-Fc density, except for Q345, which shows weaker electron density for the side chain, likely due to the intrinsic flexibility of the C-terminal tail of DAIP (Fig. 4C). In contrast to the solvent exposed and accessible residues Q39, Q298 and Q345, the two glutamines Q65 and Q144 are found in β-strands C1 and CIII, respectively, and are less solvent accessible (Fig. 4E).

**Figure 4**

**DAIP structure vs kinetic data**—The kinetic results largely correlate with the accessibility of glutamine positions found in the DAIP structure. The two fastest modified glutamines, namely Q39 and Q298, are presented on loops CVII-AI and BCVI, respectively. Those are located on top of the seven bladed β-propeller and are the most solvent accessible glutamines (Fig. 4C-E). However, Q345 is also solvent accessible and presented on the C-terminal loop, but modification efficiency by MTG is at comparably low level as for the buried Q65. The least efficiently modified substrate, Q144, is on the opposite side of the propeller in reference to the four clustered glutamines. In search for structural determinants for specificity and modification efficiency, we identified Q65 and Q144 to be located on β-strands and hence to be less solvent accessible, whereas Q39, Q298 and Q345 are located in loop regions and solvent exposed. The location in β-strands and consequently the reduced flexibility and accessibility are likely to be partially responsible for the low labeling efficiency. As previously shown, the active site residues of MTG are located in a cleft that can be accessed from two sides, of which the front vestibule, which is in close proximity to the catalytic cysteine, is mainly hydrophobic with patches of weak positive charge, whereas the back vestibule is strongly negatively charged (Fig. 5). The position of the catalytic cysteine residue and charge distribution in the front vestibule suggests an accommodation of the acyl donor (glutamine substrate), while the negative charge and presence of the catalytic His/Asp suggests accommodation of the positively charged acyl acceptor (lysine substrate), resulting in the formation of a ternary complex (9). Thus, in line with the previous suggestion by Kashiwagi and coworkers, we observe that, compared to substrate glutamines in β-strands, glutamines in an accessible and flexible loop are more efficiently modified, which are likely to be more easily accommodated in the cleft to reach the active site cysteine.

**Figure 5**

However, since DAIP is a purely β-strand protein, we need structural and biochemical characterization of substrates with glutamines in α-helical secondary structure elements to analyze the impact of helical structures on MTG mediated modification. In order to identify additional structural features that may guide efficient modification of Q39 and Q298 in particular, but also Q345, we generated a structural overlay of the three glutamines present in loop regions using the main chain and Cβ-atoms (Fig. 4D). By comparing the conformation of the glutamines and the two residues flanking both sides, we identified similarities in the overall conformation of the Q39 and Q345. However, the conformation of Q298, was significantly different, while modification efficiency was shown to be between the one for Q39 and Q345. This suggests that flexibility and accessibility are a prerequisite for efficient modification, but are modulated by additional properties of the substrate sites. We thus analyzed and compared the sequence and amino acid...
composition (+/- 5 amino acids) of the glutamine sites surrounding Q39, Q298 and Q345 (Table 1 and Fig. 4F). Q39 is mainly flanked by small polar or hydrophobic residues (with the exception of one aromatic residue at position +4), lacking charged residues. Q298 is flanked by a total of three aromatic/bulky residues, whereas Q345 is preceded by two negatively charged glutamic acids. In agreement with compatibility to the mostly hydrophobic front vestibule, the Q39 site with mainly small polar or hydrophobic residues (lacking charged residues) is most efficiently modified, whereas acidic, or in general charged residues are apparently the least favorable. The presence of bulky/aromatic residues in close proximity does reduce the efficiency compared to small polar or hydrophobic residues, but seems to be more favorable than the presence of charged residues. This is also in line with the inefficient modification of β-glucosidase, where glutamines located in predicted loop regions are flanked by aromatic/bulky and/or charged amino acids (Fig. 3C). Two DAIP variants further supported our conclusions. To mimic the N-sided flank of Q345 and Q298, G36/T37 and T37/L38 of Q39 (Q1 variant) were replaced for two glutamates or tyrosine/phenylalanine, respectively. While insertion of the negative charges reduced the biotinylation rate by 97 percent, the effect of the bulky/aromatic amino acids was slightly smaller, with reduction in velocity by 85 percent compared with the unmodified Q39 variant (Fig. 2D and 2E). Interestingly, we were able to use the native MTG recognition site Q298 positioned in the β-hairpin for the design of an engineered MTG-tag to efficiently conjugate a reporter cargo onto a therapeutic monoclonal antibody (2). In this study, the β-hairpin was mimicked by the use of a disulfide bridge, which significantly improved modification efficiency by MTG compared to the variant lacking the disulfide bridge. In our biochemical data we observe a 3-fold higher cross-linking activity for Q39 compared to Q298, suggesting that the use of the Q39 sequence in combination with the β-hairpin structure might further improve efficiency of that system.

