Structural Studies on N-acetylglucosaminidase Enzyme-Inhibitor Complexes

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The substrate of N-acetylglucosaminidase, the disaccharide di-N-acetylchitobiose, is the primary product of chitinolysis by bacterial chitinases. Purified recombinant enzyme from the soil bacterium *Serratia marcescens* was used in a crystallographic analysis. Based on the structure solution of an abortive enzyme-substrate complex with intact substrate bound in the active site, a reaction mechanism with general acid catalysis is proposed. Only one protein carboxyl side chain is involved, the intermediate is stabilised by the acetamido group of the substrate (anchimeric assistance). A feature of substrate binding is a remarkable distortion of the inner, non-reducing sugar ring towards the reaction transition state. A comparison of the chitobiose complex with complexes using other compounds such as the inhibitors 2-acetamido-2-deoxy-D-gluconhydroximo-1,5-lactone (Lognac) and O-(2-Acetamido-2-deoxy-D-glucopyranosylidens)-amino N-phenylcarbamate (Pugnac) as well as the analogous binding of glucose give further insight into the mechanism of this enzyme. The tri- and tetrasaccharides, chitotriose and chitotetraose, cannot be bound to the enzyme under crystallisation conditions, but the disaccharide cellobiose binds to two further affinity sites outside the active site pocket. As the two sites align with the chitobiose binding sites, higher oligosaccharides may well be bound and degraded by the enzyme.

Keywords: N-acetylglucosaminidase, chitobiase, Serratia marcescens, inhibitor, oxazoline, lactone, phenylcarbamate, hydrolysis, catalysis, mechanism, structure, 3-D structure.

Introduction

The key enzymes of the chitinolytic enzyme cascade from the soil bacterium *Serratia marcescens* are chitinases, of which chitinase A dominates, and at least one chitobiase or N-acetylglucosaminidase. *Serratia marcescens* is one of the most active chitinolytic bacteria possessing enzymes with high activity over a wide pH range (1). The enzymes are of particular biotechnological interest with potential application as biological control agents (2).

The structure solution of chitinase A (3) and chitobiase (4, 5) which have been presented before show an eight stranded βα-barrel as catalytic domain for both enzymes. Chitinase A has three domains, chitobiase four. The enzymes are classified into glycosyl hydrolases families 18 and 20, respectively (6, 7). Though the reaction mechanisms by which chitinase A cleaves the chitin polysaccharide and chitobiase hydrolyses the disaccharide are related, the nature of the active site is different. Chitinases posses a groove through which the substrate slides, chitobiase, an exo-enzyme, possesses a pocket in which the substrate docks with its non-reducing end. A comparison is presented elsewhere in this book and relates to other known chitinase structures (8).

Protein-substrate and protein-inhibitor binding studies in the actual protein crystal are the key to understanding an enzymatic reaction mechanism at the structural level. Alongside the structure solution, this is presented for chitobiase with the rare finding of an apparently abortive enzyme substrate complex in which the substrate is bound in the active site pocket of the enzyme with an intact scissile bond (5, 9). There may be several reasons for this: the high salt concentration of 65% ammonium sulphate and the low pH of 5.6 present in the crystallisation buffer or the restricted flexibility of the crystal lattice.

Methods

Crystals were grown as previously described. For this study, native enzyme crystals were briefly transferred to crystallisation mother liquor to which the desired compound has been added. The crystals were kept in this buffer for about 2 to 10 minutes. Longer soaks reduce the crystal order and lower the diffraction limit. The presented high resolution data were collected to a resolution of 2 Å or better, low resolution data extend to 4 Å. Single crystals were used with an EMBL imaging plate scanner at the EMBL beamlines, HaSyLab, DESY, Hamburg. The electron-density maps shown here were calculated as the difference between the native protein structure and the complexes.

