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The structure of a family GH25 lysozyme from *Aspergillus fumigatus*

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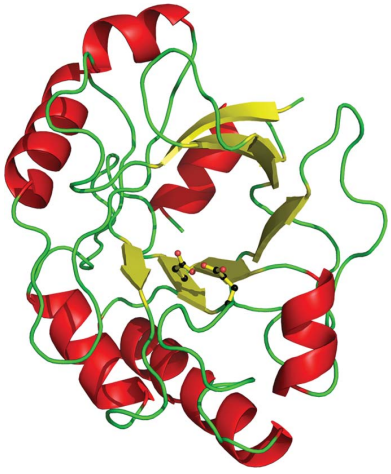
Lysins are important biomolecules which cleave the bacterial cell-wall polymer peptidoglycan. They are finding increasing commercial and medical application. In order to gain an insight into the mechanism by which these enzymes operate, the X-ray structure of a CAZy family GH25 'lysozyme' from *Aspergillus fumigatus* was determined. This is the first fungal structure from the family and reveals a modified α/β-barrel-like fold in which an eight-stranded β-barrel is flanked by three α-helices. The active site lies toward the bottom of a negatively charged pocket and its layout has much in common with other solved members of the GH25 and related GH families. A conserved active-site D*X*E motif may be implicated in catalysis, lending further weight to the argument that this glycoside hydrolase family operates *via* a 'substrate-assisted' catalytic mechanism.

1. Introduction

Lysins are important biomolecules that cleave the bacterial cell-wall polymer peptidoglycan at a variety of different positions. The term 'lysozyme' is broadly used to describe enzymes that cleave the β-1,4-glycosidic bond between *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) (or *vice versa*) in the carbohydrate backbone of peptidoglycan (Fig. 1*a*). There is increasing interest in the potential of such lytic enzymes as antimicrobial agents. This reflects their exquisite efficiency (Nelson *et al.*, 2001) and potential specificity for specific bacteria. In this context, lysozyme activity against medically relevant pathogens has been shown to include *Streptococcus pneumoniae* (Loeffler *et al.*, 2003), *Bacillus anthracis* (Schuch *et al.*, 2002) and *Enterococcus faecium* (Yoong *et al.*, 2004). Lysins have also found applications in cheese manufacture (de Ruyter *et al.*, 1997) and in the killing of *Listeria* in food preparations (for a recent example, see Soni *et al.*, 2010).

In nature, the β-1,4 bonds of peptidoglycan are cleaved by a structurally diverse set of enzymes in terms of biochemical and structural properties which have been classified into five homology families (CAZy; http://www.cazy.org; Cantarel *et al.*, 2008): hen egg-white lysozyme (HEWL; GH22 family), goose egg-white lysozyme (GEWL; GH23), bacteriophage T4 lysozyme (T4L; GH24), *Sphingomonas* flagellar protein (FlgJ; GH73; Hashimoto *et al.*, 2009) and *Chalaropsis* lysozymes (GH25). A sixth family GH108 is likely to emerge, although it remains largely biochemically uncharacterized. The first three types share common structural features but with very low sequence identities: these enzymes consist of a constant core of two helices and a three-stranded β-sheet that accommodates the substrates in the inter-domain cleft (Monzingo *et al.*, 1996).

The *Chalaropsis* 'class' of lysozymes, family GH25, are structurally unrelated to the other lysozyme folds. These enzymes display a modified α/β-barrel-like fold in which an eight-stranded β-barrel is flanked by just three α-helices (Rau *et al.*, 2001). This family exhibits both β-1,4-*N*-acetylmuramidase and β-1,4-*N*,6-*O*-diacetylmurami-



