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Recent Advances in Sortase-Catalyzed Ligation Methodology

John M. Antos[1], **Matthias C. Truttmann**[2], and **Hidde L. Ploegh**[2]

[1]Department of Chemistry, Western Washington University, 516 High Street, Bellingham, WA 98229 (USA)

[2]Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge, MA 02142 (USA)

Abstract

The transpeptidation reaction catalyzed by bacterial sortases continues to see increasing use in the construction of novel protein derivatives. In addition to growth in the number of applications that rely on sortase, this field has also seen methodology improvements that enhance reaction performance and scope. In this opinion, we present an overview of key developments in the practice and implementation of sortase-based strategies, including applications relevant to structural biology. Topics include the use of engineered sortases to increase reaction rates, the use of redesigned acyl donors and acceptors to mitigate reaction reversibility, and strategies for expanding the range of substrates that are compatible with a sortase-based approach.

Introduction

The manipulation of protein structure in ways beyond the reach of standard genetic approaches is a critical activity in the modern biochemical sciences. Among the numerous reported approaches for protein derivatization, the transpeptidation reaction catalyzed by bacterial sortases, a process referred to as *sortagging*, has attracted attention because of its ease of use and broad scope with respect to both protein targets and the types of modifications installed. In its most common form, sortagging involves the pairing of sortase A from *Staphylococcus aureus* ($SrtA_{staph}$) with an LPXTG-containing substrate (Figure 1). In the presence of Ca^{2+}, the active site cysteine of $SrtA_{staph}$ cleaves between threonine and glycine to generate a thioester-linked acyl enzyme intermediate. This intermediate is then intercepted by an aminoglycine nucleophile, resulting in the site-specific ligation of the acyl donor and acceptor. Since the introduction of this strategy in 2004, a remarkably diverse set of components has been shown to be compatible with this system [1]. This includes acyl donors and acceptors such as proteins and synthetic peptides, as well as similar types of molecules displayed on solid supports and on the surface of live cells. Recent examples

Corresponding Authors: John M. Antos (john.antos@wwu.edu), Hidde L. Ploegh (ploegh@wi.mit.edu).

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Nothing declared for JMA and MCT. HLP is a co-founder of and owns stock in 121Bio, a company that uses sortase for protein modification.

include the synthesis of camelid-derived antibody fragment conjugates for the treatment of B-cell lymphoma, the installation of non-isotopically labeled protein domains to facilitate NMR analysis of proteins with limited solubility, the construction of immuno-PET reagents for non-invasive cancer imaging, and the preparation of multifunctional protein nanoparticles [2–5]. This is by no means an exhaustive list, and we refer the reader to other excellent reviews for more comprehensive discussions of sortagging applications [6–10]. Rather than focus on applications, our goal for this review is to provide an overview of advances in sortagging methodology itself. This includes the engineering and optimization of new reaction materials and reagents, as well as novel reaction systems designed to facilitate the sortagging process. To highlight these strategies, the application of sortagging to challenges in structural biology is also discussed.

Optimizing $SrtA_{staph}$ Performance

Prior to 2011, the vast majority of sortagging applications relied on wild-type sortase A from *Staphylococcus aureus* ($SrtA_{staph}$), typically employed as a soluble fragment lacking either the first 25 or 59 residues. While this enzyme continues to see consistent use, it suffers from some notable limitations: poor reaction rates and a dependency on a Ca^{2+} cofactor. To circumvent these issues, a number of strategies have now been reported that describe ways to maximize $SrtA_{staph}$ performance, either through engineering of the enzyme itself, the use of sortase-reactant fusions, or alternate reaction protocols.

