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Structures of Chitobiase Mutants Complexed with the Substrate Di-*N*-acetyl-D-glucosamine: the Catalytic Role of the Conserved Acidic Pair, Aspartate 539 and Glutamate 540

Gali Prag[1], Yannis Papanikolau[2], Giorgos Tavlas[2] Constantinos E. Vorgias[3], Kyriacos Petratos[2] and Amos B. Oppenheim[1]*

[1]*The Department of Molecular Genetics and Biotechnology The Hebrew University-Hadassah Medical School, Jerusalem 91120, Israel*

[2]*Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology Hellas, P.O. Box 1527 71110 Heraklion, Greece*

[3]*National and Kapodistrian University of Athens, Faculty of Biology, Department of Biochemistry and Molecular Biology, Panepistimiopoli Zographou, 15784 Athens Greece*

**Corresponding author*

The catalytic domain of chitobiase (β-*N*-1-4 acetylhexosaminidase) from *Serratia marcescens*, is an α/β TIM-barrel. This enzyme belongs to family 20 of glycosyl hydrolases in which a conserved amino acid pair, aspartate-glutamate, is present (Asp539-Glu540). It was proposed that catalysis by this enzyme family is carried out by glutamate 540 acting as a proton donor and by the acetamido group of the substrate as a nucleophile. We investigated the role of Asp539 and Glu540 by site-directed mutagenesis, biochemical characterization and by structural analyses of chitobiase - substrate co-crystals. We found that both residues are essential for chitobiase activity. The mutations, however, led to subtle changes in the catalytic site. Our results support the model that Glu540 acts as the proton donor and that Asp539 acts in several different ways. Asp539 restrains the acetamido group of the substrate in a specific orientation by forming a hydrogen bond with N2 of the non-reduced (−1) sugar. In addition, this residue participates in substrate binding. It is also required for the correct positioning of Glu540 and may provide additional negative charge at the active site. Thus, these biochemical and structural studies provide a molecular explanation for the functional importance and conservation of these residues.

Keywords: Serratia marcescens; crystal structure; hexosaminidase; β-*N*-acetylhexosaminidase; catalytic mechanism

Introduction

Chitin, a widely spread carbohydrate, is composed of *N*-acetyl-D-glucosamine (NAG) linked by β-1-4-glycosidic bonds. The degradation of chitin by *Serratia marcescens* proceeds *via* two steps by the action of glycosyl hydrolases (Warren, 1996). First, chitinases hydrolyze the chitin to oligosaccharides and disaccharides (diNAG, chitobiose). This is followed by the action of chitobiase which hydrolyzes the β-1-4-glycosidic bond between two *N*-acetyl-D-glucosamine residues. Chitinase and chitobiase enzymes are widespread in nature and have been found in bacteria, fungi, plants, invertebrates and vertebrates (Perrakis *et al*., 1994; Vorgias *et al*., 1993).

S. marcescens chitobiase belongs to glycosyl hydrolases family 20 (Tews *et al*., 1996; Henrissat *et al*., 1991) in which the catalytic domain is an α/β TIM-barrel and the active site lies at the center of the barrel convex side. This structure is probably conserved in all members of family 20, including the human β-hexosaminidase that catalyzes the cleavage of terminal β-*N*-acetylglucosamine or β-*N*-acetylgalactosamine residues from glyco conjugates (Fernandes *et al*., 1997; Tews *et al*., 1996; Mark *et al*, 1998; Gravel *et al*., 1995).

It was shown that hydrolysis by chitobiase proceeds *via* a substrate-assisted mechanism, in which the configuration of the anomeric carbon atom is

Abbreviations used: NAG, *N*-acetyl-D-glucosamine; X-NAG, 5-bromo-4-chloro-3-indolyl-NAG; pNp-NAG, *p*-nitro-phenyl-NAG.

E-mail address of the corresponding author: ao@cc.huji.ac.il

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retained. In this reaction glutamate 540, located in loop 4 of the catalytic domain, acts as a proton donor and the acetamido group acts as the nucleophile. (Koshland Jr, 1953; McCarter & Withers, 1994; Tews *et al*., 1996; Knapp *et al*., 1996; Drouillard *et al*., 1997).

