Structural aspects on the catalytic mechanism of Chitinases, Hevamine, and Chitobiases "Far away and yet so close?"

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Abstract

The structure determination of Chitinase A, Hevamine and Chitobiase have provided a wealth of information about these enzymes. Structural and biochemical data on substrate/inhibitor - enzymes interactions have given new insights on the catalytic mechanism of these chitin hydrolases. Three non-homologous families of glycosyl hydrolases "*Far away*", have evolved for the cleavage of chemically the same bond, but in different substrates; yet the two of these families 'invented' the same molecular machinery, unique among known glycosyl hydrolases, "*so close*"

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chitinases, chitobiases, catalytic mechanism.

Introduction

Chitinases, hevamine, endo-β-*N*-acetyl-glucosaminidases and chitobiases, are glycosyl hydrolysing enzymes which cleave the β-1,4 glycosidic bond between adjacent *N*-acetyl-glucosamine (NAG) residues. These enzymes, based on sequence similarities, are classified in glycosyl hydrolases families 18, 19 and 20 [1].

Family 18 proteins consist of viral, bacterial, fungal, insect, reptile and plant chitinases. They are variable in length and some of them have a multidomain structure. Other members of that family are Endo-β-*N*-acetyl-glucosaminidases, which hydrolyse the glycosidic linkage between the core *N*-acetyl-glucosamine residues of asparagine-linked oligosaccharides in glycoproteins, with the minimum substrate required being Man-α(1,3)-Man-α(1,6)-Man-β(1,4)-GlcNAc-β(1,4)-GlcNAc-Asn. A small subgroup of this family is comprised of eucaryotic chitobiases with a specificity for lower oligomers of β-1,4 linked *N*-acetyl-glucosamine residues. A final group contains the mammalian oviductal proteins which are secreted by ovaries and are heavily glycosylated. However no specific function has yet been assigned to them [2]. Although family 18 contains the most diverse enzymes in terms of primary structure, it is the best studied from a structural point of view.

Family 19 comprises a group of well conserved plant chitinases, which can be differentiated in two distinct subgroups, one of them bearing an N-terminal „cysteine-rich" domain. Plant chitinases are part of a group of proteins designed to restrict the growth of plant pathogens (elicitors).

Family 20 is a relatively small family and contains bacterial chitobiases and eucaryotic hexosaminidases. The reaction catalysed by hexosaminidases is the hydrolysis of the β-1,4 linkage between *N*-acetyl-galactosamine and galactose, whereas chitobiases mainly hydrolyse two β-1,4-linked *N*-acetyl-glucosamine moieties (chitobiose). The bacterial chitobiases are rather conserved with 42% to 54 % overall sequence identity. Most of the eucaryotic hexosaminidases are highly related with up to 80% overall identity.

Current structural data

The crystal stuctures of chitinase from *Serratia marcescens* [3], Hevamine from *Hevea brasiliensis* [4] and Endo-β-*N*-acetyl-glucosaminidases F1 [5] and H [6] from *Flavobacterium meningosepticum* reveal an $(\alpha\beta)_8$-barrel common fold for family 18 enzymes. The crystal structure of a family 19 Chitinase [7] indicated a completely different fold, resembling remotely that of lysozyme. The high resolution structures of Chitobiase from *Serratia marcescens* (family 20) both in its native form and in complex with the natural substrate, a substrate analog and an inibitor were more recenty determined [7,8]. The fold of family 20 proteins is similar to that of family 18 and most other glycosyl hydrolases, i.e an $(\alpha\beta)_8$-barrel. The major difference resides in the absence of the catalytic groove observed in family 18 enzymes, which is substituted with a pocket. This reflects the difference in substrate specificty (polymeric chain versus short oligomer and exochitinase versus endochitinase).

Structural data on carbohydrate binding: How would a substrate bind?

The mode of binding of a carbohydrate substrate on its hydrolase is difficult to establish directly by X-ray crystallography since the substrate is rapidly degraded. The first insight was provided by the X-ray structure of a$(NAG)_3$ (a strong competitive inhibitor of lysozyme) with lysozyme. The results of this study provided the initial model for protein-carbohydarte interactions. The first protein-carbohydrate review appeared in the middle of '80s. Since then, the number of well refined proteins with bound carbohydrates is constantly increasing. We are going to summarise the available structural data on the enzymes of family 18, 19 and 20 and try to explain their substrate specificities at the molecular level.