With regard to engineered sortases, Chen and co-workers used a directed evolution screen to identify sortase variants with enhanced catalytic activity [11•]. The integration of five underlying mutations (P94R/D160N/D165A/K190E/K196T) in a single gene resulted in the so-called $SrtA_{staph}$ pentamutant, which exhibited a ~120 fold increase in k_{cat}/K_M relative to wild-type $SrtA_{staph}$. These mutations are localized near the LPXTG-binding groove, likely improving substrate binding. The pentamutant was subsequently enhanced by adding two additional mutations known to eliminate Ca^{2+} dependency [12]. Two $SrtA_{staph}$ heptamutants have been described which add either E105K/E108A mutations or E105K/E108Q mutations to the $SrtA_{staph}$ pentamutant backbone [13•, 14, 15•]. These variants no longer require Ca^{2+}, and also retain much of the enhanced rate characteristics of the $SrtA_{staph}$ pentamutant. In the most recent example of $SrtA_{staph}$ directed evolution, an *in vitro* compartmentalization strategy was employed to identify a new Ca^{2+}-independent $SrtA_{staph}$ mutant [16]. A variant with 12 mutations was identified, including E105V and E108G, which are positions known to be involved in Ca^{2+} binding, and are also mutated in the $SrtA_{staph}$ heptamutants. In the absence of Ca^{2+}, this particular derivative displayed slightly improved activity relative to wild-type $SrtA_{staph}$ in the presence of Ca^{2+}. Overall, evolved versions of $SrtA_{staph}$ offer substantial advantages over the wild-type enzyme, and the heptamutants may represent the most universally potent sortase variants described to date. The pentamutant and heptamutant versions have also seen increasing use in demanding processes such as cell surface labeling and intracellular ligations [11•, 13•, 17, 18]. However, the pentamutant and heptamutant might not be optimal for all applications. While direct comparisons of these mutants to wild-type $SrtA_{staph}$ have clearly shown enhanced reaction rates, the wild-type enzyme was actually observed to give higher overall yields in the ligation of GFP to triglycine-coated polystyrene beads [19]. Furthermore, the pentamutant was prone to higher levels of

undesired hydrolytic and oligomeric side products if reaction progress was not carefully monitored [20]. In addition to evolved $SrtA_{staph}$ mutants, other notable engineered derivatives include a cyclized $SrtA_{staph}$ analogue that exhibited improved resistance to chemical denaturation, as well as semisynthetic analogues containing selenocysteine (Sec) or homocysteine (Hcy) in the active site [21–23]. While both Sec and Hcy derivatives showed impaired catalytic activity, this study does provide a compelling route for accessing unconventional $SrtA_{staph}$ derivatives. A summary of the engineered $SrtA_{staph}$ variants discussed in this section is provided in Table 1.

In addition to alterations of the enzyme itself, the use of fusions involving $SrtA_{staph}$ and either the aminoglycine acyl acceptor or the LPXTG acyl donor have also been reported. Both N- and C-terminal fusions have been described, typically in the context of new approaches for recombinant protein expression and purification [3, 24–30]. Of these, fusions at the N-terminus are particularly intriguing as it has been suggested that the N-termini of these constructs can access the enzyme active site in an intramolecular fashion, thereby driving transpeptidation due to the increase in local reactant concentration [3, 27, 30]. As an example, Amer et al. demonstrated an increase in ligation rates when a construct consisting of wild-type $SrtA_{staph}$ fused at its N-terminus to an aminoglycine-containing SUMO module was reacted with a separate LPXTG substrate [3]. In this case, reaction rates involving the SUMO-$SrtA_{staph}$ fusion were significantly enhanced relative to a control reaction involving separate $SrtA_{staph}$, aminoglycine SUMO, and LPXTG substrate.

A final area of note concerns the use of affinity purification/immobilization strategies to streamline sortagging protocols. It is well established that the removal of reaction by-products or residual sortase enzyme can be achieved through the strategic inclusion of His_6 affinity handles, or the use of the elastin-like polypeptide as a controlled solubility switch [3, 26, 27, 31]. $SrtA_{staph}$ has also been covalently immobilized on sepharose or PEGA resins to facilitate enzyme removal and enzyme recycling [14, 32]. A useful extension of sortase-immobilization has been the construction of flow-based systems in which reactants are passed over immobilized sortase columns [14, 33]. The flow-based reactor described by Pentelute and coworkers is particularly noteworthy in that the authors demonstrated that flow-based sortagging increased isolated product yields, and reduced contamination by hydrolytic, cyclic, or oligomeric by-products relative to analogous reactions performed using a standard solution-phase protocol [33].

Driving Ligation Product Formation

Sortagging reactions are reversible: the desired ligation product encloses an intact LPXTG acyl donor motif and the released nucleophilic fragment contains an aminoglycine acyl acceptor. To achieve high yields of the ligation product, a significant surplus of one of the reactants is typically added [31, 34]. While effective, this strategy is problematic if the ligation partner used in excess is challenging to synthesize, expensive or only available in limited quantities. To circumvent the need for excess reagents, a growing number of strategies are now available that dramatically improve sortagging yields when ligation partners are used at nearly equimolar concentrations.