Conclusions-In this work, recombinant production of the dispase autolysis inducing protein (DAIP) and its glutamine-deficient variants allowed the determination of the first natural glutamine-binding sites for microbial transglutaminase (MTG) from Streptomyces mobaraensis. The sequence motifs containing Q39, Q298, and Q345 clearly differed from those predicted by phage display and mRNA display peptide libraries (26, 27). Specifically, aromatic amino acids (position -3), arginines (-3, +1), and proline (+1) are not available in the DAIP glutamine motifs. Q pairs are also missing. Thus, small peptides appear to reflect the properties of natural MTG modification sites insufficiently, suggesting that for physiological substrates such as DAIP, approach of MTG to glutamine sites must be completely different. Therefore, in addition to the experimental determination of natural glutamine-binding sites, we determined the seven bladed β-propeller structure of the first physiological MTG substrate DAIP to 1.7 Å resolution by X-ray crystallography. Interestingly, four glutamines, which include the three efficiently modified glutamines, cluster on one side of the propeller, giving rise to the notion that cross-linking of DAIP occurs in a site directed and orientation specific manner. We further provide evidence that solvent accessibility and location outside of β-strands is a prerequisite and main determinant for efficient modification of glutamines in DAIP. Combining the structural data with the determined modification efficiencies, sequence of DAIP substrate glutamine sites and data from mutagenesis experiments, we propose that the properties of amino acids flanking the Q-sites favour efficient modification in the following order: small/polar/hydrophobic/uncharged > bulky/aromatic > charged. However, additional structural and biochemical experiments are needed in the future to identify more natural MTG-sites and their properties in order to further validate and improve our current model regarding the influence of amino acid composition of physiological glutamine sites on MTG-mediated modification efficiency.

EXPERIMENTAL PROCEDURES

Materials—Microbial transglutaminase (MTG) from Streptomyces mobaraensis, strain 40847 (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany), and 2-N-(4-[4’-N,N’-dimethylamino)phenylazo]benzoyl-L-serinyl-L-phenylalamylamido)-N’'-ethylamino...
naphthalene-5-sulfonic acid (Dabcyl-Ser-Phe-EDANS, dabSFans) were prepared as published (33, 34). The sequence determination of the genome of *Streptomyces mobaraensis* 40847 was performed by LGC Genomics (Berlin, Germany). (Mono)biotin cadaverine (5-N-biotinamido pentyl amine, MBC), streptavidin-alkaline phosphatase conjugates, and dispase were purchased from Zedira (Darmstadt, Germany), Sigma (Deisenhofen, Germany), and Worthington (Lakewood, NJ, USA), respectively.

**Codon optimization, plasmids, cloning, transformation and culture**—The *Streptomyces* gene encoding mature DAIP was adapted to *E. coli* codon usage by GenScript (Hongkong, China) to improve gene translation (Fig. S1). The sequence encoding daip<sub>opt</sub> was obtained by *Neol* and *XhoI* hydrolysis of the shuttle vector. The purified fragment was then ligated into plasmid pET22b(+), and chemically competent *E. coli* XL1 blue cells were transformed with the resulting vector construct. Positive clones containing daip<sub>opt</sub> were identified by colony PCR and sequence analysis. For the production of recombinant rDAIP as fusion protein, the coding sequence was cloned via the restriction sites *NdeI* and *XhoI* into pET21a-CPD-His<sub>10</sub> bearing the His-tagged cysteine protease domain of *V. cholerae* (35). *E. coli* BL21 (DE3) pLysS were transformed with the construct using the heat-shock procedure. The cells were cultured at 37 °C overnight. Production was carried out overnight using 1 mM IPTG in 1 L LB/SBamp at 28 °C. Utilization of a C-terminal His<sub>10</sub>-tag allowed purification by immobilized metal ion affinity chromatography (IMAC).

