

# The lysozyme mechanism sorted — after 50 years

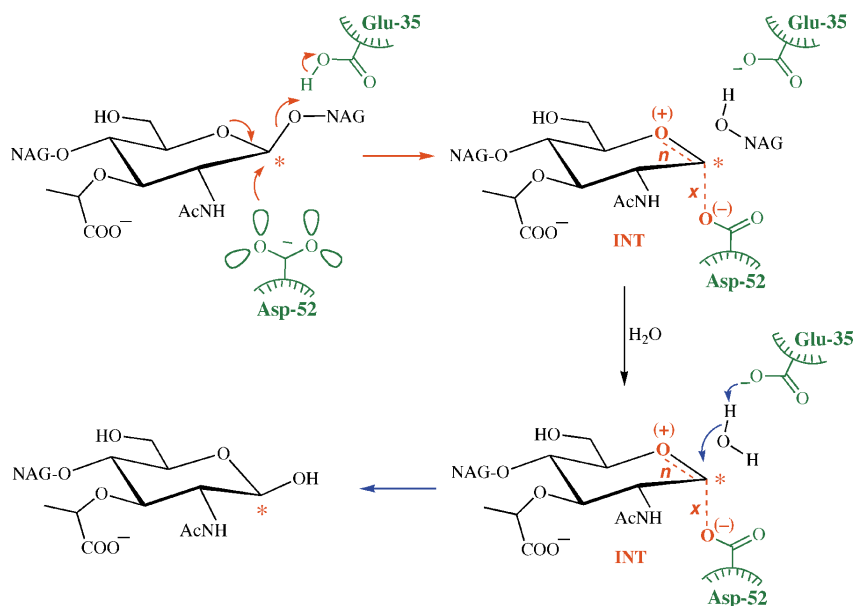
Anthony J. Kirby

Unambiguous evidence for a glycosyl-enzyme intermediate on the lysozyme reaction pathway has recently been reported, finally settling what kind of mechanism this textbook enzyme uses.

The publication in 1965<sup>1</sup> of the hen egg white lysozyme crystal structure — the first such structure of any enzyme — was a major landmark, offering the prospect of detailed explanations of enzyme mechanisms at the molecular level. Such mechanisms involve some of the most subtle relationships between structure and function in all of biology, as enzymes have to recognize and thus stabilize transition states, which probably exist for only femtoseconds. Because the structure of lysozyme was a first, and because of the coherent messages the structure seemed to provide, lysozyme has been a textbook example of enzyme mechanism ever since. Now, in a recent issue of *Nature*, Vocadlo *et al.*<sup>2</sup> report new evidence about the mechanism of lysozyme, information that has been sought after for almost 50 years.

Lysozyme is the most prominent member of the very large class of glycosidases or glycohydrolases, enzymes that catalyze the transfer of a glycosyl group to water. *In vivo* lysozyme catalyzes the hydrolysis of a polysaccharide component of the cell wall of Gram-positive bacteria. To do this it accelerates enormously the extraordinarily slow cleavage<sup>3</sup> of a glycosidic C–O bond (Fig. 1). The early crystal structure work<sup>4</sup> showed that the enzyme binds the substrate in such a way that the atoms of the target C–O bond come within reach of two, and only two, potential catalytic groups, Glu 35 and Asp 52. Once the relative geometries of all the participating groups were defined by crystallography, the scene seemed set for a simple, clear-cut mechanistic conclusion.

In practice, even in this particularly favorable case where the three-dimensional dispositions of the reacting groups were clearly defined, not even the basic reaction pathway could be agreed upon. Bioorganic chemists had — reluctantly — to face the fact that the availability of three-dimensional structural information does not necessarily provide more than the structures of the starting materials for the reaction concerned — information expected to be available for any mechanistic



**Fig. 1** The reaction catalyzed by lysozyme. The substrate is bound so that the leaving group oxygen, the 4-OH group of an N-acetylglucosamine (NAG) residue, is protonated as it leaves by the COOH group of Glu 35. Groups on the enzyme are colored green, electron movements and the key developing bonds and charges in red. Only one of the dashed *exo* and *endo* (*x* and *n*) bonds of the intermediate (INT) is actually present: which one defines the mechanism. Thus *n* is missing in mechanism (i), *x* in mechanism (ii).

investigation. The uncertainty in the lysozyme case involves the timing of bond-making at the reacting glycosidic center (marked with an asterisk in Fig. 1). The cleavage reaction goes with retention of configuration (Box 1) at the glycosidic center — that is, the incoming water molecule becomes attached to the same face of the sugar as the N-acetylglucosaminyl group (NAG-OH) it replaces (defining lysozyme as a retaining glycosidase). A concerted ( $S_N2$ -type) substitution at the reactive carbon would not lead to products with retention of configuration, so the mechanism must involve two steps — and thus an intermediate (Box 1).