The natural substrate chitobiose

Binding of the chitobiose substrate to the enzyme leads to a distortion of the disaccharide in which the two sugar planes are twisted around the glycosidic

linkage by 90°, and also tilted around this bond so that the two sugar rings are nearly perpendicular to one another. This has an important consequence for the sugar residues: they cannot form the hydrogen bond from OH3 of one ring to O5 of the other ring, an interaction known to stabilise extended polysaccharide fibres in β-1,4-linked polysaccharides. A second, even more severe distortion is observed at the inner, non-reducing sugar ring which is in a conformation close to the energetically unfavourable half-chair. This distortion is forced on the substrate by tight sandwiching of this inner sugar ring with three tryptophans and well defined polar interactions with all polar atoms - except for O5 - between this sugar residue and the enzyme. In contrary, the reducing sugar ring which is found undistorted in the usual $^4C_1$-chair packs only against one tryptophan and does not form any direct hydrogen bond with the protein.

Of particular interest are the distortions of the N-acetyl-group of the non-reducing sugar moiety. This group is bent under the plane of the sugar towards the anomeric C1 to aid catalysis through the nucleopilic carboxyl-group. Two polar interactions and the tight sandwiching between two of the aforementioned three tryptophans from the enzyme stabilise this conformation.

Figure 1 shows the difference electron density between the native structure and the derivatised enzyme-substrate complex. Three amino acids are shown: on the left the arginine to which the substrate docks with the OH3 and OH4 groups of the non-reducing sugar residue, in the middle the catalytic glutamate Glu540, and on the right tyrosine 669 which is one of the two residues forming polar interactions with the N-acetyl group provoking its distortion. These residues will appear throughout this paper. For a complete picture of substrate binding please refer to (5) and (9).

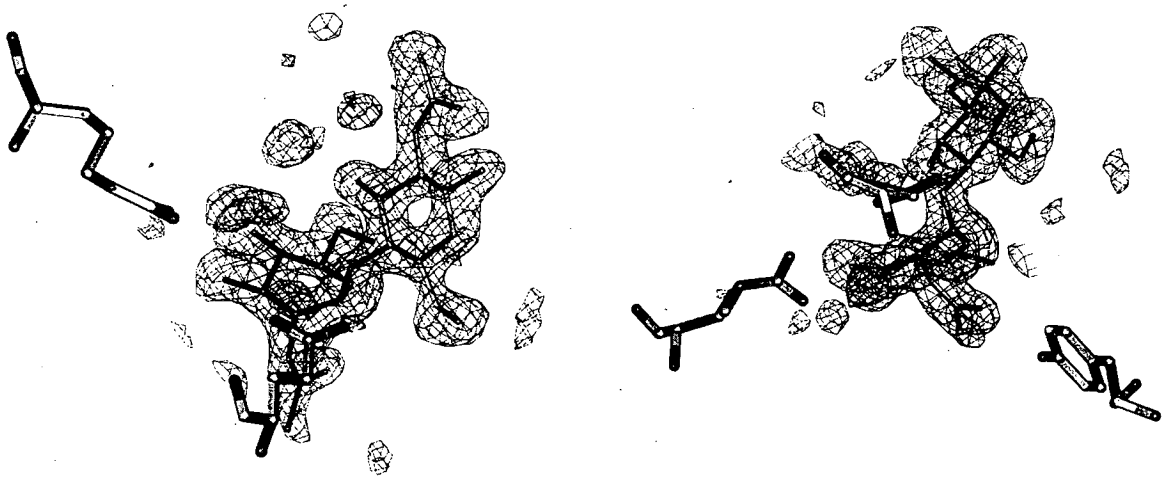

Figure 1: Difference density map between the native protein and the chitobiose-enzyme complex viewed in two orienations. The refined sugar is overlaid, the amino acids shown (from left to right) are Arg349, Glu540 and Tyr669.

Reaction mechanism of N-acetylglucosaminidase

The interpretation of the experimental data is aided by knowledge from organic chemistry. The different reaction kinetics of C2 sugar derivatives as supposed to underivatised substrate are well established (10). The acetamido-group has the particular feature of being able to form an oxazoline intermediate, from which, for stereo chemical reasons, the reaction proceeds with retention of the anomeric configuration. This fact is often utilised in organic chemistry syntheses (11).