A common cause for reversibility in standard sortagging reactions is the accumulation of the released aminoglycine peptide fragment. The physical removal of this by-product thus provides a simple way to limit reaction reversibility. Indeed, sortagging reactions conducted under dialysis conditions or in centrifugal filtration units can significantly enhance reaction conversion through selective removal of low molecular weight aminoglycine by-products [35–38]. Similarly, affinity immobilization strategies combined with sortase-substrate fusions or the aforementioned flow-based sortagging platform have been shown to minimize the need for excess reagents through the selective removal of various reaction components [27, 33]. All of these approaches are straightforward, and typically do not require changes in the sortase substrate recognition site. Notably, some of these techniques have proven particularly useful for the construction of segmentally labeled proteins for NMR analysis [36–38].

In addition to separation strategies, the designs of both the LPXTG acyl donor and aminoglycine acyl acceptor have been revisited to improve ligation yields. Conceptually, these systems involve selective deactivation of either the sortagging ligation product or other by-products to prevent reverse transpeptidation (Figure 2). Deactivation of the ligation product has been achieved through the formation of an unreactive β-hairpin at the LPXTG ligation site (Figure 2b) [13•, 39]. This secondary structure element inhibits $SrtA_{staph}$ recognition of the reassembled LPXTG motif, allowing accumulation of the desired ligation product. This approach requires the installation of several additional residues flanking the LPXTG motif, and boosts ligation yields both *in vitro* and in the cytoplasm of *E. coli*. With respect to by-product deactivation, LPXTG variants have been designed that release unreactive fragments during formation of the desired sortagging product (Figure 2c). Wiliamson et al. reported the synthesis of a depsipeptide that releases a relatively unreactive hydroxyacetyl moiety instead of a nucleophilic aminoglycine [40, 41• 42]. A second depsipeptide was synthesized by Liu and coworkers that involves release of a fragment that spontaneously deactivates via diketopiperazine formation [43]. These systems, which are best suited for N-terminal labeling, give excellent ligation yields with a variety of protein and peptide targets using only 1.0–3.0 equivalents of depsipeptide acyl donor. A final strategy for by-product deactivation involves Ni^{2+} chelation. The extension of the LPXTG motif with glycine and histidine (LPXTGGH) results in the release of a GGH-containing fragment that binds with high affinity to bivalent metal ions such as Ni^{2+} [44]. Coordination of the nitrogen lone pair by Ni^{2+} minimizes nucleophilicity, thereby limiting the reverse reaction. Due to the fact that this system relies on natural amino acids, it is compatible with both N-terminal and C-terminal sortagging, and has been shown to enhance ligation yields for both peptide and protein model systems at near equimolar concentrations of LPXTG substrate and aminoglycine nucleophile.

Broadening the Substrate Scope

The selective activation of the LPXTG motif by wild-type $SrtA_{staph}$ or engineered $SrtA_{staph}$ mutants is the foundation of the majority of sortase-mediated applications. While this selectivity is critical to the success of sortagging applications, in particular ligations performed in complex lysates or on the surface of live cells, it also restricts the technique to substrates that inherently possess the LPXTG motif or those that have been engineered to

display this peptide sequence. To address this limitation, a handful of strategies have been reported for expanding sortagging beyond the LPXTG sequence.

