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Report:

Penicillin acylases are industrially important enzymes that hydrolyse penicillin substrates to yield 6-aminopenicillanic acid, the beta lactam nucleus of many semi-synthetic antibiotics. The *pac* gene of *E. coli* penicillin G acylase encodes a precursor enzyme that is activated when the linker peptide that joins the A- and B- domains is autocatalytically excised to expose the N-terminal nucleophilic serine. Similarly, penicillin V acylase which is encoded by the *B. sphaericus pac* gene is also activated by an autocatalytic mechanism whereby the first four N-terminal amino acids are removed to create the catalytically active enzyme. The *in vivo* role of these enzymes has yet to be elucidated but it has been observed that regulation of Penicillin G acylase expression is coordinated by both temperature and phenylacetic acid. These observations have given rise to suggestions that the enzyme may function during the free living mode of the organism to metabolise aromatic compounds to generate a carbon source.

Penicillin V acylase has no detectable sequence homology to penicillin G acylase nor are the protein structures similar yet both are members of the structural superfamily, Ntn (N-terminal nucleophilic) hydrolases. Despite lacking any discernible sequence similarity (and variations in domain architecture) the Ntn hydrolases are characterised by a remarkable fold that, in the activated enzyme, results in the single active site nucleophile and associated catalytic site residues occupying equivalent positions. The Ntn-hydrolase superfamily includes the 20S proteasome, glutamine PRPP amidotransferases, glycosylasparaginases and the recent addition, the conjugated bile acid hydrolases. Whilst the enzymes share a similar arrangement in their catalytic environment, the nucleophilic residues differ *e.g.* in Penicillin G Acylase the nucleophile is a serine, whereas in Penicillin V Acylase it is a cysteine.

Structures of both wild type penicillin acylases have been solved by our laboratories. Penicillin G acylase has been extensively studied over a number of years to investigate both substrate stereospecificity and initiation of maturation by autocatalytic processing. However penicillin V acylase is less well characterised. In order to investigate key residues in the active site, the catalytic mechanism of penicillin V acylase and precursor processing, a number of mutants have been constructed where the nucleophilic cysteine has been altered. One catalytically inactive mutant (Cys1Ala) has been crystallised, in a different space group to wild type, in order to study substrate binding. X-ray data have been collected on ID14-4 from two experiments where penicillin V acylase crystals were soaked with substrates, namely penicillin V and cephalosporin. A third data set was collected from an unsoaked crystal. The non-soaked structure was solved by molecular replacement and the two soak experiments were analysed by difference Fourier methods. All three were found to contain uninterpretable stretches of positive difference electron density in the region of the active site. It is thought that the presence of these 'ligands' might be a consequence of the purification procedure, crystallisation and/or cryoprotection conditions. Another possibility is that the molecule is a naturally occurring substrate for penicillin V acylase. In any event the molecules have high affinity for binding in the environment of the active site. It has not yet proved possible to displace these unexpected and uncharacterised molecules. Further constructs have been prepared in order to investigate these phenomena.