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Identification of sortase substrates by specificity profiling

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ABSTRACT

Sortases catalyze the attachment of surface proteins to the peptidoglycan layer of gram-positive bacteria and further represent powerful tools of protein chemistry. During catalysis sortases cleave a donor substrate containing the LPxTG (x = any amino acid) sorting motif under formation of an enzyme-bound thioester and ligate this intermediate to an acceptor protein containing an N-terminal glycine residue. In addition to the well-established sortase A of *Staphylococcus aureus* several homologs of this enzyme have been identified in the genomes of gram-positive bacteria. We have profiled the specificity of seven sortases of Staphylococci and Streptococci origin and observed that sortases of the latter class displayed a more relaxed specificity for donor and acceptor substrates than their Staphylococci counterparts. Streptococci sortases prefer an LPKLG donor substrate sequence compared to the canonical sorting motif LPKTG. These findings might facilitate the use of Streptococci sortases as tools of protein chemistry.

1. Introduction

The attachment of surface proteins to the peptidoglycan layer is an important strategy of many pathogenic bacteria for escaping the immune response of the host. The anchoring reaction is catalyzed by bacterial transpeptidases of the sortase family. Surface proteins contain a so-called sorting motif are recognized and cleaved by sortases.[1,2] The sequence LPxTG (x: any amino acid residue) constitutes the canonical sorting motif of sortase A from *Staphylococcus aureus* (SAu-sortase).[3] The sorting motif is cleaved at the threonine residue, which occupies the P1 position when adopting protease nomenclature (Fig. 1a).[4] The glycine residue in P1′ is expelled and the intermediate is bound as thioester to the active site cysteine of the enzyme.[5–9] In the second step the sortase-bound intermediate is resolved by the N-terminus of a penta-glycine acceptor at the cross-bridge of the peptidoglycan layer. SAu-sortase has also been established as widely used tool in protein chemistry because the transpeptidation reaction requires only the sorting motif in a donor substrate and an acceptor with an N-terminal glycine residue.[10] This sortase-mediated ligation (SML) has found many applications including modification of proteins with small molecules and dyes, cyclization of proteins, probing of protein-protein interactions, protein immobilization, display and modification of phages and even *in vivo* ligation reactions.[11–16] Furthermore, SML has been used for multi-fragment assemblies and combined with other ligation strategies.[5,17,18]

In addition to SAu-sortase, sortase A of *Streptococcus pyogenes* (SPy-sortase) has found application in protein labeling approaches.[5,18] In contrast to SAu-sortase, SPy-sortase catalyzes transpeptidation reactions at significantly slower rates but does not require Ca^{2+} for catalysis.[19] Importantly, SPy-sortase accepts acceptor substrates with N-terminal alanine residues. The latter feature is particularly useful for dual-labeling approaches that combine SAu- and SPy-sortase for SML.[5,18] The observed differences in acceptor specificity of SPy-sortase and SAu-sortase most likely stem from altered compositions of the peptidoglycans. In case of *Staphylococcus aureus* five Gly residues extend from the Nε amino group of the central Lys residue at the cross-bridge.[20] The penta-glycine unit can be replaced by two or three alanine residues in *Streptococcus pyogenes* and other Streptococci strains even possess acceptors with N-terminal Gly, Ser, or D-Asp residues, or the free Nε amine of the lysine cross-bridge.[21] Furthermore, bioinformatic analyses of bacterial surface proteins indicate that variations of sorting motifs do exist.[3,22] For example, Staphylococci strains encoding proteins with potential LPxAG and LPxSG have been reported. Consequently, SAu-sortase was shown to ligate LPxAG donor substrates with, however, low activity.[3]

Sortases have been subjected to protein-engineering in order to improve their catalytic efficiency or to alter their specificity.[23–28] Sortases that ligate altered donor and acceptor substrates will facilitate SML and allow for more flexibility in the design of the

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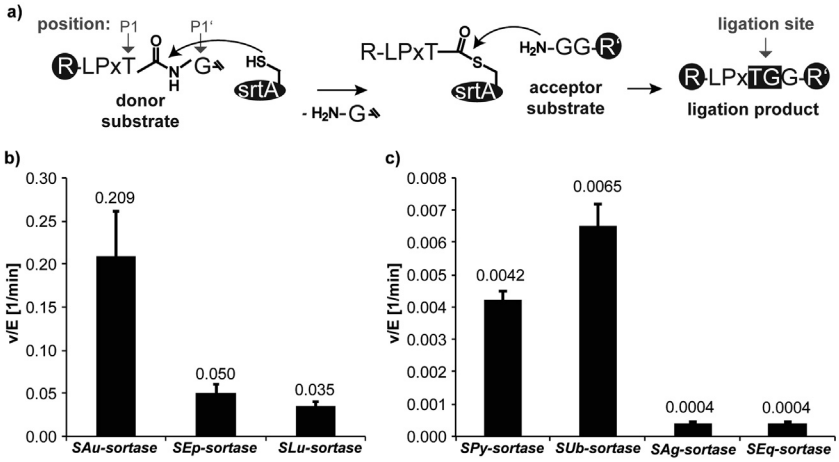

Fig. 1. Sortase reaction. a) During sortase-mediated ligation (SML) donor substrates containing an LPxTG sorting motif are cleaved at the threonine residue at the P1 position resulting in a sortase-bound thioester, which is subsequently ligated to an *N*-Gly acceptor substrate. The newly connected Thr of the donor and the *N*-Gly of the acceptor form the ligation site of the product. b) The rate of ligation product formation was determined at an early state of the reaction, below 10% of substrate consumption. Staphylococci sortases were assayed at 200 μM of donor, 1 mM of acceptor and enzyme concentration of 5 μM. c) Streptococci sortases were assayed with the same donor and acceptor concentration and 10 μM of SPy- and SUb-sortase or 50 μM for SAg- and SEq-sortase. Rates were calculated from the signal intensities of the LC analysis and normalized to the respective enzyme concentrations.

ligation schemes. While these approaches are promising the potential flexibility of wild-type sortases is only poorly explored.