One approach to broadening substrate scope involves the use of naturally occurring sortase homologs. To date, two sortase homologs in addition to $SrtA_{staph}$ have been explored for sortagging-type applications. Sortase A from *Streptococcus pyogenes* ($SrtA_{strep}$), which is Ca^{2+}-independent, can recognize an LPXTA substrate in addition to LPXTG, and accommodates N-terminal alanine residues as acyl acceptors. This subtle difference in substrate tolerance for $SrtA_{strep}$ has been exploited for applications such as dual labeling of the N-and C-termini in the same polypeptide, and orthogonal labeling of different proteins in the M13 viral particle [45–47]. Due to the lack of Ca^{2+} dependency, this enzyme has also proven effective for catalyzing ligations in live cells [48, 49]. In addition to $SrtA_{strep}$, there has been a single report on the use of sortase A from *Lactobacillus plantarum* ($SrtA_{plant}$) [50]. While the yield of ligations using $SrtA_{plant}$ was not reported, this enzyme was shown to catalyze transpeptidations involving non-amino acid primary amine nucleophiles and model proteins possessing LAATGWM, LPKTGDD, and LPQTSEQ sequences. As a final comment, it is also important to note that wild-type $SrtA_{staph}$ tolerates select deviations from the LPXTG motif. Low to moderate reaction conversions have been observed with the alternate substrates IPKTG, MPXTG, LAETG, LPXAG, LPESG, LPELG, and LPEVG [26, 30, 51]. While these substrates give reduced reaction rates relative to LPXTG, this feature has actually proven beneficial in modulating the rate of self-cleavage in a ternary sortase fusion protein designed as part of a novel strategy for recombinant protein purification [26].

In addition to natural sortases, gains in substrate scope have been achieved using $SrtA_{staph}$ mutants. Schwarzer and coworkers used a phage display selection system to identify a $SrtA_{staph}$ mutant (designated F40) with the ability to tolerate a range of XPKTG motifs [30]. The mutant exhibited a slight preference for APKTG, DPKTG, and SPKTG, though trace levels of reactivity were observed for a wide range of other XPKTG substrates, as well as FAKTG. In an elegant demonstration of the utility of this increased substrate scope, the authors reported a semisynthesis of histone H3 involving transacylation at an APATG ligation site. A more recent example of sortase engineering was reported by Dorr et al., who employed a yeast display system to identify two $SrtA_{staph}$ mutants with altered selectivity profiles [52••]. Starting from the $SrtA_{staph}$ pentamutant, which itself has some ability to accept LPEXG (X = A, C, S) and LAETG in addition to LPXTG, the authors were able to evolve one mutant (designated 2A-9) with excellent selectivity for LAETG and a second mutant (designated 4S-9) with a preference for LPEXG (X = A, C, S) [20, 52••]. Notably, both engineered sortases showed dramatically reduced activity toward the LPXTG motif, indicating that the mutants were not simply more promiscuous, but rather exhibited substrate selectivity profiles that were orthogonal to wild-type and pentamutant $SrtA_{staph}$. The value of these orthogonal sortase mutants was subsequently demonstrated in a variety applications, including site-specific labeling at the N- and C-termini of FGF1 and FGF2, the labeling of endogenous fetuin (which naturally possesses a LPPAG motif) in human plasma, and selective surface modification. A summary of alternate sortase substrates discussed in this section is provided in Table 2.

Application Highlight: Construction of Protein Fusions for Structural Characterization

The ability of sortases to site-specifically ligate complex polypeptide fragments has made them ideal tools for generating unique protein fusions for crystallographic and NMR characterization. For example, sortase-mediated ligation of HLA-DM to peptide-conjugated HLA-DR1 enabled determination of the X-ray crystal structure of the entire complex, providing new insight into the mechanism by which HLA-DM stabilizes empty HLA-DR1 and catalyzes the exchange of peptides bound by HLA-DR1 [53]. Notably, cocrystallization of separate HLA-DM and HLA-DR1 was unsuccessful, and suitable crystals were obtained only after covalent ligation of these molecules via sortagging. With regard to NMR, sortagging has found use in segmental isotope labeling, wherein an isotopically-enriched protein fragment is ligated to an NMR “silent” fragment. Examples include the attachment of unlabeled solubility enhancing tags (GB1, SUMO), as well as the construction of multidomain proteins (MecA, TIA-1, Hsp90, BRD4) where only certain domains are isotopically labeled to simplify the complexity of NMR spectra [3, 36–38, 54]. Interestingly, the particular challenges of segmental labeling have provided excellent opportunities for refining sortagging methods. Specifically, to boost the yields of ligations involving costly isotopically labeled proteins, nearly all segmental labeling applications using sortase have employed strategies for biasing reaction equilibrium through either dialysis or centrifugal filtration to remove the low molecular weight aminoglycine by-products [36–38, 54]. In addition, to avoid aggregation or degradation of isotopically labeled proteins that may occur during long reaction times, new approaches for increasing sortagging reaction rates have been developed that include the use of centrifugal filtration units, or the use of a novel sortase construct involving the fusion of an aminoglycine acyl acceptor at the N-terminus of wild-type $SrtA_{staph}$ [3, 38].

