Illuminating the Ancient Retainer

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The first crystal structure of a glycosyl enzyme intermediate provides a detailed look at the mechanism of a glycosidase.

On page 149 of this issue of *Nature Structural Biology* Rose and Withers and their co-workers (RWC)[1] report the crystal structure of a glycosyl enzyme—the first detailed structural information on what is turning out to be a common type of intermediate in enzyme-catalysed glycosyl transfer. This is the latest piece in a fascinating multi-dimensional jigsaw that relates structure and mechanism in a major group of enzymes—the glycosidaseswhich begin to rival the familiar proteinases in the range of mechanistic and structural information available. (Another new glycosidase structure, of an antifungal chitosanase from a *Streptomyces*, is reported by Marcotte *et al.* on page 155[2].)

Pieces of a mechanistic puzzle

The first structure in this series—indeed the first enzyme ever to have its crystal structure determined—was lysozyme (from hen egg white, thus HEWL[3]), familiar to successive generations of students of biochemistry and bioorganic chemistry as a textbook example of the productive interaction of structural, enzymological and mechanistic investigations[4]. To summarize: HEWL binds six sugar residues of its substrate (an alternating [(NAGNAM)$_n$] polysaccharide component of bacterial cell walls) to bring the target glycosidic linkage into close proximity with the two carboxyl groups of the active site. Of these, one is active as COOH, the other as carboxylate, lying on opposite faces of the target sugar ring.

The suggested mechanism had the COOH group of Glu 35 acting as a general acid to assist the departure of the leaving group OR (the rest of the sugar chain in Fig. 1), and the COO$^-$ group of Asp 52 stabilizing the positive charge left behind. Exactly how is still not entirely clear[5]: simplest, and chemically most convincing, is nucleophilic participation (Fig. 1) though in the case of HEWL the distances involved, and the failure to observe what ought to be a rather stable glycosyl-enzyme intermediate, made this difficult to accept[6]. Nevertheless the double nucleophilic displacement mechanism is the simplest explanation of the observed stereochemistry of the reaction (overall retention of configuration, as shown in Fig. 1), and intermediates clearly are involved in the mechanisms of other glycosidases using two active-site carboxyl groups.

The most direct evidence for this mechanism comes from work with modified substrates. The one used by RWC[1] (2,4-dinitrophenyl β-cellobioside with the 2-hydroxyl group replaced by fluorine-2FDNPC; Fig. 2) is typical. It has a good leaving group, which needs little or no assistance from the active site general acid, so that the first glycosyl transfer (reaction i in Fig. 2) is relatively rapid and effectively irreversible. In addition, the electronegative fluorine in the 2-position inhibits all nucleo-philic substitutions at the glycosidic centre. (All such substitutions go through transition states with significant build up of positive charge at this centre). The hydrolysis of the glycosyl enzyme (reaction ii in Fig. 2) can thus be slowed sufficiently for it to be observed in favourable cases, and this has been achieved by the Withers group for several different glycosidase reactions, using ^{19}F NMR and electrospray mass spectrometry.

The ^{19}F NMR evidence is good enough to confirm the α-stereochemistry at the glycosidic centre, but not of course to provide details of binding, or of the conformation of the ester-acetal group. The new evidence contained in a crystal structure of the glycosyl-enzyme intermediate shown in Fig. 2, at 1.8 Å resolution, provides these details, and is a key piece of the mechanistic jigsaw. (The only comparable crystal structure available is of a glycosyl derivative of a mutant of the inverting glycosidase T4 lysozyme[7]. Replacing the active-site Thr 26 by glutamate produced a modified enzyme which catalysed the first step of glycoside cleavage, much as in Fig. 1; but the modified T4L was not equipped for efficient hydrolysis of the glycosyl enzyme produced).

Structure of the intermediate

The enzyme under consideration is a β-1,4-xylanase/glucanase from *Cellumonas fimi*, known to cleave β-glycosides with retention of configuration. The crystal structure of the catalytic domain was already available, and the mechanism was known to involve two carboxyl groups, Glu 127 COOH as a general acid and Glu 233 COO$^-$.

The structure of the glycosyl enzyme (shown to be catalytically competent) confirms the α-configuration at the glycosidic centre. The disaccharide lies in a deep cleft in the enzyme, occupying two sugar-binding subsites and covalently attached to the carboxyl group of Glu 233.