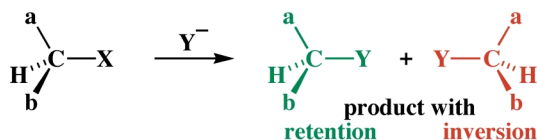
There are two feasible mechanisms involving intermediates. The first, mechanism (i), is a double displacement, in which the carboxylate group of Asp 52 acts in the first step as a nucleophile to form the glycosyl-enzyme intermediate, in

a concerted  $S_N2$ -type reaction — necessarily with inversion of configuration (Box 1). This enzyme carboxylate is then itself displaced from the glycosyl-enzyme intermediate (Fig. 1) by water in a second step, the second inversion restoring the original configuration. The second, mechanism (ii), is a dissociative ( $S_N1$ -type) process (Box 1): in the first step of this mechanism the leaving group departs to leave an intermediate glycosyl cation, with its positive charge stabilized by the lone pair of electrons of the ring oxygen, and electrostatically by the negative charge of the Asp 52 carboxylate. Water then simply adds in the second step to the only accessible face of the cation. The problem is that mechanism (i) would itself be largely dissociative, and involve a substantial build-up of positive charge in the same places as in mechanism (ii), because the ring oxygen plays a similar, crucial role in either

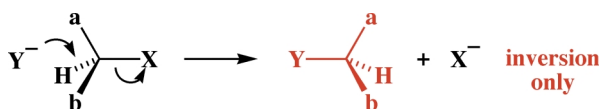
## news and views

**BOX 1 Stereochemistry of substitution is a guide to mechanism.**

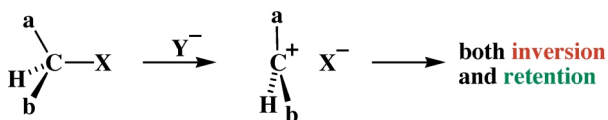
$S_N1$  and  $S_N2$  ( $S_N$  = substitution, nucleophilic) refer here to the mechanisms by which one substituent at a tetrahedral carbon center can replace another.



In principle the product can have the same configuration as the reactant (retention) or its mirror image (inversion). Which is observed depends on the mechanism.  $S_N2$  is a concerted displacement, with 2 molecules coming together (thus associative): it gives only the product with inversion. Retention requires two consecutive inversions, and thus an intermediate that has undergone inversion.



Alternatively, when the structure and conditions are favorable, the reactant can dissociate, unilaterally to a cation/anion pair.



This is the  $S_N1$  mechanism. Because carbocation now bears only three substituents, it is planar; a nucleophile can add from either face — at least if the cation is free in solution. In the active site of an enzyme one face may well be inaccessible. This is the alternative explanation (mechanism (ii)) for the observation of retention of configuration in the lysozyme reaction.

mechanism — without it no reaction would be observed. As a result the usual mechanistic probes, such as secondary kinetic isotope effects, cannot distinguish them. The crucial difference that might settle the debate about which mechanism lysozyme uses is thus the identity of the intermediate, and for over 40 years some of the best groups in the business have tried — and failed — to identify or trap the glycosyl-enzyme intermediate of mechanism (i) or the cation intermediate of mechanism (ii).