dase activities (Felch *et al.*, 1975), and its evolutionary spread is diverse, comprising bacterial, viral (mainly phage) and eukaryotic representatives. To date, four members have been structurally characterized: *B. anthracis* BaGH25c (Martinez-Fleites *et al.*, 2009), *Streptomyces coelicolor* cellosyl (Rau *et al.*, 2001), the bacteriophage lysin PlyB (Porter *et al.*, 2007) and Clp-1 lysozyme from a *Streptococcus pneumoniae* phage (Hermoso *et al.*, 2003; Perez-Dorado *et al.*, 2007), with only the latter being in a complex with peptidoglycan fragments (Perez-Dorado *et al.*, 2007). There is still a lack of experimental studies on the catalytic mechanism of GH25 enzymes, with no three-dimensional structure of a eukaryotic representative having been obtained to date; the properties of eukaryotic representatives have been inferred from those of bacterial homologues together with mutagenesis data. Here, we report the crystal structure of the fungal GH25 from *Aspergillus fumigatus* (hereafter referred to as AfGH25) at a resolution of 1.7 Å. We show that the enzyme is similar to viral and prokaryotic GH25 enzymes (Martinez-Fleites *et al.*, 2009) with an active site that is consistent with catalysis occurring *via* a 'neighbouring-group' mechanism (Vocadlo & Davies, 2008) with net retention of anomeric configuration.

2. Materials and methods

2.1. Cloning, expression and purification

The open reading frame encoding the *afgh25* gene (http://www.uniprot.org/uniprot/A4DA29.html) was amplified with the inclusion of the wild-type secretion signal (MKFSIVAIATIAGLASA) from an *A. fumigatus* strain Af293 cDNA pMWRAfum25C plasmid library (provided by Alfredo de Lopez, Novozymes Biotech Inc.) and