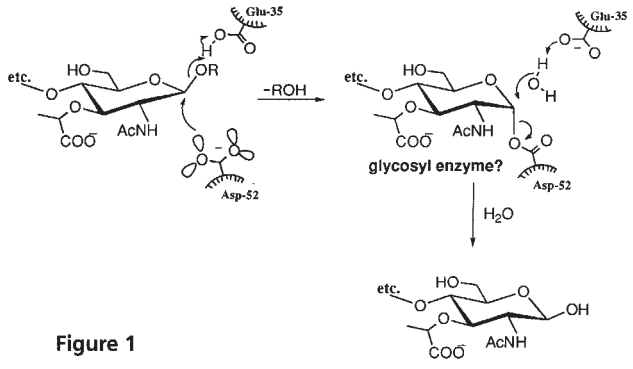

Figure 1

The properties of the new C–O bond (marked with an arrow in Fig. 2) are of special interest: its length is given as 1.6 Å, intriguingly long (thus implying high reactivity, with implications for the HEWL mechanism[6]—though the accuracy is unlikely to be better than 0.1 Å). Further, the conformation about it is also that expected from stereoelectronic considerations, with the (in plane) sp^2 lone pair of the ester oxygen antiperiplanar to the ring C–O bond. The carboxyl group of the active site general acid (Glu 127) lies close to the glycosidic centre, on the side opposite the new C–O bond, apparently in the carboxylate form as shown by its interactions with hydrogen-bond donors at both oxygens. The full set of substrate-binding interactions is mapped out for the disaccharide residue. Most interesting is the environment of the fluorine atom, which replaces the hydroxyl group of the natural substrate, thus removing possibly important hydrogen-bonding interactions, perhaps including one with Glu 233.

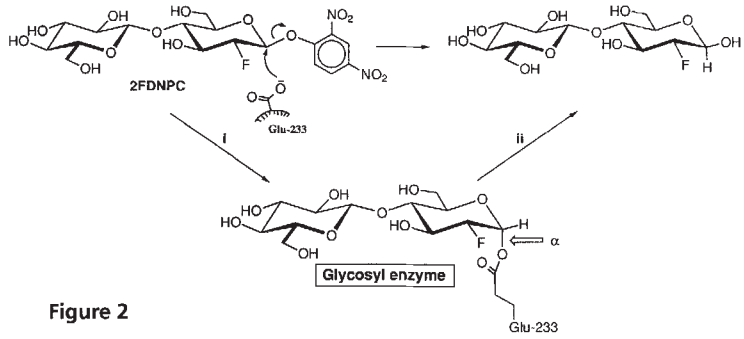

Figure 2

Binding, mechanism and family groups

Binding interactions at the 2-position are of particular interest for enzymes catalysing the hydrolysis of chitosan (Fig. 3), which has a charged NH_3^+ group in this position.

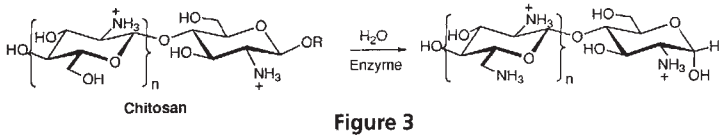

Figure 3

Marcotte *et al.*[2] report the crystal structure of an antifungal chitosanase which shows little sequence homology with familiar glycosidases. However its three-dimensional structure reveals gross similarities to T4 lysozyme (which is itself structurally homologous to HEWL). Thus two roughly globular domains are connected to form a cleft, rich in negatively charged carboxylate residues. Like T4L this glycosidase appears to be an inverting enzyme, and model building using hexaglucosamine identifies a likely active site, with two of the many carboxyl groups lining the cleft in the relative positions associated with inverting glycosidases.

Crystal structures are now available for something like 25 glycosidases. Not surprisingly, given the vast range of substrates involved, these enzymes have evolved a range of different binding modes, clearly reflected in their three-dimensional structures. Thus exoglycosidases like β-glucosidase, operating on the terminal residue of a chain (or, by definition, on a monosaccharide) need only a binding pocket; whereas endoglycosidases like lysozymes, operating in-chain on polysaccharide substrates, bind their elongated substrate in a cleft. There are even instances where loops appear to wrap around a bound polysaccharide substrate, to form a sort of tunnel. The pattern is the now-familiar one, with three-dimensional structures conserved more frequently than primary sequences, and the classification of these structures into groups or families is of great topical interest (for a good introduction to this area see ref. 8).

The common mechanistic problem—how to break a strong bond to a poor leaving group at a highly unreactive glycosidic centre—has a more general solution than those revealed by the binding modes disclosed above. The poor leaving group demands a general acid. Since carboxyl is the strongest general acid available on amino-acid side chains it is no surprise that the COOH group of an aspartic or glutamic acid residue is to be found in the region of the aglycone oxygen. But general acid catalysis by itself is not enough, because it leads to a high-energy oxocarbenium ion, which has to be stabilized or preferably bypassed: either solution requires a second catalytic group, which is often a second carboxyl group, active in the carboxylate form. This may act as a nucleophile (as in the case described by RWC, shown in Fig. 2); or alternatively (presumably, but not simply[6]) as a general base assisting the attack of a molecule of water, as suggested for the chitosanase reaction referred to above[2] and for various other enzymes giving products with retention of conformation. Other groups may also participate, but exactly how they, or even carboxylate, contribute to the extraordinary efficiency of these enzyme-catalysed reactions is still a major unresolved question. The emerging pattern of relationships between binding mode and mechanism—particularly inversion and retention—in glycosidases adds a new dimension to the discussion. But so far even lysozyme, the Ancient Retainer of the title, still has its secrets.

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