The lack of success is perhaps not surprising for the cation intermediate, because such species are known to be very short-lived in the presence of any sort of nucleophile — short-lived enough, at  $\sim 10^{-12}$  seconds (close to the timescale of a single vibration), for some to doubt the existence of such an intermediate in the lysozyme active site on these grounds alone — it simply would not exist long enough to participate in catalysis. The gly-

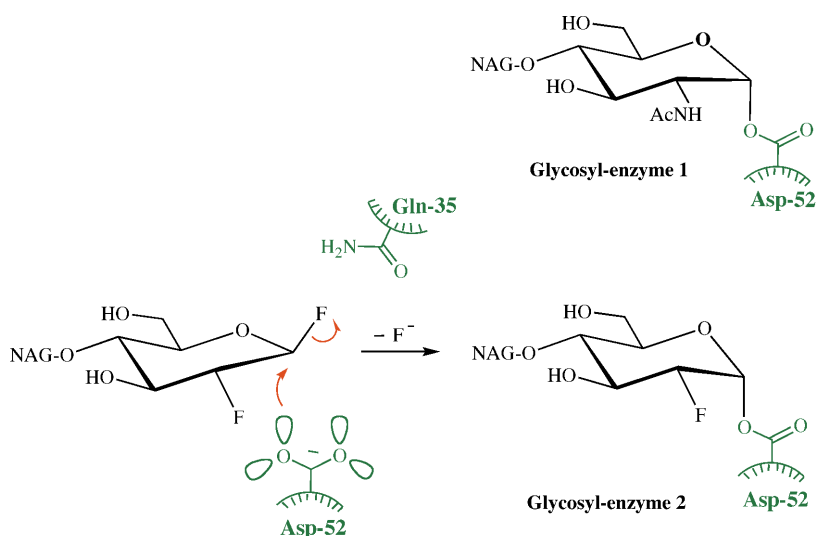
cosyl-enzyme intermediate, on the other hand, is an acyl glycoside, which would be relatively stable in solution (pentaacetyl glucose, for example, is commercially available, and needs no special storage precautions). However, the glycosyl-enzyme has never been observed in reactions of the wild type enzyme; so if it is an intermediate, the enzyme must process it very efficiently. One logical way to raise its

concentration would be to slow down the reactions that remove it.

Vocadlo *et al.*<sup>2</sup> have done this successfully by mutating the active site glutamic acid to glutamine. According to mechanism (i), this would remove the general base catalyzing the attack of water on the intermediate (blue arrows in Fig. 1), and the intermediate should thus survive longer in the modified active site. However, according to mechanism (i), this same group is involved as a general acid in the initial formation of the intermediate, so the formation step has to be artificially accelerated to observe the intermediate. This was done simply by using fluorine, a leaving group good enough to depart without assistance. Thus, when the mutant lysozyme (E35Q) is incubated with NAG<sub>2</sub>-fluoride, the steady state concentration of a new intermediate, which has a mass number consistent with glycosyl-enzyme 1 (Fig. 2), is high enough to be observed by electrospray ionization mass spectrometry (ESI-MS).

Further stabilization of the intermediate and corroboration of mechanism (i) were obtained by replacing the group in the 2-position (NHAc in the natural and most model substrates) of the substrate disaccharide with fluorine. When placed at that position, the highly electron-withdrawing fluorine strongly disfavors reactions at the glycosidic center, allowing glycosyl-enzyme 2 (Fig. 2) to be observed (again by ESI-MS) in the reaction of the wild type enzyme.

Finally, Vocadlo *et al.*<sup>2</sup> combined the two approaches in a study of the reaction of the mutant enzyme (E35Q) with the doubly fluorinated substrate. In this study, the ESI-MS experiment shows stoichiometric formation of the intermediate correspond-



**Fig. 2** Structures of the glycosyl-enzyme intermediates discussed in the text. NAG is the N-acetylglucosaminyl group: its basic structure appears (in black) in glycosyl-enzyme 1, which has a second NAG group attached to the 4-oxygen.

ing to glycosyl-enzyme 2 (Fig. 2), which now lives long enough for a crystal structure determination to be possible. The crystal structure confirms the structure of the intermediate assigned from the ESI-MS results: and the starting enzyme can be regenerated by allowing the reaction of the intermediate to proceed to completion.

The combined results of these three experiments add up to convincing evidence for the covalent intermediate pathway of mechanism (i) in the natural reaction of wild type hen egg white lysozyme. The details of the crystal structure allow further insights into the way the

enzyme adapts to accommodate first the substrate and then the intermediate, and the stereoelectronic requirements of the reaction.