Family 18 chitinases

The structures of hevamine with $(NAG)_3$ and allosamidin [9] and Chitinase A with $(NAG)_2$ and allosamidin have been determined [unpublished results]. The binding of the sugars to these three proteins exhibit numerous differences as well as striking similarities. These enzymes, together with Endo-β-*N*-acetyl-glucosaminidases F1 and H, share a common evolutionary origin and subsequently a common fold, but their specificities were adapted to slightly different substrates (Chitinase A and Hevamine in contrast to Endo-β-*N*-acetyl-glucosaminidase F1 and H). They have also acquired differences that led to changes in specificity and efficiency of catalysis (Lysozyme activity of Hevamine in contrast to Chitinase A which in turn is a more efficient enzyme for the cleavage of chitin).

From the elucidation of the structure of Hevamine and Chitinase A with $(NAG)_3$ and $(NAG)_2$ oligomers, respectively and allosamidine, several subsites have been defined for the binding of the sugar substrate in the cleft (groove) around the catalytic site. We describe them briefly in an attempt to explain the differences and the features realated to the specificity of the enzymes of family 18.

The (+2) subsite in Chitinase A is mainly defined by the stacking of the aromatic residue Phe396 against the hydrophobic face of the sugar. Two direct hydrogen bonds are present: (i) between the carboxylate oxygen of Asp391 and the N2 of the N-acetyl group of the sugar and (ii) between the Nζ atom of Lys320 and O8 atom of the sugar. The Phe396 ring which is characteristic for the (+2) site in Chitinase A is absent in Hevamine. This explains the apparent higher affinity of Chitinase A for chitobiose in this subsite as observed in the crystal of this complex. In contrast, in Hevamine no sugar was bound in this subsite. The (+2) site is completely missing in Endo-β-*N*-acetyl-glucosaminidase F1, reflecting the difference in substrate specificity, since in that position a linkage with an Asn residue is expected.

The (+1) subsite in Chitinase A is defined by the aromatic ring of Trp275 which is packed against the hydrophobic face of the sugar. No direct hydrogen

bonds exist with this sugar and the affinity is modulated via 3 water molecules by mediating hydrogen bonds betwen the O3 and O6 sugar atoms and the protein.

The Trp275 ring of Chitinase A is not present in either of the enzymes. This supports the assumed higher affinity of this site in Chitinase A compared to Hevamine. Upon cleavage of peptigoglycan from Hevamine a (*N*-acetyl-muramate=NAM) NAM residue is expected to occupy that site. However, there are no obvious structural reasons why a NAM residue could not be accomodated at that position in Chitinase A. The fact that this ring in Endo-β-*N*-acetyl-glucosaminidase F1 can form more favourable hydrogen bonds, reflects the need for the tight binding of this sugar, since this is the only interaction for the docking of the 'product' part of the substrate.

The distance of the O4 atom of the sugar from the carboxyl oxygen of the catalytic residue Glu315 is 3.2 Å in Chitinase A. This distance is longer than expected for nucleophilic attack. Upon binding of the rest of the substrate at the (-) sites, a slight movement of the sugars bound in the (+) sites is to be expected without influencing the hydrophobic packing. It can lead to the formation of more direct hydrogen bonds with that sugar ring.

In Chitinase A and Hevamine complexed with allosamidin, an aromatic ring (Trp539) is packed against the hydrophobic face of the double ring of allosamidin, defining **subsite (-1)**. This Trp residue is present in all three enzymes. The interactions of the oxazoline group in the Chitinase A-allosamidin complex are similar to the ones described for the complex with Hevamine. They are also well conserved in Endo-β-*N*-acetyl-glucosaminidase F1.

The second sugar in **subsite (-2)** forms hydrogen bonds to the carboxyl oxygens of Glu540 and Glu473 with the O7 and N2 atoms of the *N*-acetyl group respectively. The O6 atom of the sugar hydrogen bonds with the main chain nitrogen of Thr276.