**Site directed mutagenesis**—Mutagenesis was performed by PCR using the respective primer pairs (Table S1), daip<sub>opt</sub> in pET-22b(+), and Pfu DNA polymerase (Thermo Scientific) or Q5-HF DNA polymerase (New England Biolabs). Upon digestion of parental DNA by *DpnI* for 1 h at 37 °C, *E. coli* XL1 blue cells were transformed with the resulting constructs daip<sub>opt</sub> in pET21a-CPD-His<sub>10</sub>. Successful mutagenesis was carried out until four out of five glutamine codons were replaced by asparagine codons. A control plasmid contained daip<sub>opt</sub> without any residual glutamine codon (Q<sub>0</sub>).

**Purification of rDAIP and variants**—Induced *E. coli* BL21(DE3) pLysS cells containing rDAIP were precipitated by centrifugation (17,000 *g*, 15 min, 4 °C) and lysed by sonication or by high pressure homogenization (2.72 kbar). Cellular debris was removed by centrifugation (20,000 *g*, 30 min, 4 °C). Purification was performed by immobilized metal ion affinity chromatography using Chelating Sepharose Fast Flow (CV: 7 ml), 15 mM imidazole in 50 mM Tris/HCl pH 8.0 with 300 mM NaCl. For the purification of the tag-free variants, elution of the cleaved target protein was performed after incubation for 60-90 min with 50 μM phytic acid and subsequent release of CPD by a linear imidazole gradient of 15-500 mM. Fractions were separated by 12.5 % SDS polyacrylamide gel electrophoresis and Coomassie-stained. The combined IMAC fractions were purified by Fractogel EMD SO<sub>3</sub>ᵀ, phenylsepharose, and size exclusion chromatographies.

**DAIP activity measurements**—The activity of DAIP was determined indirectly using several protease assays (29, 34). Upon pre-incubation with dispase, residual protease activity was monitored by hydrolysis of casein, azocasein or dabSFans. In brief, DAIP (0-330 nM), dispase (330 nM) and 2 mM CaCl<sub>2</sub> in 200 μl 100 mM Tris/HCl pH 7.5 were incubated for 30 min at 37 °C before 200 μl alkali-soluble casein or azocasein (1 % w/v each) was added. Reaction was continued for 10 min and terminated by the addition of 600 μl 10 % trichloroacetic acid. Upon centrifugation, supernatants were used to monitor the absorbance at 280 nm (casein) or 440 nm (azocasein). Mixtures without DAIP (100 % dispase activity) and without dispase and DAIP (0 % dispase activity) were used as controls.

For continuously running measurements, DAIP (0-200 nM), dispase (100-200 nM) and 2 mM CaCl<sub>2</sub> in 80 μl 50 mM Tris/HCl pH 7.5 were pre-incubated for 30 min at 37 °C before 80 μl substrate solution containing 86.6 μM dabSFans was added. The increase in fluorescence was continuously monitored at 535 nm (λ<sub>exc</sub> of 340 nm) using a Genios SpectraFluor plus fluorimeter (Tecan) or Polarstar multi-mode microplate reader (BMG Labtech). The data, obtained in triplicate, were evaluated by weighted least square minimization using GraphPad Prism.

**Alkylation of microbial transglutaminase**—Purified transglutaminase from *S. mobaraensis* (10.3 μM), 1 mM N-lauroylsarcosine, 500 μM...
methylamine in ethanol (or ethylamine and ethanolamine) in 50 mM HEPES pH 8 were allowed to react up to 17 h at 37 °C under shaking (300 rpm). Reagents were removed by dialysis against water.