Multiple alignment of family 20 of glycosyl hydrolases, based on 17 sequences extracted from the SwissProt bank, shows the complete conservation of both Asp539 and Glu540. in loop 4 (positions of the amino acid residues are those of *S. marcescens* chitobiase). It was previously shown that changing the catalytic glutamate of chitobiase from *Streptomyces plicatus* and β-hexosaminidase A from humans inactivated the enzyme (Mark *et al*, 1998; Fernandes *et al*, 1997). Here, we chose to change Asp539 and Glu540 of *S. marcescens* chitobiase, the only member for which the structure of the enzyme and the enzyme-substrate complex were solved. The availability of inactive mutant proteins allowed us to achieve co-crystallization with the native substrate. The structures of the enzyme-substrate co-crystals of the mutant proteins allowed us to gain insight into the function of these conserved residues in the active site.

Results and Discussion

Biochemical characterization of *chb* mutants

The kinetic parameters of the purified wild-type and mutant proteins D539A, E540A and E540D were determined (Table 1). The residual level of activity of the D539A/E540A protein was too low to allow kinetic analysis. Mutation D539A was found to increase K_M and to decrease K_{cat}, suggesting that D539 is involved in both substrate binding and catalytic activity. This mutational change caused a decrease of over 1000-fold in the enzymatic efficiency (K_{cat}/K_M). Mutations E540A and E540D were found to decrease K_M by about fivefold and to decrease K_{cat}, suggesting that E540 functions in catalysis. These mutations led to a decrease in enzymatic efficiency of about 30 and 120 fold respectively (Table 1). Although no structural data are available, we suggest that the somewhat higher activity of the E540A mutant over that of E540D could be due to the participation of a water molecule accommodated between the scissile bond and the Cβ group of the mutated Ala540 residue. A similar explanation was previously suggested for a mutant in lysozyme (Sanz *et al*., 1992). Our results demonstrate that the conserved Asp539-Glu540 pair plays an important role in the catalytic site.

Co-crystal structures of chitobiase mutants with diNAG

To investigate the structural consequences of the amino acid substitutions, the structures of D539A and E540D mutants complexed with diNAG were determined. By using mutants defective in catalysis we were able to co-crystallize these purified mutant enzymes with diNAG and to solve their structures (Table 2). Diffraction data were collected to 1.9 Å and 1.8 Å resolution. The RMS (root-mean-square) deviations (of the backbone coordinates) between the wild-type enzyme and the D539A and E540D mutants, were calculated to be 0.31 Å and 0.37 Å, respectively. These findings show that the structure of the mutant proteins did not change. The detailed structures of the α/β-barrel domains of mutant proteins and the position of the substrate is shown in Figure 1. The conformation of the diNAG bound to the enzyme is similar to that described (Tews *et al*., 1996). However, the two mutants reveal subtle changes in both amino acid residues and in the substrate.

The analysis of the D539A-diNAG complex reveals, surprisingly, that although the position of the substrate is maintained, the acetamido group is flipped by 175° (the dihedral angle of the N2-C7 bond) with respect to the wild-type and E540D complexes (Figures 2 and 3). It appears that the flipped acetamido group occupies part of the space that is filled by the side-chain of D539 in the wild-type. Thus, it appears that one function of D539 is to restrain the acetamido group in a specific orientation by forming a hydrogen bond with N2 of the −1 sugar. The loss of this hydrogen bond is probably responsible for reduced level of affinity of the mutant protein to the substrate. In both wild-type and mutant complexes, residues Trp616 and Trp639 form hydrophobic interactions with the acetamido group of the −1 sugar. The altered conformation of the acetamido group may be favored by its hydrogen bond to Glu540 (Figure 3).

The mutant protein D539A resulted in additional changes at the active site. The electron density of the side-chain of residue E540 is poor ($\rho = 0.65 \times \sigma$). This is reflected by increased atomic temperature factors (*B*-factor). The average *B*-factor

Table 1. Kinetic constants of wild-type and mutant chitobiase proteins

Enzyme	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($s^{-1} \times \mu M^{-1}$)
WT	0.063	827.0	1.3×10^4
D539A	1.991	17.0	0.8×10^1
E540A	0.014	6.0	4.2×10^2
E540D	0.01	1.4	1.1×10^2

Kinetic assays were preformed with pNp-NAG as described in Materials and Methods. Standard errors were less than ±15% for K_M and less then ±18% for K_{cat} determinations.