The (-3) subsite is defined in Chitinase A by Trp167, which packs almost perfectly against the sugar ring as modelled in Hevamine, where that Trp is absent. The two direct hydrogen bonds in Hevamine are disrupted and presumably substituted by the hydrophobic interaction. This is a very interesting

subsite since in Chitinase A it should only bind NAG, in Hevamine either NAG or NAM and in Endo-β-*N*-acetyl-glucosaminidase F1 a mannose.

The sugar in **the (-4) subsite** of Chitinase A binds in a different manner than in Hevamine. On moving further from the active site it might be expected that the mode of binding is less well conserved and this sugar ring could adopt a different position. This site is completely lost in Endo-β-*N*-acetyl-glucosaminidase F1 reflecting the difference in substrate specificity. Of interest is the 'exchange' of the two direct hydrogen bonds in Hevamine for the hydrophobic interaction with Trp in Chitinase A.

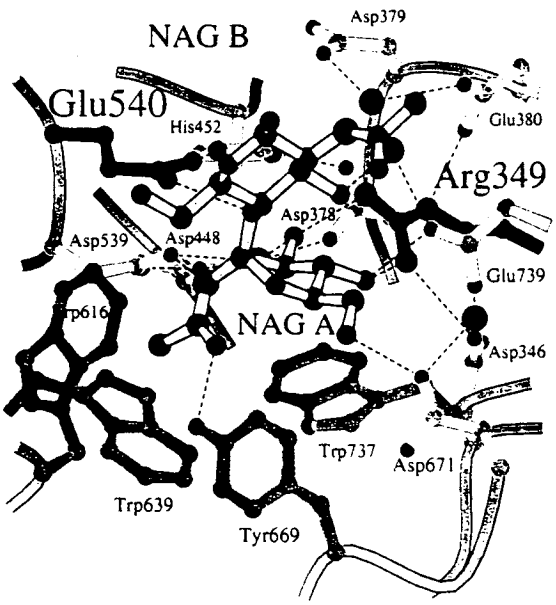
From our modelling studies on Chitinase A and allosamidin it is possible that there is one more **binding site (-5)** which does not exist in Hevamine or Endo-β-*N*-acetyl-glucosaminidase F1, H.

The molecular surface of the area of the catalytic grrove of chitinase A; gray shades represent hydrophobic and aromatic residues. The bound 2-NAG (magenta) and allosamidin (dark blue) in the two complex structures are both drawn as stick models. (Drawn by GRASP)

Family 20 chitobiase

The complex of Chitobiase with ist natural substrate $(NAG)_2$ is a rare example of a complex of an enzyme with its intact substrate. The binding/active site of chitobiase for it's substrate $(NAG)_2$ lies at the C-terminal end of the $(\alpha\beta)_8$-

barrel and is a boot-shaped pocket. A major feature of the bound $(NAG)_2$ is that the two sugar planes are tilted around their glycosidic linkage by about 90° which disrupts the intramolecular H-bond between O5A and OH3B stabilising the extended chain conformation in β-1,4 linked glycosides. The details of this binding of $(NAG)_2$ to Chitobiase can be summarised as follows: (i) All polar atoms– except O5A –of the non-reducing sugar ring NAGA make direct H-bonds with the protein. The reducing sugar ring NAGB, accessible from the surface, makes only indirect H-bonds with protein groups via bridging water molecules. (ii) While NAGB is in the typical $^4C_1$ chair, NAGA is disorted to a conformation close to the energetically unfavourable 4 sofa. (iii) Four tryptophans line part of the binding pocket complementary to the hydrophobic surfaces of the sugar rings. (iv) NAGA is 'docked' to the enzyme by H-bonds from OH3A and OH4A to Arg 349 which sits at the base of the binding pocket. (v) The catalytic residue Glu 540 forms an H-bond to the glycosidic oxygen (vi) The acetamido group of the non-reducing sugar ring NAGA is bent towards the anomeric C1A. This distortion is stabilised by hydrophobic interactions and by polar interactions with Asp 539 and Tyr 669.



Schematic view of the active site of chitobiase complexed with its natural substrate chitobiose.