As there are usually two protein carboxyl groups participating in enzymatic glycosyl hydrolysis (12) and as there are is no second group identified in N-acetylglucosaminidase at an appropriate distance, the proposed reaction mechanism under anchimeric assistance of the N-acetyl-group proceeds with overall retention of the anomeric configuration via formation of an oxazolinium ion as shown in Figure 2. Experimental support for retention comes from the homologous hexosaminidases for which the N-acetyl-group has also been shown to play a role in catalysis (13) and for *Serratia marcescens* N-acetylglucosaminidase itself (Armand, Henrissat, Davies, unpublished data).

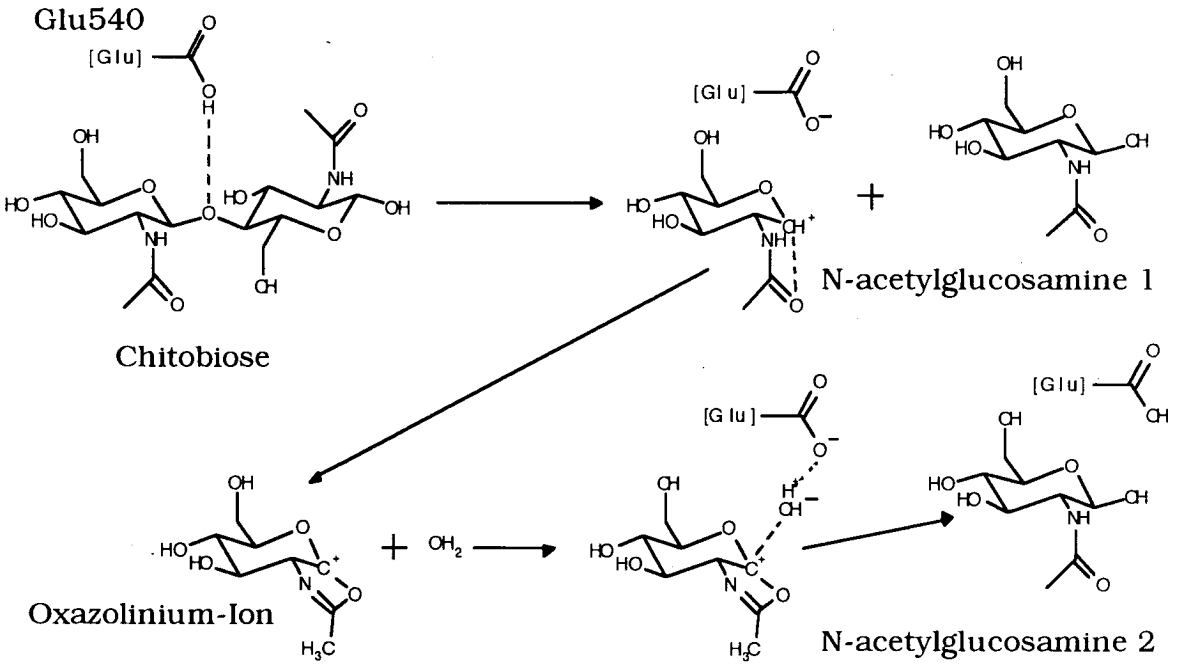

Figure 2: The reaction mechanism of chitobiose hydrolysis by N-acetylglucosaminidase involves only one catalytic residue, Glu540.

The initial event in the reaction mechanism as shown in Figure 2 is the formation of a hydrogen-bond between the catalytic acid Glu540 and the oxygen of the glycosidic linkage, followed by cleavage of this bond. While the first N-acetylglucosamine diffuses out as product, the second one is stabilised as oxazolinium ion in the enzyme. A water can diffuse in, its OH- group completes hydrolysis, and the proton restores the catalytic acid Glu540 while this second sugar leaves the enzyme as product.