As always, absolute proof of a mechanism is not possible. In this case, the introduction of the fluorine at the 2-position on the substrate not only stabilizes the covalent intermediate, it will also destabilize an intermediate cation (the putative intermediate of mechanism (ii)): the modification thus favoring the one pathway over the other. But lysozyme now sits comfortably in the general class of retaining glycosidases<sup>5</sup> (Box 1), for which a great

deal of evidence supports the nucleophilic mechanism. Textbook authors who favored the S<sub>N</sub>1-type mechanism must now make a note for their next editions.

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## Action at the assemblin dimer interface

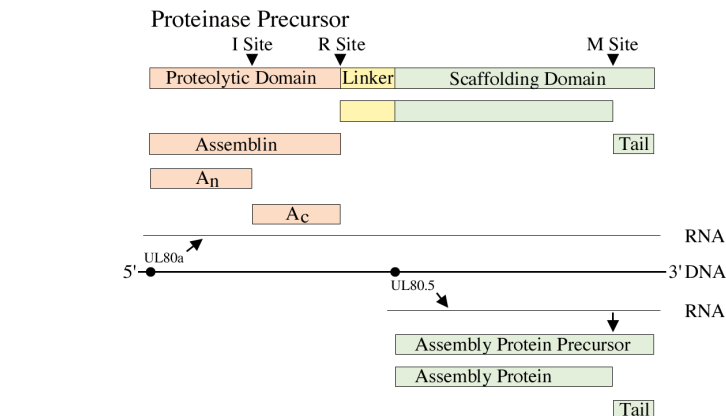
Wade Gibson

A recent study of cytomegalovirus assemblin proteinase suggests that dimer-induced stabilization of the oxyanion hole may activate the enzyme.

Notably unusual from the outset<sup>1–3</sup>, the herpesvirus maturational proteinase continues to provide surprises and new challenges. Essential for the production of infectious virus<sup>4,5</sup>, and the prototype of a new 'clan' of serine proteinases<sup>6</sup>, this enzyme is of specific interest as a potential target for antivirals and of general interest as a model for broadening understanding of the serine proteinase family<sup>7–9</sup>. Propelled by curiosity and the dramatic clinical benefit resulting from inhibitors of the HIV aspartyl proteinase<sup>10</sup>, biochemical, enzymatic and structural studies of the herpes proteinase moved fast. On page 810 of this issue Batra, Khayat and Tong<sup>11</sup> add to our understanding of this intriguing enzyme through studies probing the mechanism of its activation. They have applied a combination of kinetic, biophysical and crystallographic methods to characterize structure-based mutants in the dimer interface of assemblin, the proteolytic domain of the cytomegalovirus proteinase. Based on their data, they propose what may prove to be a new mechanism of dimer-induced activation — indirect stabilization of the oxyanion hole.

### Structure of assemblin

The herpesvirus proteinase is made as a precursor that has been difficult to purify, so most studies have been done with its



**Fig. 1** CMV assemblin is derived from a precursor. The proteinase precursor is encoded by open reading frame UL80a and is autoproteolytically cleaved at the maturational (M), release (R), and internal (I) sites. M site cleavage removes the carboxyl 'Tail' and R site cleavage releases the proteolytic domain, assemblin. I-site cleavage converts assemblin monomers to dimers composed of its amino (A<sub>n</sub>) and carboxyl (A<sub>c</sub>) halves. UL80.5 encodes the proteinase substrate, assembly protein precursor, and is nested, in frame, and 3'-coterminal with UL80a. The amino acid sequence of the assembly protein precursor is identical to that of the proteinase scaffolding domain.

comparatively soluble and well-behaved proteolytic domain, called assemblin. X-ray crystallography revealed that assemblin is structurally distinct from all previously studied serine proteinases<sup>12–15</sup>, enough so to be distinguished as the prototype of a new subclass<sup>6</sup>. Although the spatial relationship of its catalytic triad is conserved with those of other serine proteinases, the triad itself (Ser-His-His) differs from all others characterized by having His as the third mem-

ber in place of Asp or Glu. Also different from other serine proteinases, assemblin has an induced fit mechanism that affords some flexibility in binding substrate, but the binding pocket is shallow and offers few leads for inhibitor design. Important to the work of Batra, Khayat and Tong<sup>11</sup> presented here, the oxyanion hole at the active site, essential for catalysis, involves two adjacent and absolutely conserved arginines. Dimerization is required for the catalytic activity