Catalytic mechanism

Enzymatic hydrolysis of the glycosidic bond takes place via general acid-base catalysis that requires two critical residues: a proton donor and a nucleophile base. This hydrolysis can give rise to either an overall retention or an inversion of anomeric configuration.

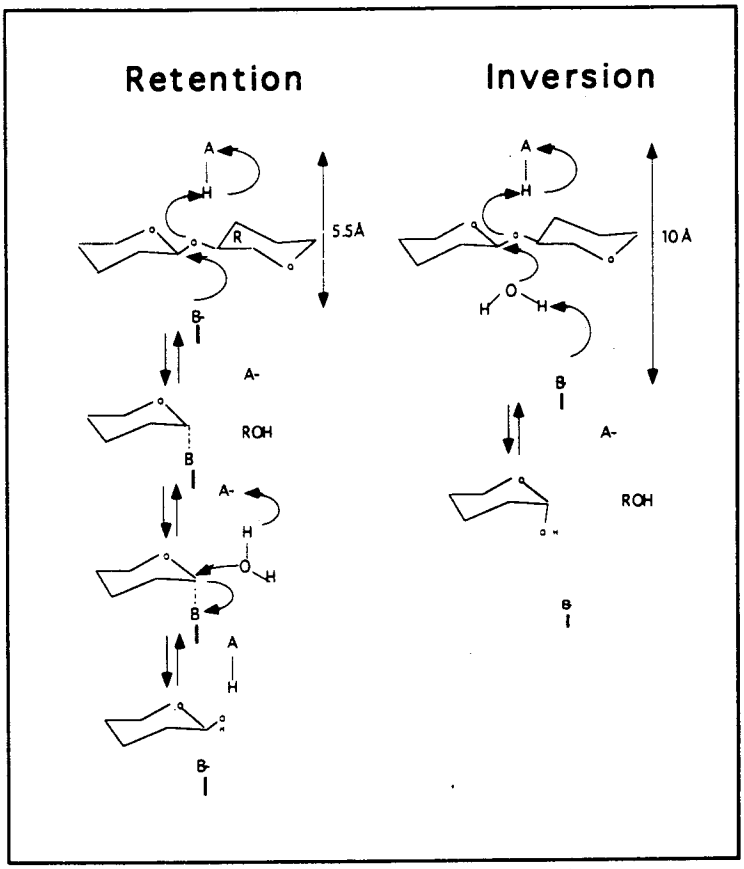
There are two variations of the classical acid-base catalysis mechanism which is common for glycosyl hydrolases.

The first variation involves a protonated acidic residue as proton (H+) donor which is the -COOH group of a Glu residue and a second negatively charged amino acid which electrostatically stabilises the positive charge at C1 formed during catalysis. The -COOH of the Glu donates a H+ to the glycosidic oxygen causing it to be a better leaving group due to the polarisation of the scissile bond. The product leaves and the remaining sugar acquires a positive charge, called *carbonium ion* or *oxocarbonium* intermediate. The second negatively charged residue is thought to stabilise this oxocarbonium ion intermediate, or to act as a nucleophile to form a covalent intermediate (glycosyl-enzyme). Then the carbonium ion reacts with an activated H_2O (OH-) from the equatorial side leading to **retention** of the anomeric configuration of the C1.

In the second variation, the stabilising base (negative charged residue) is too far from the C1 atom to stabilize the positively charged carbonium ion intermediate. That residue facilitates the polarisation of a water molecule from the solution, which attacks directly from the free axial side, leading to **inversion** of the anomeric configuration.