MTG mediated labeling procedure and kinetics—Labeling of DAIP was performed according to ref. 20 with minor modifications. DAIP or its variants (8 μM) and 0.2 mM MBC in 100 mM HEPES pH 8 were incubated with 0.5 μM MTG at 37 °C for up to 16 h (Fig. 2C) or the indicated time (Fig. 2D). DAIP without MBC or MTG were the controls. The reaction mixtures were separated by SDS-PAGE (12.5 %) and, where applicable, blotted onto PVDF membranes for DAIP detection and biotin signaling. Coomassie-blue (Coomassie), or primary polyclonal rabbit antibodies against DAIP, secondary goat IRDye® 680LT anti-rabbit IgG (DAIP-IgG) were used for the detection of protein in general or DAIP. The degree of biotinylation (Biotin) was determined using fluorescent IRDye® 800CW Streptavidin conjugates (33 ng/mL) in combination with the Odyssey® Sa Infrared Imaging System (LI-COR Biotechnology, Bad Homburg, Germany). Fluorescence was monitored at 693 nm (λexc of 676) and 789 nm (λexc of 774 nm) for IRDye® 680LT (DAIP-IgG) and IRDye® 800CW (Biotin), respectively. Equal areas of the fluorescent protein bands were integrated to evaluate biotinylation rates, expressed in terms of rfu/min, and corrected by the amount of unspecific streptavidin binding using the Q0 variant as reference. Rates for mutants Q39-GT36/37EE and Q39-TL37/38YF were normalized using the data for Q39.

Crystallization and data processing—Purified protein from S. mobaraensis culture broth (as described in ref. 29) was used for crystallization. DAIP crystals grew in 15 % (v/v) glycerol, 8.5 % (v/v) 2-propanol, 85 mM HEPES pH 7.5 and 17 % (w/v) PEG 4k in drops with equal volume of precipitant and protein solution at a concentration of 10 mg/mL. The crystals reached full size (~120x130x100 μm) after one week at 20 °C. Crystals were soaked with 100 mM Yb-DOTA according to the manufacturer’s recommendations (Jena Biosciences) and cryo-protected using mother liquor. Yb-SAD data set was collected at a wavelength of 1.386 Å on a PILATUS 6M detector at BL14.1 (HZB-BESSYII). Data reduction and scaling was carried out with the XDS package (36). Phasing and initial model building was done in HKL2MAP (37). Coot (38) with iterative refinement cycles in Phenix.refine (39) has been used for manual model building. Figures were created using PyMol (https://www.pymol.org/).

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CONFLICT OF INTEREST
The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS
Conceived and designed the experiments: DF, SS, SZ, SF FP, HK, HLF, AS. Performed the experiments: DF, SS, VE, JB, AE, ME. Analyzed the data: DF, SS, SZ, SF, FP, HK, HLF, AS. Contributed reagents/materials/analysis tools: FP, HLF, AS. Wrote the paper: DF, SS, HLF, AS. All authors approved the final version of the manuscript.

DATA DEPOSITION
The atomic coordinates and structure factors (code: 5FZP) have been deposited in the Protein Data Bank (http://www.pdb.org/).
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FOOTNOTES
The abbreviations used are: βGlc, Beta-Glucosidase; dabSFans, 2-N-(4-[4′-N′,N′-dimethylamino)phenylazo]benzoyl-L-serinyl-L-phenylalanylamido)-N′'-ethylaminonaphthalene-5-sulfonic acid; DAIP, dispase autolysis inducing protein; MBC, (mono)biotin cadaverine; MTG, microbial transglutaminase from *Streptomyces mobaraensis*.
FIGURE LEGENDS

FIGURE 1. Modification of the dispase autolysis inducing protein (DAIP) mediated by microbial transglutaminase from Streptomyces mobaraensis. A: Formation of the primary acyl enzyme complex. B: Products resulting from cross-linking, side-chain hydrolysis (in the absence of primary amines), and incorporation of biotin cadaverine.

FIGURE 2. Q39, Q298 and Q345 are preferred substrates of MTG. A: Sequence of the dispase autolysis inducing protein. Amino acids determined by Edman degradation (29) or mass spectrometry are underlined. Cysteines and potential binding sites of microbial transglutaminase are highlighted in green, orange (glutamines) and blue (lysines). B: Protein patterns of the tag-free dispase autolysis inducing protein. Lanes: M, molecular mass marker mixture; lane 1, applied supernatant; lane 2, flow-through; lane 3, IMAC fraction containing rDAIP; lane 4, CPD; lane 5, SEC fraction containing rDAIP. C: Transglutaminase mediated biotinylation of tag-free glutamine-deficient DAIP. D: Biotinylation rate of tag-free DAIP mediated by microbial transglutaminase. E: Increase in fluorescence mediated by enzymatic biotinylation of tag-free glutamine-deficient rDAIP variants: ● Sm-Q5; ● Q39; ● Q298; ● Q345; ● Q65; ● Q144; + Q39-EE (GT36/37EE); + Q39-YF (TL37/38YF).