Here we report cloning and characterization of new sortases of the genus Streptococci and Staphylococci. We investigated the specificity of these enzymes with respect to the residues that form the ligation site, in particular the P1 position of the sorting motif and the N-terminal residue of the acceptor. We observed that sortases of the genus Streptococci displayed a broader specificity in general. Analysis of the transpeptidase activity of Streptococci sortase indicated preference for an alternative LPxLG sorting motif, which is preferred over the canonical LPxTG sequence. The preference also translates into more efficient protein labeling by SPy-sortase when using LPKLG donor substrates.

2. Materials and methods

2.1. General methods and reagents

Amino acid (AA) derivatives were purchased from GLS (Shanghai, China), coupling reagents were purchased from Merck Novabiochem (Darmstadt, Germany), Tentagel R RAM resin from Rapp Polymere (Tübingen, Germany). All other chemicals and oligonucleotides were purchased from Sigma-Aldrich (Steinheim, Germany). Analytical RP-HPLC was performed on a Shimadzu LC-10AT HPLC system with a Nucleosil C18 column (5 μm, 4.6 × 250 mm, Machery-Nagel, Düren, Germany), with 0.1% TFA in water (A) and 80% ACN, 0.1% TFA in water (B), as eluents. The analytical gradient was 5–95% B over 20 min with a flow rate of 1.5 mL/min. Preparative purifications were conducted on a Varian ProStar 210 HPLC system equipped with a Reprosil 100 C18 (5 μm, 20 × 250 mm, Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) and a flow rate of 13 mL/min. Mass analysis was conducted on a LCMS2020 (Shimadzu) equipped with a Kinetex C18 column (2.6 μm, 100 × 2.1 mm, Phenomonex) 0.1% formic acid in water (LCMS-A) and 80% ACN and 0.1% formic acid in water (LCMS-B) with a flow rate of 0.2 mL/min.

2.2. Solid-phase peptide synthesis

All peptides were synthesized by Fmoc-based solid-phase peptide chemistry using a Syro_I synthesizer (MultiSynTech GmbH). The synthesis was performed on TentaGel R RAM resin (0.37 mmol/g). Amino acid side-chains were protected as follows: Abz(Boc), Arg(Pbf), Asn(Trt), Asp(OtBu), D-Asp(OtBu), Dap(Boc), Gln(Trt), Glu(OtBu), Lys(Boc), Lys(Dnp), Ser(tBu), Thr(tBu), D-Thr (tBu), Trp(Boc) and Tyr(tBu). Fmoc-Met-OH was replaced by Fmoc-Nle-OH since preliminary experiments indicated sulfoxide formation under the selected reaction conditions. In order to achieve high purity of crude peptides, each residue was incorporated by double couplings using diisopropylcarbodiimide (DIC)/Oxyma Pure in the first coupling step and 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium-hexafluorophosphate (HBTU)/*N*-methylmorpholine (NMM) for the second coupling reaction. Each amino acid was coupled in 4-fold molar excess for 40 min. Removal of the Fmoc group was carried two times with 40% piperidine in DMF for 3 min and 12 min. Dansyl was coupled to the acceptor peptide with 4 eq. dansyl chloride with 10 eq. DIPEA in DMF for 2 h. 5,6-Carboxyfluorescein was coupled manually with 4-fold molar access for 2 × 1 h. All peptides were deprotected and cleaved from the resin with TFA/TIPS/H_2O/Phenol (85/5/5/5) for 3 h. Peptides used for the initial screens were precipitated in cold diethyl ether followed by two washing steps with cold ether. The average purity of the obtained peptides was 85% and this material was used for fluorescent screens of sortase substrate specificity without further purification (Supporting Figs. S1 and S2). Peptides for HPLC-based assays and protein labeling were further subjected to preparative HPLC resulting in an average purity of 95% (Supporting Figs. S3–S5).

2.3. Construction of sortase expression vectors

Synthetic sortase genes of *Staphylococcus epidermidis* (UniProt: A0A0E1V9Y6) Lys57-Asn203, *Staphylococcus lugdunensis* (UniProt: A0A1J4IES2) Asn55-Lys201, *Streptococcus agalactiae* (UniProt: Q8DZY1) Ile80-Leu247, *Streptococcus equinus* (UniProt: E8JNC5) *Val81-Phe248, Streptococcus pyogenes* (UniProt: Q1JGU0) Val82-Thr249 and *Streptococcus uberis* (UniProt: B9DS55) Val84-Glu252 were purchased from life technologies. The DNA was codon-optimized for expression in *E. coli*. Synthetic sortases were subcloned in pET23b and pET28a expression vectors via *Nde*I and *Xho*I restriction sites. The sortase genes from Staphylococci were cloned into pET23b resulting in C-terminal His6-Tag, whereas Streptococci