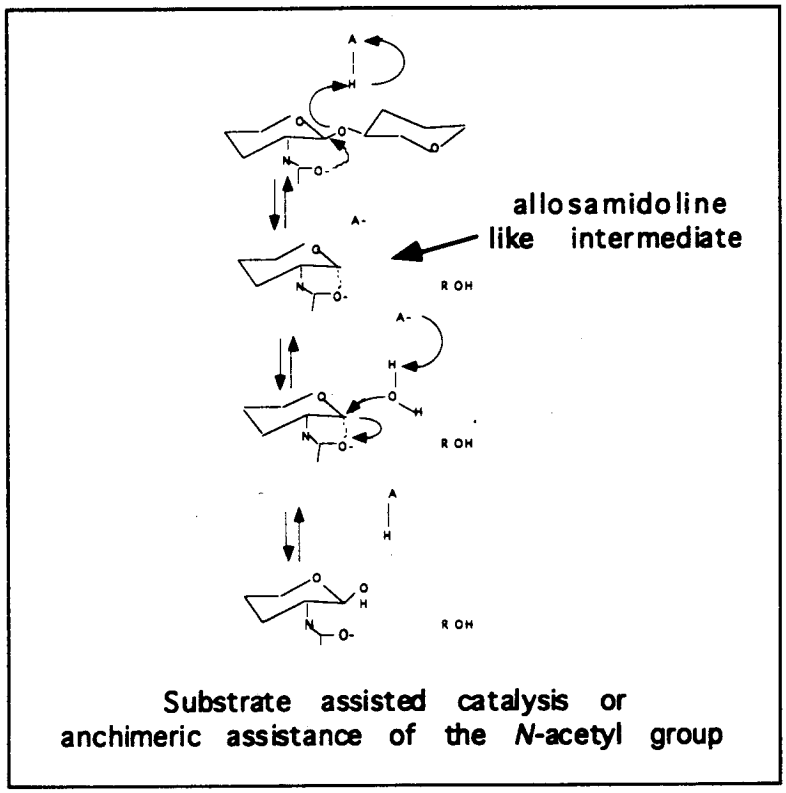
The distance between the two catalytic residues is characteristic of the mode and stereochemistry of the reaction mechanism: 4.8 - 5.3 Å is typical for hydrolysis with retention of anomeric configuration (via a double displacement mechanism), and roughly 9 - 9.6 Å for inversion (single displacement), where an

additional water is positioned between the anomeric carbon and the second protein carboxylate.



Biochemical experiments have clearly shown that Hevamine and several other bacterial chitinases are retaining enzymes [10,11]. A structurally conserved Glu has been assigned to be the proton donor in acid-base catalysis (i.e Glu315, Glu127 and Glu132 in the Chitinase A, Hevamine and Endo-β-*N*-acetyl-glucosaminidase F1, respectively). An Asp residue which located in the n-2 position from Glu is also important for the enhancement of the acidic character of the Glu residue by donating a hydrogen bond to the second carbonyl oxygen. In all three structures no negatively charged residue can be unambiguously assigned to stabilise the carbonium ion intermediate. Therefore, the positive charge at C1 is proposed to be stabilised at its axial side through the O7 carbonyl oxygen of the *N*-acetyl group, which has a partial negative charge. In this way a water molecule can attack C1 from the equatorial side, resulting in retention of

the anomeric configuration. This kind of stabilisation is in contrast to many other retaining glycosyl hydrolases, however it was prosoped a few decades ago by Koshland [12,13] and called **substrate assisted catalysis** or **anchimeric assistance of the *N*-acetyl group**. The current crystallographic data provide strong evidence for this mechanism: (i) in Hevamine and Chitinase A allosamidin complexes, allosamidin resembles the structure of an intermediate involved in substrate assisted catalysis; (ii) in Chitobiase the *N*-acetyl group seem to be deformed and is in close proximity with the C1 atom, as discussed in the next paragraph.



Substrate assisted catalysis or anchimeric assistance of the *N*-acetyl group

The structure of the complex of Chitobiase with its natural substrate provided the strongest evidence supporting this mechanism. It is the first complex of a glycosyl hydrolase with its intact natural substrate, i.e. with an intact glycosidic linkage between sugar moieties in the -1/+1 sites (either side of the cleavage point). The carboxylate residue acting as the catalytic nucleophile is Glu 540 which lies at a distance of 2.9 Å to the glycosidic linkage. In chitobiase again no second protein carboxyl group at an appropriate distance for either a

classical retaining or for an inverting mechanism is observed. However, due to the distortion of NAGA, its acetamido O7A atom lies 3.0 Å from C1A and is ideally poised to act as the nucleophile (base). In the first step of catalysis, Glu540 transfers its proton to the glycosidic oxygen and the C1A-O4B bond between the two sugars is split. NAGB leaves a potential carbonium ion on NAGA. In practice this intermediate can be stabilised by the *N*-acetyl group of NAGA itself, either through formation of a covalent bond between O7A and C1A resulting in an oxazolinium ring or by electrostatic stabilisation of the oxocarbonium ion by the partial negative charge of the carbonyl oxygen of the acetamido group. In both cases, this would stabilise the reaction intermediate for a sufficient time so the NAGB can diffuse out and the reactant, an incoming water molecule can diffuse in. This water adds to C1A and completes hydrolysis. The double displacement mechanism results in retention of anomeric configuration.