Inhibitors of N-acetylglucosaminidase

Two well characterised synthetic inhibitors (14) have been tested with the enzyme for their mode of binding: the inhibitors LOGNAC (2-acetamido-2-deoxy-D-gluconhydroxymo-1,5-lactone and PUGNAC (O-(2-Acetamido-2-deoxy-D-gluco-pyranosylidens)-amino-N-Phenyl-carbamate) as shown in Figure 4.

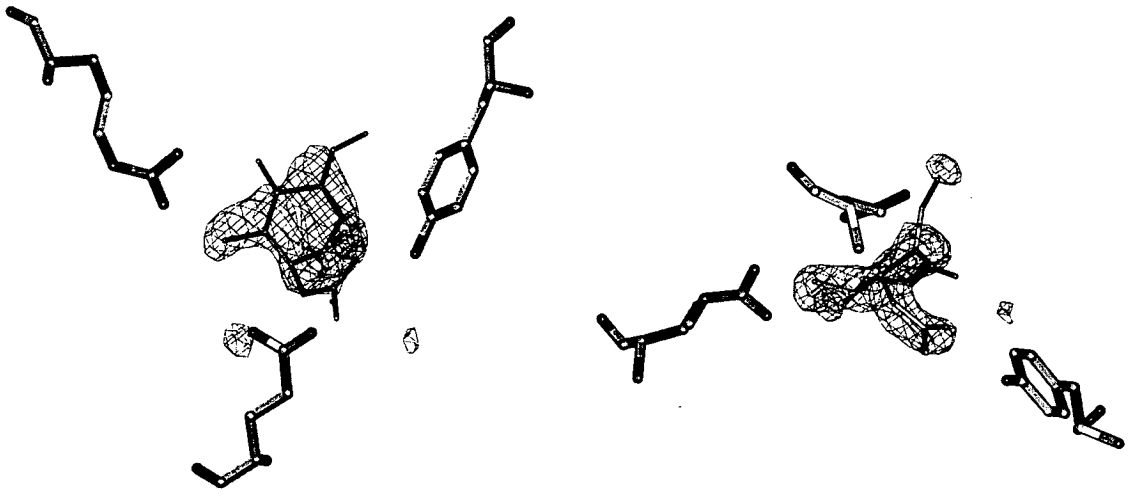

Figure 3: Difference density map between the native protein and the inhibitor-enzyme complexes. The refined compound LOGNAC is overlaid with the electron density.

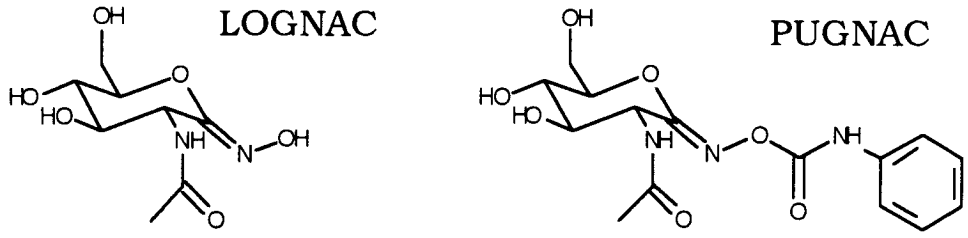

Figure 4: N-acetylglucosaminidase inhibitors.