Table 2. Diffraction data processing and refinement statistics

	D539A	E540D
A. *Diffraction data*		
Unit cell		
a (Å)	109.8	109.2
b (Å)	100.0	99.4
c (Å)	86.3	86.6
Space group symmetry	$P2_12_12$	$P2_12_12$
Diffraction limit (Å)	1.75	1.85
Observations	937,195	1,001,880
Unique reflections	76,047	71,983
Completeness (%)[d]	86.9 (90.8)	96.9 (94.0)
R_{merge} (%)[a,d]	2.4 (9.5)	5.5 (21.6)
Overall I/σ	30.8	27.8
B. *Refinement*		
Resolution limits	15-1.8	10-1.9
R-factor (%)[b]	17.1	19.1
R_{free} (%)[c]	22.3	24.6
Number of residues	857	857
Number of water molecules	823	834
C. *RMS deviations from ideality*		
Bond length (Å)	0.022	0.029
Bond angle (deg.)	2.29	2.89
Aromatic planar groups (Å)	0.012	0.015
D. *Average of* B-*factor values* (⟨Å^2⟩)		
All atoms	21.8	25.7
Main-chain	19.6	24.7
Side-chain	23.6	26.8
Substrate (diNAG)	15.5	24.9
Solvent (H_2O, SO_4^{2-})	31.0	34.9

[a] $R_{merge} = \Sigma|I_i - \langle I\rangle|/\Sigma|I_i|$ where I_i is an individual observed intensity measurement and $\langle I\rangle$ is the average intensity for this reflection.

[b] *R*-factor $= \Sigma|F_{obs} - F_{calc}|/\Sigma F_{obs}$ where F_{obs} and F_{calc} are the observed and calculated structure factors, respectively.

[c] $R_{free} = \Sigma||F_{obs}| - |F_{calc}||/\Sigma F_{obs}$ calculated from 5% of the reflections selected randomly and omitted from the refinement process.

[d] Values in parentheses refer to the corresponding values of the highest resolution shell (from SCALEPACK output, e.g. 1.95-1.8 Å).

for the E540 side-chain was refined to 41.1 Å^2, as compared to the total averaged *B*-factor of all side-chains in the molecule 23.6 Å^2 ($\sigma = 8.4$ Å^2, Table 2). Similar analysis of the wild-type complex (PDB code: 1qbb) yielded a *B*-factor for the E540 side-chain of 26.4 Å^2 and total averaged *B*-factor of 16.4 Å^2 ($\sigma = 2.8$ Å^2). These results suggest a greater flexibility of E540 in the mutant. Furthermore, E540 assumes an altered conformation in which the distance between the Oε1-carboxylic group of E540 and the glycosidic oxygen atom increases from 2.8 Å to 5.3 Å (Figures 2 and 3). These results suggest that D539 acts to restrain the movement of E540.

The conserved D539 residue may have additional functions. It possibly aids in the contact between the acetamido O7 atom of the −1 sugar and the anomeric carbon C1 atom of the substrate and in stabilizing the partial positive charge of the acetamido group while forming the oxazoline ring (Terwisscha-van-Scheltinga *et al.*, 1994, 1995). D539 may also improve proton donation by forming an electrostatic interaction with E540. Thus, it is difficult at present to separate the various contributions of the D539 residue to enzymatic activity. Attempts to obtain the D539N mutant, which could have helped in the above analysis, failed.

The analysis of the E540D-diNAG co-crystal is simpler, since the structural difference from the wild-type enzyme is confined to the absence of one CH_2 group. In the E540D-diNAG complex the distance between the carboxylic end and the glycosidic oxygen atom was found to be 4.3 Å (Figure 3). Thus, increasing the distance between the carboxylic end and the glycosidic oxygen atom from 2.8 Å, as found in the wild-type enzyme, to 4.3 Å is sufficient to reduce the catalytic activity of the mutant (Table 1). These findings support the hypothesis that E540 acts as the proton donor. It is not clear why the mutations changing the E540 residue appears to increase the degree of affinity of the enzyme to the substrate.

Our detailed structural analysis of two mutants at the catalytic site allows a more comprehensive analysis of chitobiase mechanism of action. It is clear from our results that in the complex the structure of the substrate is distorted. As was previously observed (Tews *et al.*, 1996) the planes of the two sugars are tilted around the glycosidic linkage by about 90° with respect to one another. This distortion is stabilized by hydrophobic and polar interactions.

It was previously shown that the cleavage of diNAG proceeds *via* a substrate-assisted mechanism, in which the configuration of the anomeric carbon is retained (Drouillard *et al.*, 1997). Our results support the proposal that Glu540 donates a proton to the glycosidic bond. This results in the cleavage of diNAG and to the release of the +1 sugar from the active site. Our results show the importance of the conserved Asp539 residue. As discussed above, this residue performed multiple functions.