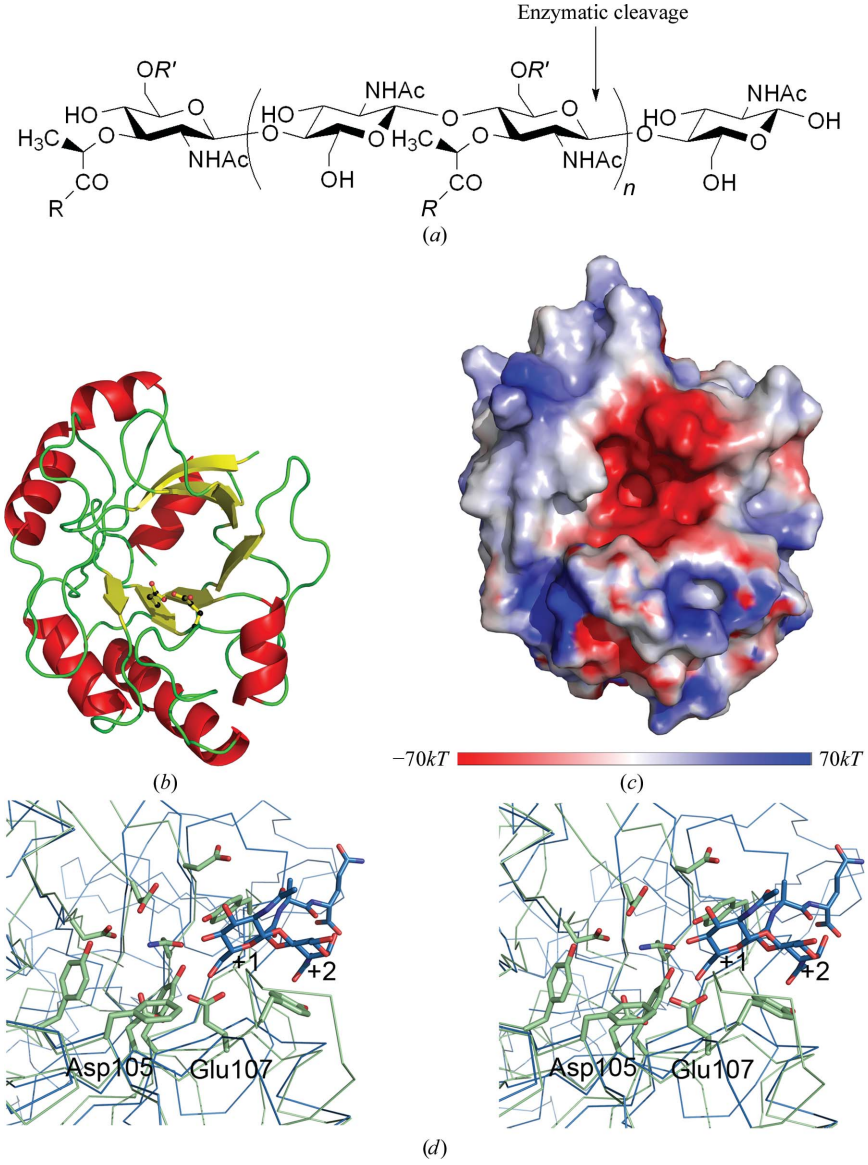


Figure 1
(*a*) Generic reaction of a 'lyzozyme': cleavage of one of the β-1,4 glycosidic bonds in the backbone of peptidoglycan (*R* indicates possible peptide cross-links and *R*′ indicates possible *O*-acetylation). (*b*) Three-dimensional protein cartoon of AfGH25 with the putative catalytic residues Asp105 and Glu107 shown in ball-and-stick representation. (*c*) Electrostatic surface representation of AfGH25 in the same orientation as in (*b*). The active-site residues are located towards the bottom of a highly negatively charged 'pit'. (*d*) Divergent ('wall-eyed') stereo representation of the overlap of AfGH25 (pale green) with the *Streptococcus pneumoniae* phage enzyme in complex with peptidoglycan fragments (blue; Hermoso *et al.*, 2003; Perez-Dorado *et al.*, 2007). The negatively charged and aromatic residues of AfGH25 referred to in the text are shown, with the putative catalytic residues Asp105 and Glu107 labelled.

Table 1
AfGH25 X-ray data and refinement statistics.

Values in parentheses are for the outer shell.

Data processing	
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 80.7, b = 111.8, c = 119.2
Wavelength (Å)	0.97600
Molecules in asymmetric unit	6
Resolution range (Å)	40.75–1.70 (1.79–1.70)
R_{merge}	0.088 (0.371)
$\langle I\rangle/\langle\sigma(I)\rangle$	13.0 (3.0)
Completeness (%)	99.9 (99.5)
Redundancy	6.3 (3.9)
Refinement statistics	
No. of reflections	18715
R_{cryst}	0.17
R_{free}	0.21
Mean B values	
Protein atoms (Å^2)	13
Ligand/ion atoms (Å^2)	11
Solvent atoms (Å^2)	24
R.m.s.d. bonds (Å)	0.018
R.m.s.d angles (°)	1.57
Ramachandran statistics (%)	
Preferred regions	96.28
Allowed regions	3.12
Outliers	0.61
PDB code	2x8r

the primer pair SteD1597NotI, 5′-ATA**GCGGCCGC**ACCATGA-AGTTCTCTATCGTTGCCATTGCCAC, and SteD1594XhoI, 5′-AAT**CTCGAG**TTAACCACTGGCTAACTTCCTCAACTGG, using PCR. The reaction was performed using Pwo polymerase (Boehringer Mannheim) and yielded a single PCR product of the predicted 745 base-pair size. The PCR product was purified and digested with the restriction endonucleases *Not*I and *Xho*I (New England Biolabs) and the fragment was subsequently ligated into the pre-digested *A. oryzae* expression vector pENI1898. The ligation was transformed into competent *Escherichia coli* DH10B cells and DNA purified from selected colonies. The integrity of the construct was verified by DNA-sequence analysis and the plasmid was transformed into *A. oryzae* ToC1512 for expression purposes. Transformants were spore-purified twice and submitted to fermentation. Protein expression was targeted towards the culture medium by the secretion signal and was confirmed by SDS–PAGE of the medium, which showed a protein band migrating at the expected rate for the molecular size.