FIGURE 3. β-glucosidase is not efficiently modified by MTG despite of solvent accessible glutamines. A/B: A model of the β-glucosidase from almonds (Prunus dulcis, Uniprot-ID: H9ZGE0) (prediction by Phyre2, Model PDB: 3PTK 62 % sequence identity of 478 out of 544 aligned residues). β-glucosidase is colored in light teal in cartoon mode. 13 out of 15 glutamines (Q4 is located in the signal peptide and is therefore removed from the mature protein; Q543 is not included in the Phyre2 model) are highlighted in orange, of which at least 10 are predicted to be solvent accessible. N- and C-termini are highlighted with a blue and red sphere, respectively. C: Flanking residues of the 14 glutamines of β-glucosidase. Aromatic/bulky residues are colored yellow, positively and negatively charged residues in blue and red, respectively. The only glutamine that partially meets our postulated requirements for MTG modification is Q272, which however still contains a bulky/aromatic amino acid at position -1 adjacent to Q.

FIGURE 4. The crystal structure of DAIP reveals a seven-bladed β-propeller fold and provides insights into structural features of efficient MTG substrate sites. A: Top view of the seven bladed β-propeller in cartoon mode. Blades I to VI are formed by four β-strands A-D. Blade VII is formed by two β-strands from the C-terminus (A-B) and one from the N-terminus (C). Lysines (yellow) and glutamines (orange) are shown in stick representation. N- and C-termini of DAIP are highlighted with blue and red spheres, respectively. B: Side view of DAIP (rotated by 70° related to panel A). Four large loops are highlighted in purple. Loop CVI-AI and BCVI contain Q39 and Q298, respectively. N- and C-terminal loops are highlighted in blue and red, respectively. C: Close up on the five glutamines (sorted by substrate preference for MTG). Refined 2Fo-Fc map with contour level of 1.0 σ is displayed in grey for each glutamine. The MTG substrate preference is indicated by green to red colour gradient on top of the close ups. D: Superposition of Q39, Q298 and Q345 loop region based on the respective glutamine backbone.
E: Cartoon and surface representation of DAIP (grey), showing the clustering and solvent accessibility of DAIP glutamines (orange). While Q39, Q298 and Q345 are exposed to solvent, Q65 and Q144 are buried, and less accessible.

FIGURE 5. Glutamine binding in the front vestibule of MTG is likely to be controlled by flexibility and amino acid composition of the substrate sequence. A-C: Electrostatic surface representation of transglutaminase from *Streptomyces mobaraensis* (PDB: 1IU4). The front vestibule (black triangle) carries a weak positively charged patch, which is likely to bind the acyl donor (glutamine substrate). The back vestibule (white triangle) is strongly negatively charged and is likely to accommodate the positively charged acyl acceptor (lysine substrate). D: Catalytic triad residues (Cys64, Asp255, His274) positions are superposed in the close up. The front vestibule is narrowed towards the active site cysteine, suggesting that substrate glutamines in flexible loops are more easily accommodated and reach the active site cysteine for efficient modification. In addition to flexibility, small/polar/hydrophobic/uncharged residues are preferred for efficient modification, whereas bulky/aromatic and/or charged residues are likely to interfere with accommodation of the substrate glutamine in the cleft, resulting in inefficient MTG-mediated modification.
### TABLES

**Table 1**

**Amino acids flanking glutamine and lysine residues of the dispase autolysis inducing protein**

| Glutamine sequences | Lysine sequences |
|---------------------|------------------|
| -5 -1 +1 +5         | -5 -1 +1 +5      |
| TTGTL Q39 SVSYT     | APSCT K14 VTGDG  |
| HNDEL Q65 RSTDA     | GCTWT K76 VATLG  |
| AGSDG Q144 LYDST    | AFAWE K100 NGGYL |
| YGTYF Q298 AYGTD    | GRTVT K115 LSSPS |
| GLEEV Q345 IHH--    | GVGD K130 ARRDH  |
|                     | TGKVG K316 THNAH |
Table 2