Conclusions and Future Directions

Sortagging has benefitted from technical refinements that now offer the end user a range of options when implementing this strategy. We envision that the techniques described here will see increased use, and will enable the next generation of sortase-based applications. With this in mind, this review would not be complete without commenting on emerging areas for application development. One area involves the site-specific construction of isopeptide bonds. Sortagging is typically restricted to protein termini; however, in 2015 Bellucci et al. demonstrated the ability of $SrtA_{staph}$ to catalyze ligations targeted to specific lysine-containing acceptor sequences embedded in proteins, thereby providing a new route to accessing branched polypeptides [55]. A second area for innovation involves intracellular sortagging. A handful of reports have now demonstrated sortagging with Ca^{2+}-independent sortases in both eukaryotic and prokaryotic systems, although controlling side reactions and introducing exogenous reaction components (acyl donors or acyl acceptors) remains a challenge [13•, 16, 48, 49]. Finally, we also envision an increase in sortase application development to address open questions in structural biology. A particularly powerful aspect of sortagging is its ability to generate protein architectures that cannot be accessed using standard genetic methods. In addition to the isopeptide linked structures discussed above,

this also includes complexes where proteins are linked via their respective C- or N-termini to give non-natural C-to-C or N-to-N fusions, such as the HLA-DM-HLA-DR1 complex described by Pos et al. [53, 56, 57]. Going forward, we speculate that the continued development of isopeptide ligations, intracellular sortagging and novel protein fusions will benefit from many of the methodology advances described in this review.

A final notable development, apart from sortase, has been the emergence of the ligase butelase-1 isolated from *Clitoria ternatea* [58•, 59, 60]. First described in 2014, butelase-1 has been shown to promote cyclizations and intermolecular ligations analogous to those catalyzed by sortases. Butelase-1 also offers some key advantages, including a smaller acyl donor motif (NHV versus LPXTG) and substantially higher reaction rates. Given these attractive features, it is likely that this enzyme will see increased use as an important alternative to sortases, and an intriguing possibility may be the combination of sortagging technology with butelase- or intein-mediated ligation to perform sequential ligations and ultimately generate more complex protein conjugates.

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Highlights

- Sortase-catalyzed ligation is a powerful strategy for protein modification.
- Engineered sortases improve reaction rates and eliminate Ca^{2+} dependency.
- Deactivation of ligation reaction products minimizes the need for excess reagents.
- Sortase mutants and sortase homologs expand the range of compatible substrates.

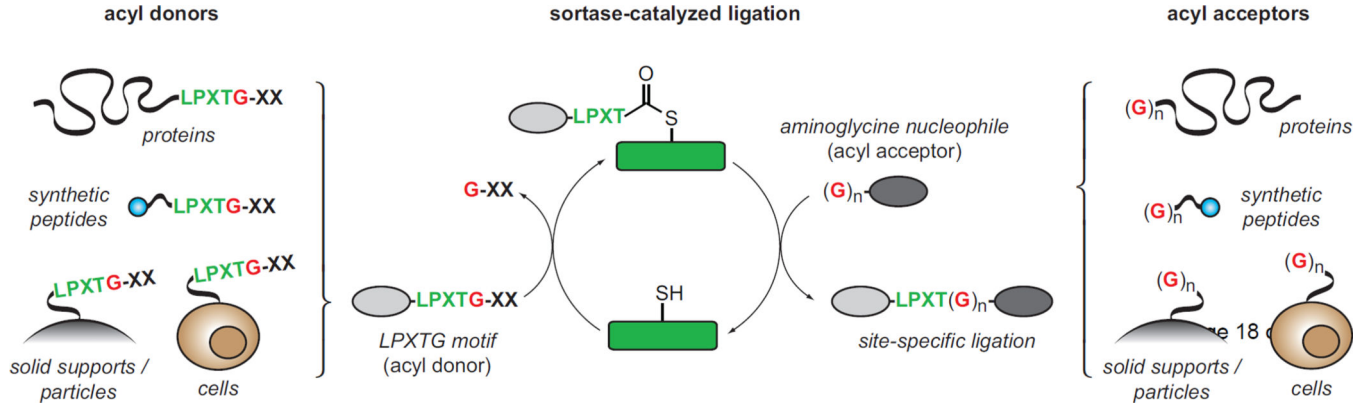

Figure 1.
Protein modification via sortase-catalyzed transpeptidation (‘sortagging’).