2.2. Purification of the GH25 lysozyme

Culture broths were filtered through a filtration cloth and subsequently through a 0.2 µm filtration unit (Nalgene) to remove the *Aspergillus* host. Solid NaCl was added to a final concentration of 400 m*M* and the pH was adjusted to pH 5.5 with 20% acetic acid. The adjusted enzyme solution was applied onto an SP Sepharose FF column (GE Healthcare) which was equilibrated in 50 m*M* acetic acid/NaOH, 200 m*M* NaCl pH 5.5. The column was thoroughly washed with equilibration buffer to remove loosely bound protein. The enzyme was then eluted using a linear NaCl gradient (200–1000 m*M*) in 50 m*M* acetic acid/NaOH pH 5.5 over five column volumes. The GH25 lysozyme eluted as a single peak and its purity was analyzed by SDS–PAGE. The resultant protein preparation was concentrated to 26 mg ml^{-1} and buffer-exchanged into 25 m*M* HEPES pH 7.5 using a Vivaspin 10 kDa cutoff concentrator.

2.3. Crystallization and structure solution

Crystals of AfGH25 were grown in 96-well MRC Crystallization Plates (Molecular Dimensions Ltd) holding a reservoir volume of 60 µl crystallization solution. Drops were set up using a Mosquito Nanodrop crystallization robot (Molecular Dimensions Ltd) by mixing 150 nl protein solution with 150 nl crystallization solution. Crystals grew in 0.1 *M* MIB (malonic acid, imidazole, boric acid) system pH 4.0 and 25%(*w*/*v*) PEG 3350, corresponding to conditions B1–B4 of the PACT premier screen (Molecular Dimensions Ltd). Crystals were harvested directly from the MRC plate and cryoprotected by bathing them in mother-liquor solution incorporating 25% glycerol prior to flash-cooling them in liquid nitrogen. Diffraction data were collected to 1.7 Å resolution at 100 K on beamline ID23-1 at the European Synchrotron Radiation Facility (ESRF) at a wavelength of 0.976 Å. Data were processed with *MOSFLM* (Leslie, 1992) and scaled in *SCALA* (Collaborative Computational Project, Number 4, 1994). The structure was solved by molecular replacement using *Phaser* (McCoy *et al.*, 2007), with the coordinates of *Streptomyces coelicolor* lysozyme (PDB code 1jfx; Rau *et al.*, 2001), which shares approximately 51% amino-acid sequence identity (Altschul *et al.*, 1997) with AfGH25, as a search model. The starting model was improved manually using *Coot* (Emsley & Cowtan, 2004) between cycles of *REFMAC* (Murshudov *et al.*, 1997). The structure was validated using *MolProbity* (Chen *et al.*, 2010) prior to deposition. Data-collection and refinement statistics are given in Table 1. The refined structure was deposited in the PDBe database (http://