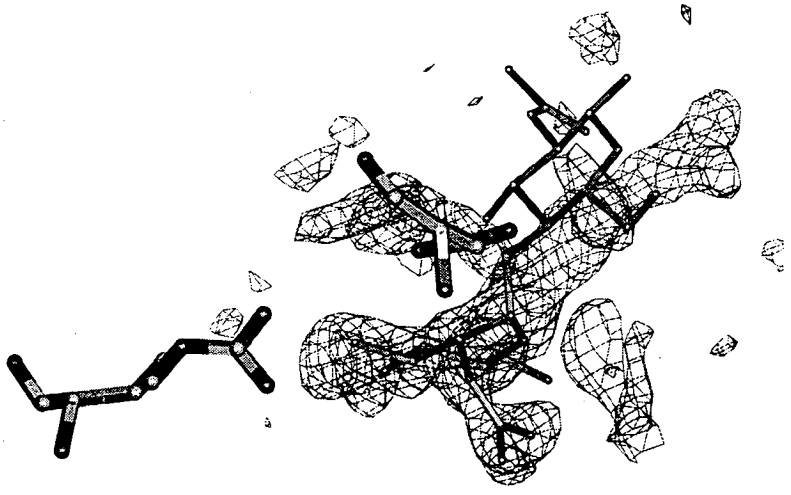

Figure 5: Difference density map between the native protein and the inhibitor-enzyme complexes. The chitobiose molecule is overlaid with the difference density for PUGNAC.

A remarkable feature of the enzyme-inhibitor complexes is the almost identical binding of the non-reducing sugar ring with the substrate: this sugar is distorted to the 4 sofa conformation with the N-acetyl group bent towards the anomeric C1 as discussed before. In LOGNAC, Figure 3, Glu540 binds to nitrogen rather than to the glycosidic oxygen, and the proximal OH group of the oxime superimposes ideally with the reducing sugar of chitobiose. No atom of this compound takes a position that is not taken by the atoms of the substrate, underlining the conclusions for the reaction mechanism as derived from the abortive enzyme-substrate complex. PUGNAC, however, is different as the amino-N-phenylcarbamate is extended and does not superimpose with the reducing sugar moiety of the substrate enzyme complex. For comparison, the difference electron density of this compound in Figure 5 is overlaid with the substrate structure.

Glucose and Cellobiose

To test whether the distortion of the sugar residue is a feature of the derivatisation with the N-acetyl group in the C2 position, underivatised sugars have been tested against the enzyme. These include studies on Glucose and on Cellobiose, Figure 6. While glucose binds in the active site pocket, Figure 7, cellobiose does bind outside this pocket, Figure 8.

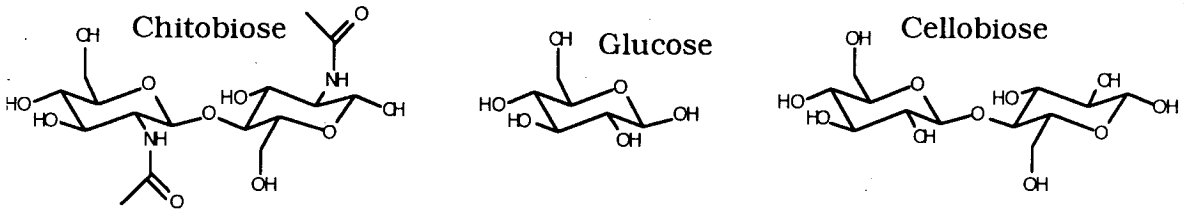

Figure 6: Saccharides that can be bound to N-acetylglucosaminidase.