Yield and activity of tag-free DAIP variants with replaced glutamines

| DAIP variant   | Yield a) (mg/l) | dabSFans hydrolysis b) EC(50 ± SD e) (nM) | DAIP/dispase (mole/mole) |
|---------------|-----------------|------------------------------------------|--------------------------|
| wtDAIP (Sm-Q5)| 26.9 ± 1.2 d)   | 0.135 ± 0.006                             |
| rDAIP (Q5)    | 13-25           | 21.3 ± 1.3 d)                             | 0.106 ± 0.007            |
| Q39 (Q1)      | 11.2            | 16.5 ± 1.5 c)                             | 0.165 ± 0.015            |
| Q39-GT36/37EE (Q1) | 2.5    | 39.8 ± 2.2 d)                             | 0.199 ± 0.011            |
| Q39-TL37/38YF (Q1) | 2.1    | 21.4 ± 0.9 d)                             | 0.107 ± 0.005            |
| Q65 (Q1)      | 8.3             | 14.6 ± 1.0 c)                             | 0.146 ± 0.010            |
| Q144 (Q1)     | 3.8             | 27.5 ± 0.8 d)                             | 0.138 ± 0.004            |
| Q298 (Q1)     | 3.7             | 25.6 ± 0.8 d)                             | 0.128 ± 0.004            |
| Q345 (Q1)     | 6.3             | 29.2 ± 0.8 d)                             | 0.146 ± 0.004            |
| Null-Q (Q0)   | 1.5             | 15.3 ± 0.4 c)                             | 0.153 ± 0.004            |

a) Calculated for 1 l culture broth and highly purified protein. – b) Dispase (100 nM c), 200 nM d)) was pre-incubated with DAIP for 30 min at 37 °C. Upon addition of dabSFans, increase in fluorescence was continuously monitored at 535 nm (λexc of 340 nm). – e) Data are means of three independent measurements.
FIGURES

FIGURE 1

A

B

\[ \text{N}^\gamma\text{-Glutamyllysine isopeptide bond} \]

\[ \text{Hydrolyzed glutamine} \]

\[ \text{Biotinylated glutamine} \]
Dispase autolysis inducing protein

FIGURE 2

A

B

C

D

E

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FIGURE 3

A

B

C

MTG substrate site Secondary structure Type within 45 Saa
ADAQ Q40 o-helix aromatic/bulky, charged
AGIQ Q47 o-helix aromatic/bulky, charged
HEITP Q169 a-helix aromatic/bulky, charged
YTEM Q269 loop aromatic/bulky, charged
GSKQ Q272 loop aromatic/bulky, charged
PAQDR Q292 o-helix charged
AMRQ Q293 o-helix charged
RABF Q300 o-helix aromatic/bulky, charged
channel Q320 o-helix aromatic/bulky, charged
MVET Q337 a-helix aromatic/bulky, charged
ESCQ Q441 loop charged
LRGEQ Q469 o-helix aromatic/bulky, charged
GAVNQ Q470 loop aromatic/bulky, charged
TEQNP Q543 ? aromatic/bulky, charged
FIGURE 4

Dispase autolysis inducing protein

| MTG substrate site | Secondary structure | Type within +/- 5aa |
|--------------------|---------------------|-------------------|
| TTGTL Q39 SVSTX    | loop                | small, hydrophobic, polar |
| YGTVP Q298 AXCTD   | loop                | aromatic/bulky      |
| GLEEY Q345 IHH--   | loop                | charged             |
| HNDEL Q65 RSTBA    | β-strand            | charged             |
| AGSRTQ Q144 LYDST  | β-strand            | charged, aromatic/bulky |
FIGURE 5

Dispase autolysis inducing protein

A top view

B front view

C back view

D

- Preferred structure and amino acid composition of Gln-site:
  - flexible loop >> β-strand
  - small/polar/hydrophobic/uncharged >> bulky/aromatic > charged

acetyl acceptor (lysine substrate) binding site

acetyl donor (glutamine substrate) binding site
Structure of the Dispase Autolysis Inducing Protein from *Streptomyces mobaraensis* and Glutamine Cross-linking Sites for Transglutaminase

David Fiebig, Stefan Schmelz, Stephan Zindel, Vera Ehret, Jan Beck, Aileen Ebenig, Marina Ehret, Sabrina Fröls, Felicitas Pfeifer, Harald Kolmar, Hans-Lothar Fuchsbaue and Andrea Scrima

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