COMMENTARY

Yet more intramolecular cross-links in Gram-positive surface proteins

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The surface of Gram-positive bacteria comprises a single membrane and, typically, a thick layer of cross-linked peptidoglycan that imparts strength and rigidity. Anchored to this cell wall are protein assemblies, such as pili, and surface proteins, such as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) that act as surface adhesins. These proteins are critical not only for bacterial binding to host surfaces in the early stages of infection but also for biofilm formation and immune evasion. In a series of studies published since 2007, these cell surface proteins and protein assemblies have been shown to contain isopeptide and thioester bonds, highly unusual intramolecular covalent linkages between amino acid side chains. These cross-links have either a structure-stabilizing role or may be directly involved in adhesion. Using an elegant combination of structural biology and mass spectrometry, Kwon et al. (1) reveal in PNAS an ester bond as yet another covalent linkage in a putative MSCRAMM from the Gram-positive pathogen *Clostridium perfringens*. Remarkably, this ester bond, joining the side chains of a Thr and Gln residue, is equivalent to an unresolved acyl-enzyme intermediate, formed on an autocatalytic pathway that resembles the mechanism of serine proteases.

Despite fundamental differences in the way they are encoded in bacterial genomes, following delivery/assembly at the cell surface, pili and many MSCRAMMs share important similarities. In such proteins, a series of repetitive domains (commonly referred to as stalk regions) are responsible for the length of the structure and allow an N-terminal region to be positioned away from the cell surface. At the C terminus, both pili and MSCRAMMs are anchored to the cell wall by sortases (2). Although many of the building blocks of Gram-positive surface proteins and pili bear some resemblance to their Gram-negative equivalents (they are variants of the Ig-like β-sandwich fold), their mechanisms of assembly are very different (3).

As mentioned above, some of the more remarkable discoveries made since the molecular characterization of Gram-positive pili (4, 5) are the prevalence of intramolecular covalent linkages in the component proteins. The first of these bonds was discovered in the stalk protein of pili from the human pathogen *Streptococcus pyogenes* (6). These intramolecular isopeptide bonds have now been characterized experimentally in a wide-range of pilus subunits from many Gram-positive pathogens (reviewed in refs. 7–9) and also in the MSCRAMM FbaB (10). Intramolecular isopeptide bonds are most commonly formed between the side chains of Lys and Asn residues (Fig. 1) [although Lys-Asp bonds also exist (10, 11)]. The residues comprising these bonds are strategically positioned to bridge the first and either penultimate or last

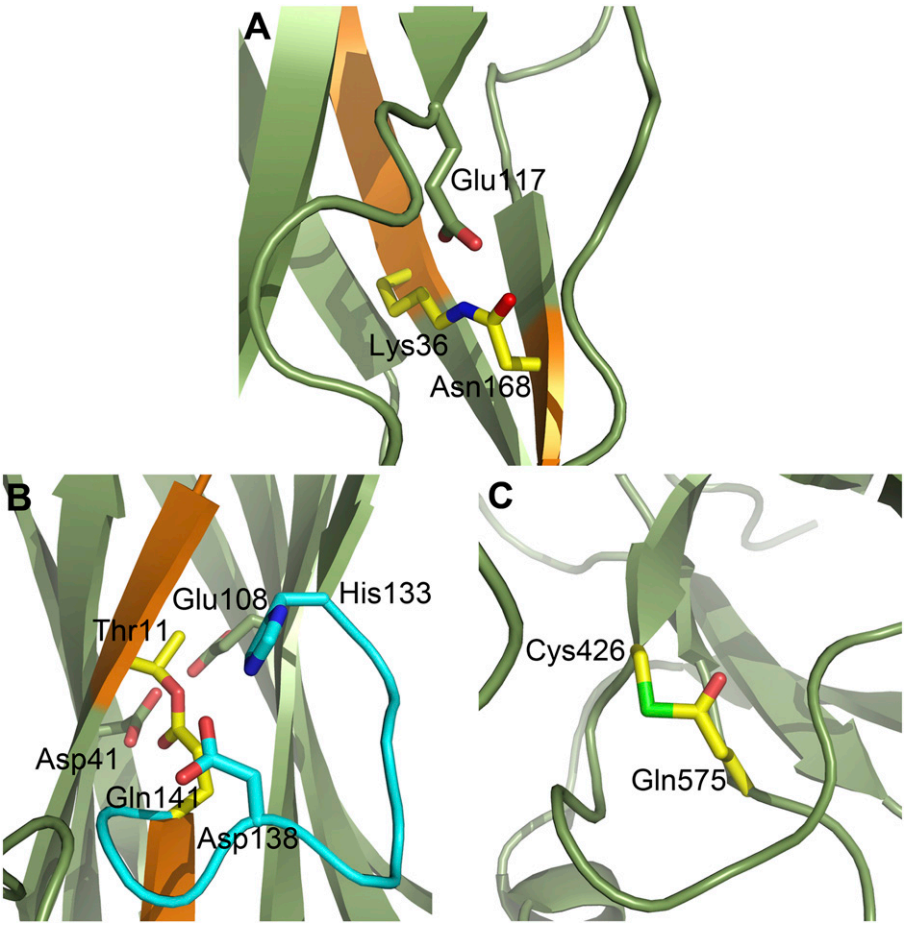

Fig. 1. Intramolecular covalent linkages in Gram-positive pili and MSCRAMMs. (*A*) Lys-Asn intramolecular isopeptide bond in Spy0128, (*B*) Thr-Gln ester bond in Cpe0147, and (*C*) Internal thioester in Spy0125. The side chains of residues are colored: yellow, residues forming the bonds; cyan, catalytic residues in Cpe0147; green, residues contributing to the proton shuttles. In orange are parts of the β-strands that are covalently linked through the bonds.