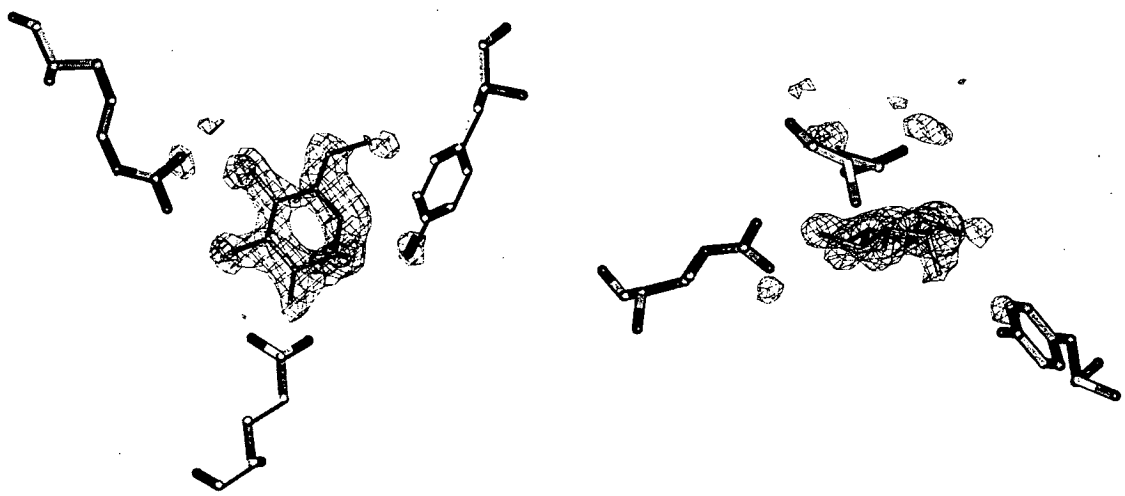

Figure 7: Difference density map between the native protein and glucose complexes. The refined sugar is overlaid with the electron density map.

The binding of glucose is similar to the binding of LOGNAC and the binding of the non-reducing sugar of the substrate chitobiose. However, the sugar is not distorted and is bound in the usual $^4C_1$-chair. It can be assumed that the interactions around the N-acetyl-group are critical to force the distortion towards the 4 sofa conformation.

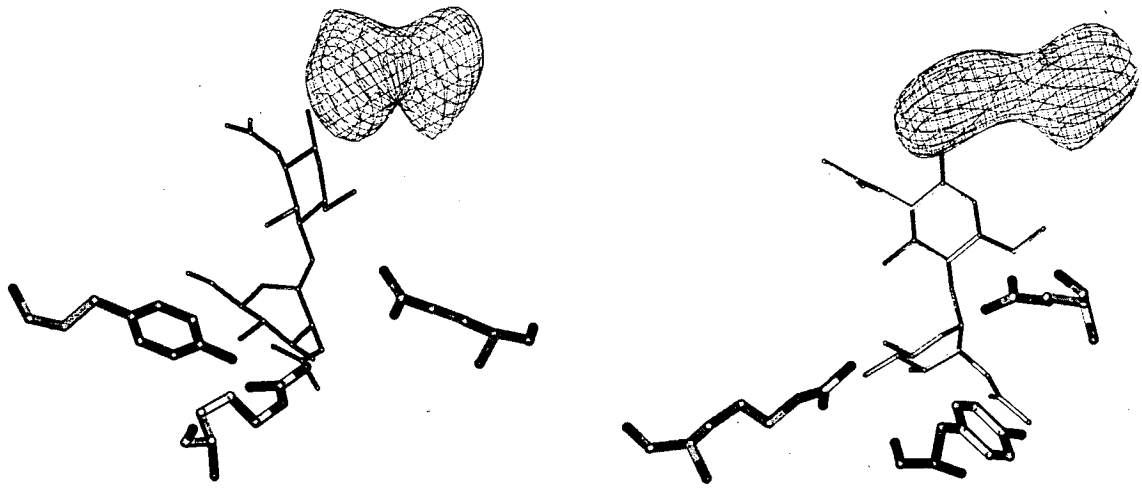

Figure 8: Low resolution difference density map between the native protein and cellobiose complexes. The position of chitobiose from the substrate complex is shown.

Cellobiose does not bind in the active site pocket. However, binding is observed in two sites outside the binding pocket that align with the two binding sites for the chitobiose molecule, as shown in a low resolution study in Figure 8. If there are affinity sites outside the binding pocket, it is possible to conclude a mode for the exo-cleavage of longer oligosaccharides than the dimer chitobiose: the terminal non-reducing end would be cleaved off such an oligomer. Binding studies with these compounds, trisaccharide chitotriose and tetrasaccharide chitotetraose, proved unsuccessful as these oligosaccharides cannot be dissolved in the crystallisation buffer in a sufficient concentration.

