Life imitates cookery: lysinoalanine crosslinks in peptides and proteins

George W. Preston

Stoller Biomarker Discovery Centre, University of Manchester, Manchester, UK. E-mail: george.preston@manchester.ac.uk

Lysinoalanine (Lal) crosslinks are modifications that arise spontaneously in proteins during cooking and food processing. They occur when dehydroalanine (Dha) residues, formed under conditions of high temperature and/or high pH, react with lysine residues (Fig. 1A). Interestingly, Lal crosslinks are also found in peptide antibiotics such as duramcyin; and accumulating evidence suggests that they are present in the flagellar 'hook' of certain spirochaete bacteria. The crosslinks observed in antibiotics and flagellar hooks seem to form in a more controlled way than the ones that occur during cooking and food processing. This article highlights recent research on Lal crosslinks and their mechanisms of formation.

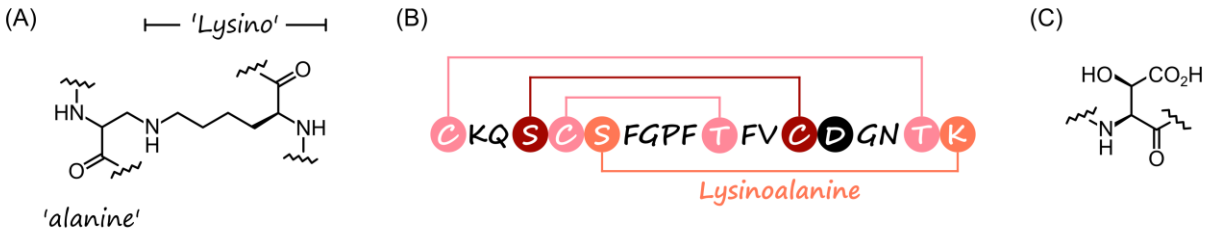

Fig. 1. Structures mentioned in the text. (A) The lysinoalanine crosslink. The 'lysino' part comes from lysine; the 'alanine' part comes from cysteine/cystine/serine *via* dehydroalanine (Dha). The configuration of the 'alanine' part varies depending on the context of the crosslink. (B) Duramycin. A filled circle/white symbol indicates a site of post-translational modification. The other types of crosslink besides Lal are lanthionine and methyllanthionine. (C) The hydroxyaspartic acid residue in duramycin.

COOKING UP CROSSLINKS

The discovery of Lal crosslinks arose from research on the effects of alkali on proteins (Bohak, 1964). Treatment of bovine ribonuclease with aqueous sodium hydroxide (pH 13) was observed to cause the partial loss of two amino acids – lysine and cystine – and the appearance of Lal. The alkali, it seemed, was initiating crosslinking by decomposing cystine to Dha, and then perhaps helping lysine to combine with the Dha. Later it was shown that heat, too, could effect the formation of crosslinks, and that Lal was present in certain processed and cooked foods (Hasegawa *et al*., 1987). Recent analyses of food processed at high pH has shown that, under this condition at least, serine and cysteine are also likely precursors to Dha (Zhao *et al*., 2016). As we will see, these two amino acids have been implicated as precursors to Dha *in vivo*.

RECIPE FOR STEREOSELECTIVITY

Duramycin is an antibiotic synthesised by certain bacteria of the genus *Streptomyces*. It has a complex structure containing not only Lal but also lanthionine-type crosslinks (Fig. 1B). Crosslinking and other post-translational modifications (PTMs) occur within a ribosomally-synthesised polypeptide called DurA. An enzyme called DurM is responsible for most of the PTMs: it dehydrates serine and threonine residues and forms the lanthionine-type crosslinks (Huo *et al*., 2017). Another enzyme, DurX, hydroxylates duramycin's only aspartic acid residue (Fig. 1B, C). Formation of the Lal crosslink is the penultimate transformation, and is followed by the removal of a long 'leader' peptide.
Writing in *Nature Chemical Biology*, An *et al*. present evidence for the participation of a third enzyme, DurN, in the synthesis of the Lal crosslink (An *et al*., 2018). Although DurN was previously uncharacterised, its gene had been cloned and expressed in *Escherichia coli* as part of an earlier study (Huo *et al*., 2017). An *et al*. used the same *E. coli*-based approach to produce DurN (this time as a fusion protein), and also a model substrate containing Dha. To test the hypothesis that DurN was responsible for Lal crosslinking, the authors combined their model substrate with the DurN fusion protein and watched for crosslinking activity. The disappearance of the substrate's electrophilic character was one indication that DurN was effecting crosslinking; the disappearance of diagnostic molecular fragments in tandem mass spectrometry analyses was another.