The structural data are supported by preliminary kinetic data which provide direct evidence for a retaining mechanism (Armand, Vorgias, Henrissat unpublished data). Furthermore, the homologous hexosaminidases, classified in the same glycosyl hydrolase family, degrade substrate with an overall retention of configuration [14].

Evolutionary aspects

The evolutionary aspects which emerged from the determinaton of the above structures are numerous. The presence of an N-terminal domain in chitinase from *Serratia marcescens*, the ChiN domain [2] is of particular interest. This domain has a fibronectin type III fold, without sharing any sequence similarity with FnIII type domains, which occur in other family 18 chitinases. The role of this domain - formed by either sequences - and the way FnIII type and ChiN domains evolved in family 18 enzymes remains unclear. Eucaryotic chitobiases also belong to family 18. Allthough they share the same substrate specificity with family 20 chitobiases and a similar fold, they seem to have evolved from different genes, demonstrating an example of convergent evolution, or „how

nature provided similar solutions to the same problem" through different pathways.

In two families (presumably evolutionary unrelated) the same mechanism was chosen for cleavage of the glycosidic bond in the chitin polymer (family 18) and the lower oligomers (family 20), although the requirements for the binding of the substrate were different. However, a different solution emerged in family 19 despite the fact that the reaction utilizes the same substrate and leads to the same products. It remains to be seen if the difference in the mechanism mirrors differences in substrate specificity or biochemical properties of the enzymes.

Conclusions

The comparison of the information revealed by the X-ray structure analysis of the two major enzymes involved in the process of the enzymatic hydrolysis of chitin, Chitinase A and Chitobiase, reflected their specificity for the polymer of chitin and lower oligomers, respectively. A similar organisation of the three-dimensional arrangement of the catalytic groups is observed; the active site is, however, marked by distinct differences necessary to modify the molecular machinery to hydrolyse the glycosidic bond in slightly different substrates. The active site cleft realised in Chitinase A has an architecture designed for a polymeric substrate. A varying set of subsites, which are similar to those in the other members of family glycosyl hydrolases (as we indicated by the comparison between Hevamine, Chitinase A, Endo-β-*N*-acetyl-glucosaminidase F1 and Endo-β-*N*-acetyl-glucosaminidase H) underline the common feature of binding the chains of linked *N*-acetyl-glucosamine residues, while the dissimalirities accounting for changed substrate affinity and specificity are minor and based on individual interactions at the amino acid level. The difference in Chitobiase, where a disaccharide is the major substrate, is the substitution of this cleft by a pocket. This is

a necessary change to accommodate better the smaller substrate. Tight binding is also essential to promote the substrate to a high energy ground state. This is also accomplished by an increased number of atomic interactions between the sugar and the amino acid residues. The molecular mechanism, unlike the used mechanisms for other glycosyl hydrolases, utilizes a single Glu residue and leads to retention of configuration of the anomeric centre, C1. This is accomplished by the use of the *N*-acetyl group of the substrate itself to provide for the necessary partial charge needed to stabilize the putative oxocarbonium intermediate. This mechanism, first directly shown for chitobiase on the unproductive enzyme substrate complex, is supported from the structural data in Chitinase A and Hevamine in complex with the strong chitinase inhibitor allosamidin. The *N*-acetyl group in Chitobiase is shown to be deformed and polarized - in an ideal conformation to stabilize the oxocarbonium. In Chitinase A and Hevamine, the allosamisolin group near the active site closely resembles the structure of the sugar ring with a deformed *N*-acetyl group. Given that allosamidin is a strong inhibitor of Chitinase A it is very likely that it resembles a transition state analogue, with the deformed *N*-acetyl group in close proximity with the C1 atom where the oxocarbonium develops during catalysis.

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