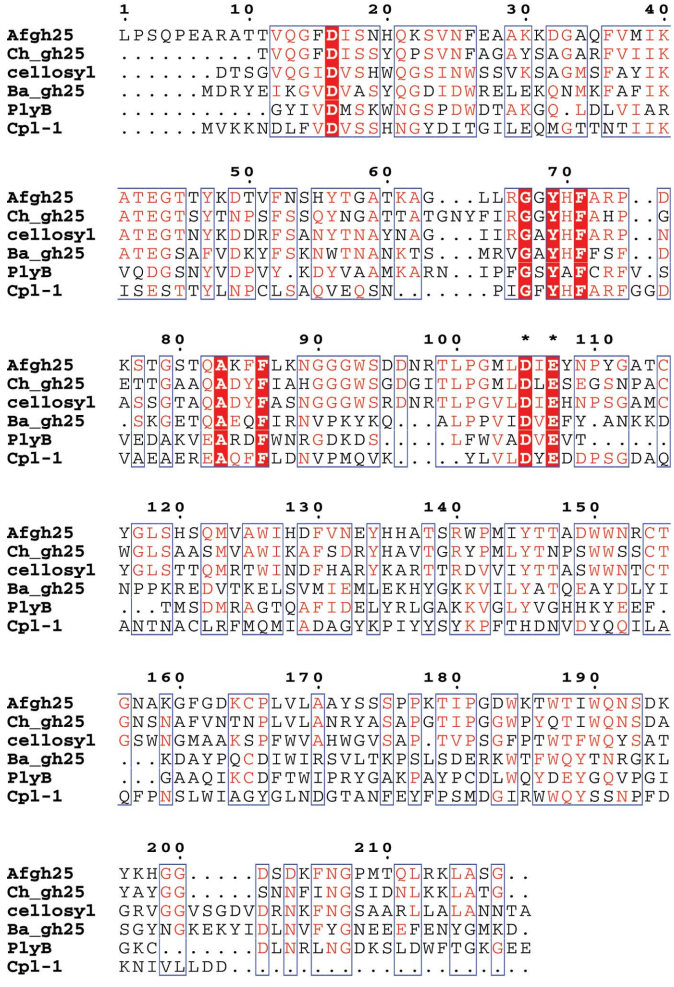


Figure 2
A *Multalin* (http://multalin.toulouse.inra.fr/multalin/) sequence alignment of known GH25 structures. Conserved residues are shown in red and asterisks denote the positions of the putative catalytic residues Asp105 and Glu107 which make up part of the D*X*E motif.

www.ebi.ac.uk/pdbe) under code 2x8r. Structural figures were produced with *PyMOL* (DeLano, 2002) and *MolScript* (Kraulis, 1991).

3. Results and discussion

The crystals of AfGH25 belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 80.7$, $b = 111.8$, $c = 119.2$ Å and six molecules in the asymmetric unit (Table 1). The chains were traced from Tyr10 through to Gly211 in all six molecules, with an ion, modelled as Cl^-, associated with each molecule. 1105 water molecules have also been modelled. As for other GH25 lysozymes (see below), the AfGH25 structure is a modified α/β-barrel fold built from eight β-strands and three α-helices (Fig. 1*b*). A structural similarity analysis against other solved members of CAZy family GH25 was carried out using *Coot*. The closest related structure was that of the bacterial lysozyme from *S. coelicolor* (1 Å r.m.s.d. over 206 C^α atoms), followed by that from *B. anthracis* (1.6 Å r.m.s.d. over 187 C^α atoms) and those of CP-1, a cell-wall endolysin from *Streptococcus pneumoniae* (2.1 Å r.m.s.d. over 169 C^α atoms), and PlyB, a bacteriophage-encoded lysin (2.0 Å r.m.s.d. over 173 C^α atoms). Despite relatively low sequence identities among the structurally characterized GH25 members (18–51%), strong conservation is observed at the C-termini of the β-strands and this location corresponds to the entrance of the catalytic cavity. In AfGH25, as in the other GH25 members, this cavity is lined by a constellation of negatively charged residues (Asp16, Asp105, Glu107, Asp194 and Asp201; Figs. 1*c* and 1*d*) and aromatic residues (Tyr69, Phe71, Tyr108, Tyr145 and Tyr172; Fig. 1*d*).

Superposition of the AfGH25 coordinates onto the structure of the complex of Clp-1 lysozyme with a peptidoglycan analogue shows no conservation around the residues that bind the sugar moieties in subsites +1 and +2 (Fig. 1*d*; the glycoside hydrolase subsite nomenclature is as in Davies *et al.*, 1997). The loops surrounding these sites adopt different conformations in the two enzymes, so that in the absence of a complex structure the structural determinants that underlie the specificity of the AfGH25 enzyme remain uncertain.