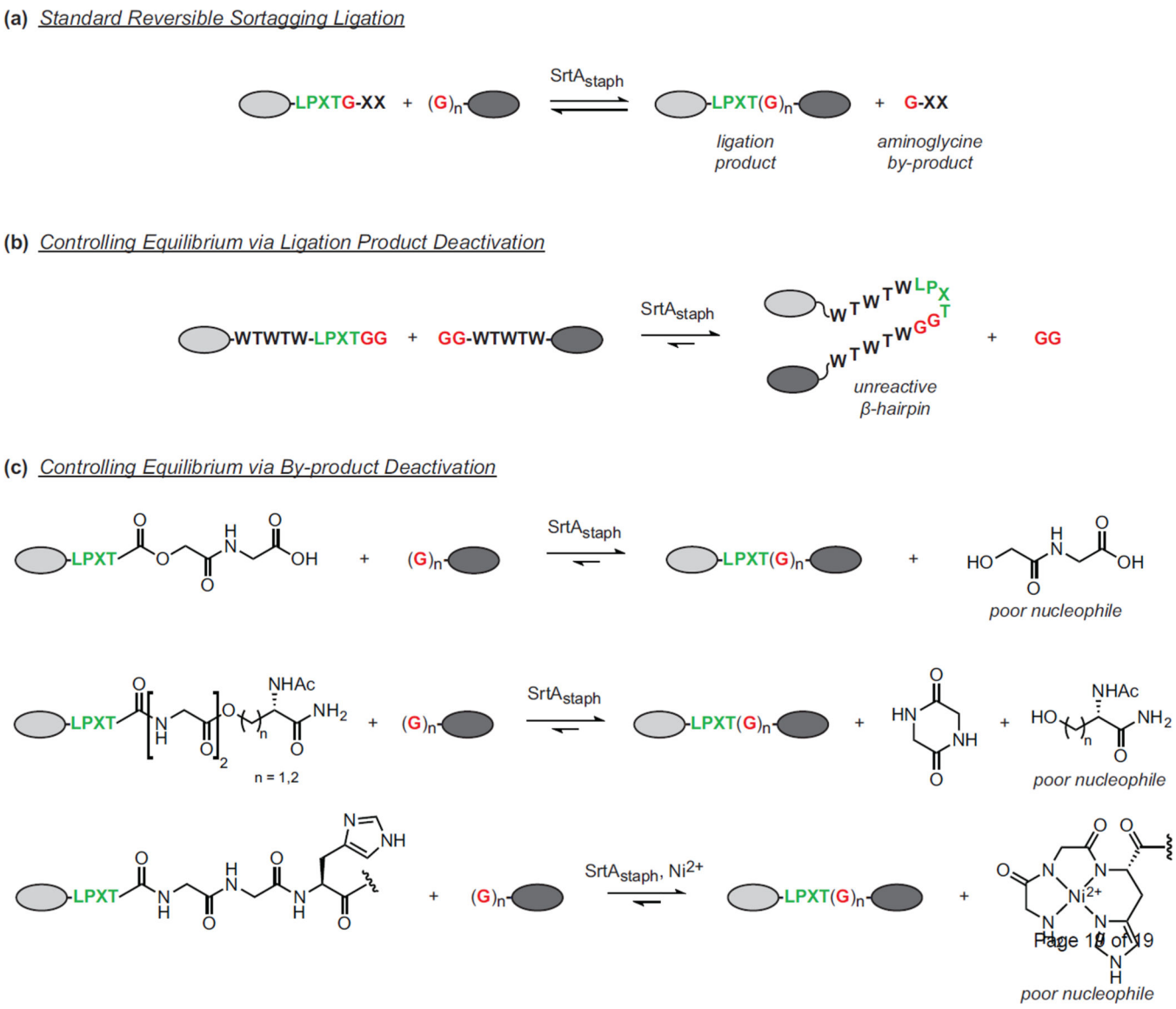

Figure 2.