Further evidence for DurN's participation in crosslinking came from X-ray crystallography of complexes with either duramycin or a substrate analogue. The crystallography not only revealed the full three-dimensional structure of DurN, but also provided clues as to the enzyme's mode of catalysis. Together with the results of single amino acid substitution

experiments, the crystal structures highlighted the importance of the hydroxylated aspartic acid residue (Fig. 1C). This residue, it seems, acts as a base to deprotonate the ammonium group of the incoming lysine residue.
It was surprising, then, to learn that Lal crosslinking could sometimes proceed in the absence of DurX (the enzyme that hydroxylates aspartic acid), and without the involvement of DurN (which requires the hydroxyl group as a sort of cofactor). Careful analysis, however, revealed an important difference between the products obtained in the presence and absence of DurX: the product obtained in the absence of this enzyme was a mixture of isomers (containing different configurations of the 'alanine' part of the crosslink); whereas that obtained in its presence contained a Lal residue of high stereochemical purity. The authors suggest that the stereochemistry of the crosslink might be important for duramycin's antimicrobial activity.

'INSTANT' CROSSLINKS

Spirochaetes, like many other bacteria, use flagella to propel themselves through liquid media (Wolgemuth, 2016). Spirochaete flagella contain structures called hooks, each of which is made from over a hundred copies of a protein called FlgE. Hooks serve to anchor the bacterium's whip-like flagellar filaments, which – unusually – are located in its periplasm (the medium between the inner and outer bacterial membranes). In 2016, Miller *et al*. reported the formation of Lal crosslinks by FlgE from *Treponema denticola*, a species implicated in periodontal disease (Miller *et al*., 2016). These crosslinks differed from the one found in duramycin in three main ways: they were inter- rather than intramolecular; they could form without the assistance of enzymes or other biological factors; and the precursor to Dha was cysteine rather than serine. In a more recent report from the same group, Lynch *et al*. describe a series of *in vitro* experiments exploring FlgE's crosslinking ability in more detail (Lynch *et al*., 2019).

Just as the authors of the duramycin paper had done, Lynch *et al.* obtained variants of their protein of interest by inserting modified genes into *E. coli*. Some of the variants contained single amino acid substitutions, while others had whole domains deleted. An assay involving separation of residual FlgE monomer from a 'high-molecular-weight complex' allowed the authors to measure the effect of changing different amino acids. Experiments involving defective variants (e.g., one containing a lysine-to-alanine substitution) provided evidence that crosslinking is dependent on the non-covalent oligomerisation of FlgE. Experiments with other variants revealed that, in addition to lysine and a Dha-precursor, various auxiliary amino acid residues (e.g., a threonine residue, Thr-334) are also necessary for successful crosslinking. Previous work had shown that the lysine and Dha-precursor (cysteine) residues were essential for proper motility of *T. denticola* (Miller *et al*., 2016).

Lynch *et al*. gained further mechanistic insights by analysing a model FlgE dimer containing a single Lal crosslink. X-ray crystallography of this dimer, and of corresponding monomers, allowed the roles of auxiliary amino acids such as Thr-334 to be inferred. The authors were keen to identify two putative bases: the one that abstracts the α-proton of cysteine and thereby triggers Dha formation; and the one that assists the reaction of lysine with Dha. Interestingly, the most plausible candidates turned out to be water molecules or hydroxide ions (the authors used a buffer of pH 8.5) rather than amino acid residues. Thr-334 and other amino acid residues appeared to form a hydrogen bonding network that enhances the reactivity of the Dha residue towards lysine.

FOOD FOR THOUGHT

Detailed investigations *in vitro* have enabled mechanisms of crosslinking *in vivo* to be inferred. Hopefully further exploration of the functional significance of Lal crosslinks *in vivo* will follow. It will be interesting to see whether any more examples of these unusual structures emerge in the future.

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