Our recent studies on the BaGH25 lysozyme suggest a mechanism for GH25 (Martinez-Fleites *et al.*, 2009) in which catalysis proceeds through an oxazoline intermediate. Evidence for this arises from the conservation of sequence similarities within the GH25 family (Fig. 2) and the resemblance that this architecture bears to other retaining hydrolases belonging to families GH18, GH20, GH56, GH84 and GH85 (Fig. 3; Martinez-Fleites *et al.*, 2009; recently reviewed in Martinez-Fleites *et al.*, 2010). These families have been shown to have an active-site DE or D*X*E sequence motif. The DE carboxylate pair promotes a double-displacement mechanism in which the nucleophile is not enzyme-derived but instead lies on the intramolecular 'neighbouring group' of the *N*-acetyl carbonyl group (Vocadlo & Davies, 2008). Catalysis occurs *via* the formation and subsequent breakdown of a covalent oxazoline intermediate. There is clear structural conservation in the putative −1 site in all structurally characterized GH25 enzymes which not only involves the presumed catalytic residues but also several aromatic residues that form a well defined pocket whose entrance is about 7 Å wide and 10 Å deep. We suggest that Asp105 and Glu107 act as the signature catalytic constellation in AfGH25. We propose Asp105 to be the catalytic acid/base, initially protonating the leaving group to facilitate its departure (general acid assistance) and subsequently acting as a general base to activate the hydrolytic water molecule. Glu107 acts to stabilize or deprotonate the oxazoline N atom (Martinez-Fleites *et al.*, 2009).

The biological role of the AfGH25 enzyme has yet to be established. The presence of an N-terminal signal secretion peptide indicates the location of AfGH25 to be extracellular, so it can be speculated that the molecule may act as a selective agent by possessing antimicrobial activity or may possibly serve as a tool for the breakdown of bacterial peptidoglycan for nutritional purposes. This may either be by the direct assimilation of bacterial cell-wall components or as part of a mechanism to improve access to the content of the bacterial cell. The growing concern related to the spread of multi-drug-resistant bacteria caused by classical small-molecule 'drug'-based antimicrobial treatments makes lysins an interesting candidate for a novel type of antibacterial agent. Since these enzymes target the bacterial cell wall, the occurrence of bacterial resistance developed owing to multiple exposures would be less likely. Thus, from an applied perspective it appears as if lysins have many of the desired characteristics required, such as efficacy, specificity and biodegradability.

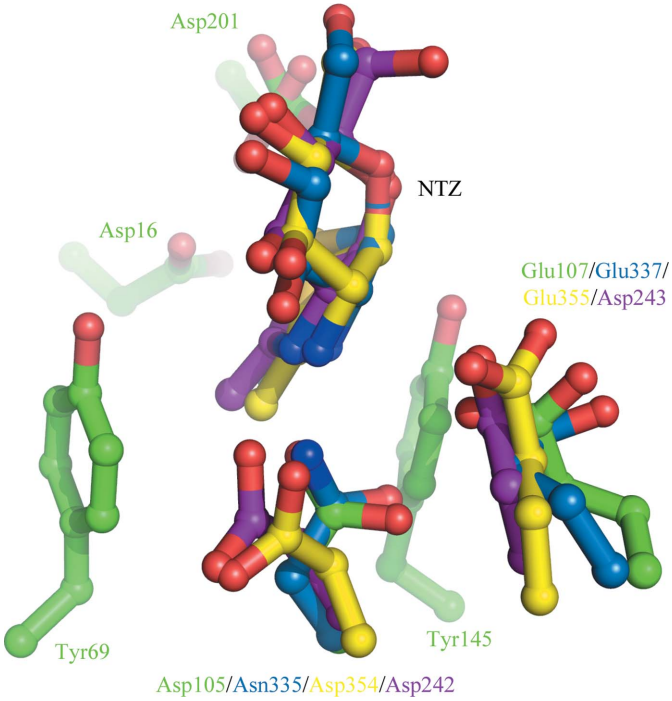


Figure 3
Superposition of the catalytic cavities of retaining hydrolases belonging to families GH20 (yellow; PDB code 1np0; Mark *et al.*, 2003), GH84 (purple; PDB code 2chn; Dennis *et al.*, 2006) and GH85 (blue; PDB code 2w92; Abbott *et al.*, 2009) and BaGH25c (green) around the coordinates of their NAG-thiazoline (NTZ) complexes.

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