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β-strand of the Ig-like domains. In this location, the cross-links impart enhanced thermal stability and resistance to proteases (10, 12–15). The residues can also confer remarkable resistance to mechanical stress (16). Site-directed mutagenesis and computational analyses have revealed a mechanism for how these bonds are formed. In the hydrophobic core of the protein domains, the pK_a for the side-chain amino group of the Lys is lowered, allowing nucleophilic attack of the Cδ atom of either Asn or Asp with an adjacent, buried Glu or Asp providing a proton shuttle (10, 17). All that is required is three appropriately positioned amino acids in a hydrophobic environment.

A second unusual intramolecular covalent linkage was discovered in the adhesin presented at the tip of *S. pyogenes* pili. A thioester bond between a Cys and a Gln residue was observed in a shallow cleft on the protein surface (Fig. 1) (11). Such internal thioesters have previously been seen in complement and complement-like proteins of the mammalian innate immune system, where they mediate covalent attachment to pathogen surfaces (18). This relationship, and the observation that mutation of the Cys in *S. pyogenes* pili reduced adherence to model host cells, suggests that this bond may be involved in mediating covalent attachment to host surfaces. Although such covalent linkages of relevance to infection remain to be determined, recent experimental evidence showing that (*i*) the internal thioester in the *S. pyogenes* pilus adhesin does not confer significantly increased thermal stability or resistance to proteases (12) and, (*ii*) internal thioesters have the capacity to react with biological amines, such as spermidine (19), suggests the role of these bonds is different to intramolecular isopeptide bonds. It is not clear from current structural and mutational data how these bonds form.

In PNAS, Kwon et al. (1) add to the list of intramolecular covalent linkages in Gram-positive surface proteins, with the identification of an unprecedented stable ester bond formed between a Thr and a Gln side chain in a putative *C. perfringens* MSCRAMM termed Cpe0147 (Fig. 1). As observed for the intramolecular isopeptide bonds in pilus subunits, the ester bonds in the repeat domains of Cpe0147 are strategically positioned to link the first and last β-strands of the protein domain. Consistent with a role in conferring protein stability, loss of the ester bond by mutation results in reduced thermal stability and increased susceptibility to proteolysis in vitro.

However, perhaps even more intriguing than their identification is the mechanism by which these stable ester cross-links form autocatalytically. This mechanism is very different from that observed for intramolecular isopeptide bonds. Instead of just three appropriately positioned amino acids in a hydrophobic environment, Cpe0147 has acquired a 7-aa insertion in the final β-strand of the repeat domain that forms a "looped-out" structure that alters the canonical Ig-like fold (Fig. 1). On this loop are a His and an Asp residue, the positions of which, when considered with the Thr destined for ester bond formation, adopt a catalytic triad conformation reminiscent of that seen in serine proteases (where the Thr is replaced with a Ser). In this arrangement, the Thr side chain can act as a nucleophile to attack the Cδ atom of the Gln side chain, ejecting ammonia and forming an ester bond (a Glu/Asp pair acts as a proton shuttle). The "active-site" is then locked in a position equivalent to the acyl-enzyme intermediate of serine proteases. Normally, such intermediates would be resolved by a water molecule, regenerating the catalytic site. However, Kwon et al. (1) highlight a neat trick used by Cpe0147. The authors show that the His and Asp residues, positioned on the inserted loop, form a hydrogen bond, and this prevents the His adopting a conformation that could support hydrolysis of the ester. Position seems to be everything as, somewhat surprisingly, even a subtle Thr/Ser substitution appears to prevent stable ester bond formation. However, it is not reported whether this result is because the ester bond never forms or because the full hydrolysis reaction has occurred, which would result in a conversion of the Gln encoded in the bacterial genome into a Glu.

Isopeptide domains are predicted to be present in thousands of Gram-positive surface proteins. Kwon et al. (1) suggest that ester domains are also produced by various bacteria, but it is not yet clear how common they are. It is intriguing that evolution has achieved multiple ways to autocatalyze the formation of intramolecular covalent linkages that confer enhanced protein stability or resistance to stress (in addition to the well-known disulphide bridge, which is rarely found in MSCRAMMs or Gram-positive pili).

The structures, secretion, and assembly mechanisms of many Gram-negative surface proteins, in particular chaperone-usher pili, are well understood (20). Ten years ago, only a little was known about the structures of MSCRAMMs, and the molecular nature of Gram-positive pili had not been described. Now we have representative 3D structures for all components of a complete Gram-positive pilus, and atomic details of the folded repeat domains in some MSCRAMMs are emerging. Because, to date, studies of each of these proteins have generated surprises, it is exciting to contemplate what future analyses will discover. For example, do such bonds exist in proteins produced by Gram-negative bacteria, or indeed, viral, archaeal, or eukaryotic proteins other than components of complement? Because it is essentially impossible to de novo predict the presence of such bonds from sequence alone, we probably have to wait for another discovery of unexpected electron density, deviating molecular mass, or unusual stability for the next breakthrough.

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