Towards binding Product

An obvious experiment from here is to try to bind the product of the reaction, monomeric N-acetylglucosamine, to the enzyme. The most surprising result of this study is the observation of bound disaccharide to the enzyme giving rise to an identical picture as in Figure 1. The reason for this can be twofold: the monosaccharide used for the study could have had disaccharide impurities, or the enzyme is able to perform transglycoslylation under the conditions in the crystal. To test this hypothesis, several crystals have been analysed in which the product has been titrated against the enzyme. Using low concentration of product, binding of a single monosaccharide in the outer site can be observed. The conclusion of this could be that whenever product is bound to the inner site, i.e. in position of the non-reducing ring of chitobiose in Figure 1, this sugar must be distorted as

discussed before. However, as this represents the high energy ground state of the saccharide, it might be restricted to the substrate disaccharide under the high-salt conditions of the crystallisation buffer and the limited flexibility of the crystal lattice. An experiment with the transition state analogue oxazoline has revealed the same result, in which bound disaccharide is observed, Figure 9.

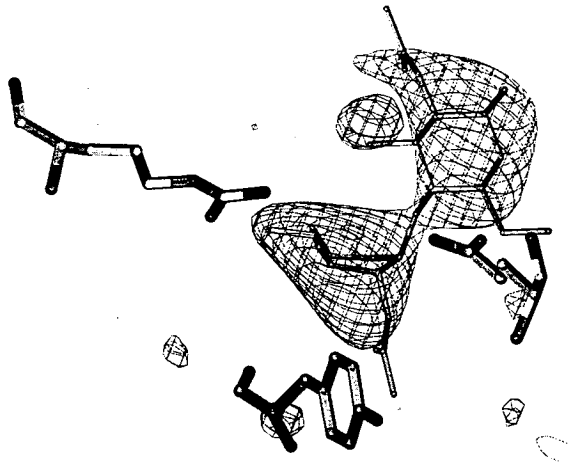

Figure 9: Low resolution difference density map between the native protein and oxazoline complexes. The position of chitobiose from the substrate complex is shown.

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1 Roberts, R.L., Cabib, E., *Analyt. Biochem.* 127, 402 (1982).

2 Oppenheim, A.B., Chet, I., *Tibtech.* 10, 392 (1992).

3 Perrakis, A., Tews, I., Dauter, Z., Oppenheim, A.B., Chet, I., Wilson, K.S., Vorgias, C.E., *Structure* 2, 1169 (1994).

4 Tews, I., Vincentelli, R., Perrakis, A., Dauter, Z., Wilson, K.S., Vorgias, C.E., 'Chitin World', Karnicki, Z.S., Wojtasz-Pajak, A., Brzeski, M.M., Bykowski, P.J. (ed.) [1993], 416-423.

5 Tews, I., Perrakis, A., Oppenheim, A.B., Dauter, Z., Wilson, K.S., Vorgias, C.E., Nature Struct. Biol., in press.

6 Henrissat, B., *Biochem. J.* 280, 309 (1991), Henrissat, B., Bairoch, A., *Biochem. J.* 293, 781 (1993).

7 Tews, I., Vincentelli, R., Vorgias, C.E., Gene 170 [1996], 63-67.

8 Perrakis, A., Tews, I., Vorgias, C.E., this volume.

9 Tews, I., Wilson, K.S., Vorgias, C.E., in: 'Advances in Chitin Science', Dormard, A., Jeuniaux, C., Muzzarelli, R., Roberts, G. (ed.) [1995], 26-33.

10 Koshland, D.E., *Biol. Rev.* 28, 416 (1953).

11 Paulsen, H., *Angew. Chem. Int. Ed. Engl.* 21, 155 (1982).

12 Sinnot, M.L., *Roy. Soc. Chem.* 259 (1987).

13 Lai, E.C.K., Withers, S.G., *Biochem.* 33, 14743 (1994).

14 Horsch, M., Hoesch, L., Vasella, A., Rast, D.M., *Eur. J. Biochem.* 197, 815 (1991).