Driving sortagging efficiency through selective deactivation of the ligation product or the ligation by-product. **(a)** Standard ligations using LPXTG substrates and aminoglycine nucleophiles are reversible, necessitating the need for excess reagents or the continuous removal of the aminoglycine by-product. **(b)** Selective formation of a β-hairpin deactivates the ligation product and prevents it from engaging in the reverse reaction. **(c)** Modified acyl donors release by-products that are unable to serve as nucleophiles in the reverse transpeptidation reaction.

Table 1

Engineered $SrtA_{staph}$ Variants with Altered Catalytic Activity / Stability

Enzyme	Advantages		Disadvantages		References
wild-type $SrtA_{staph}$	-		•	Poor *in vitro* kinetics	[1,8–9]
			•	Ca^{2+}-dependent	
$SrtA_{staph}$ pentamutant (P94R/D160N/D165A/ K190E/K196T)	•	Evolved enzyme exhibiting significant enhancement in activity (~120 fold increase in k_{cat}/K_M relative to wild-type)	•	Ca^{2+}-dependent	[11•]
	•	Variants containing a subset of these mutations also show enhanced activity			
$SrtA_{staph}$ dual mutant (E105K/E108A or E105K/E108Q)	•	Ca^{2+}-independent	•	Activity without Ca^{2+} slightly reduced relative to wild-type in the presence of Ca^{2+}	[12]
$SrtA_{staph}$ heptamutant	•	Combines rate enhancements of $SrtA_{staph}$ pentamutant with Ca^{2+}-independent mutations	•	Mutant enzyme activity slightly reduced relative to pentamutant in the presence of Ca^{2+}	[13•,14,15•]
cyclo-$SrtA_{staph}$	•	Backbone cyclized $SrtA_{staph}$	-		[21–22]
	•	Improved resistance to chemical denaturation			
$SrtA_{staph}$ mutant (12 point mutations)	•	Evolved enzyme variant	-		[16]
	•	Ca^{2+}-independent			
	•	Activity without Ca^{2+} slightly improved relative to wild-type in the presence of Ca^{2+}			
Sec/Hcy-$SrtA_{staph}$ (Sec = selenocysteine, Hcy = homocysteine)	•	Semisynthetic enzyme variants	•	Activity reduced relative to wild-type $SrtA_{staph}$	[23]

Table 2

Alternate Sortase Substrates

Enzyme	Substrates	Comments
wild-type $SrtA_{staph}$	LPXTG, IPKTG, MPXTG, LAETG, LPXAG, LPESG, LPELG, LPEVG	• LPXTG motif is optimal, other sequences exhibit reduced reaction rates • References [26,30,51]
wild-type $SrtA_{strep}$	LPXTG, LPXTA	• Ca^{2+}-independent enzyme • Accepts N-terminal alanine as acyl acceptor • References [45–49]
wild-type $SrtA_{plant}$	LAATGWM, LPKTGDD, LPQTSEQ	• Accepts non-amino acid primary amines as acyl acceptor • Reference [50]
F40 $SrtA_{staph}$ mutant	XPKTG (X = A, D, S), APATG	• Evolved $SrtA_{staph}$ variant • Reaction rates reduced relative to wild-type $SrtA_{staph}$ • In addition to substrates listed, low level reactivity observed for other XPKTG and FAKTG substrates • Reference [30]
$SrtA_{staph}$ pentamutant	LPXTG, LPEXG (X = A, C, S), LAETG	• Evolved $SrtA_{staph}$ variant • LPXTG preferred • Reaction rates increased relative to wild-type $SrtA_{staph}$ • References [20,52••]
2A-9 $SrtA_{staph}$ mutant	LAETG	• Evolved $SrtA_{staph}$ variant • Reaction rates increased relative to wild-type $SrtA_{staph}$ • Substrate selectivity orthogonal to $SrtA_{staph}$ pentamutant and wild-type $SrtA_{staph}$ • LAETG strongly preferred, low level reactivity observed for other LXETG substrates • Reference [52••]
4S-9 $SrtA_{staph}$ mutant	LPEXG (X = A, C, S)	• Evolved $SrtA_{staph}$ variant • Reaction rates increased relative to wild-type $SrtA_{staph}$ • Substrate selectivity orthogonal to $SrtA_{staph}$ pentamutant and wild-type $SrtA_{staph}$ • Reference [52••]