After breakage of the scissile bond, the positive charge of the C1 of the −1 sugar is stabilized by a nucleophilic attack from the O7 of the acetamido group, resulting in the formation of an oxazolinium ring. The oxazolinium ring is stabilized by hydrogen bonding of Asp539 with the N2 atom. Interestingly, we find that one function of Asp539 is to keep both Glu540 and the *N*-acetyl group in proper position essential for catalysis. Finally, hydrolysis of water molecule completes the reaction by the hydroxyl attack at the C1 of the −1 sugar and the reprotonation of Glu540.

The results presented here provide an explanation for the importance and conservation of the Asp-Glu in glycosyl hydrolase family 20. It is interesting that chitinases belonging to family 18, of which there are now over 50 sequenced representatives, all possess an Asp-X-Glu motive at the catalytic site. In most cases, X is found to be a hydrophobic residue. Inspection of the determined 3D structures of the chitinases suggests that the

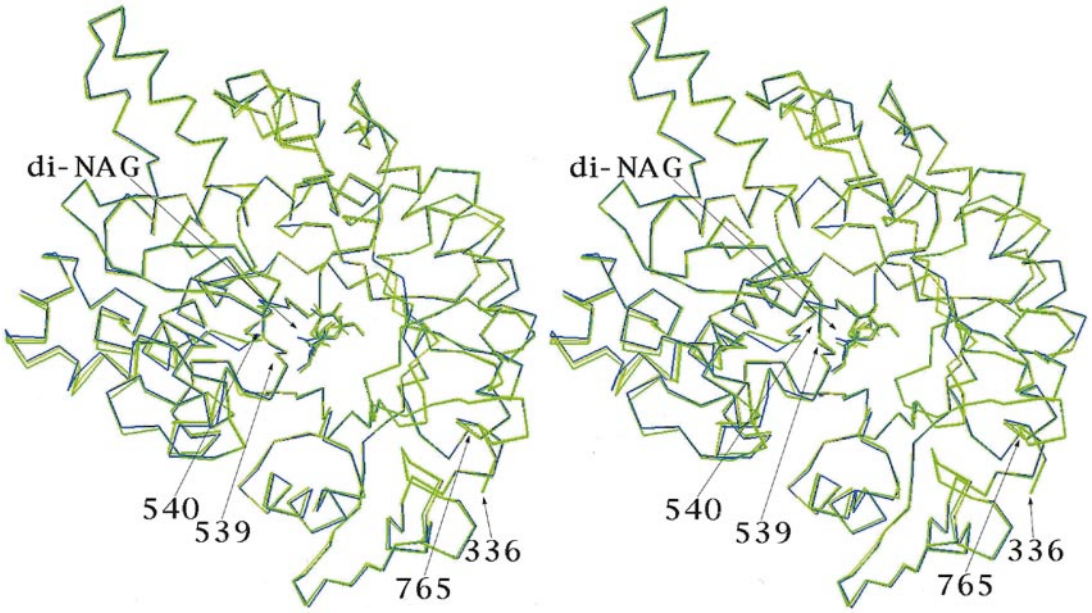

Figure 1. Comparison of the α/β-barrel catalytic domains of wild-type and mutant chitobiase structures. Stereo views of wild-type (green) mutant D539A (blue) and mutant E540D (yellow) of the C^{α} coordinates and of the diNAG atoms superimposition are shown. Location of residues at the amino (Pro336) and the carboxy (Arg765) ends of the α/β-barrel domain, and of the catalytic Asp539 and Glu540 residues are indicated. The wild-type data was taken from Tews *et al.*, 1996 (1qbb). The program *insight*II was used to generate this Figure.

aspartate and glutamate residues assume similar configuration in both chitobiase and chitinases. The function of the Asp-X-Glu motive in family 18 is currently under investigation.

Materials and Methods

Construction and expression of chitobiase mutants

The *chb* gene was subcloned into the expression vector pKK177-3 (Amann & Brosius, 1985), by PCR, using *Pow* DNA polymerase (Boehringer) and plasmid pCBII as a template (Kless *et al.*, 1989) to yield plasmid pGPchb. Substitutions were introduced into the *chb* gene using a modified protocol of QuickChange site directed mutagenesis kit (Stratagene). The PCR step was performed with 0.5 μM of each primer (33-36 bp), 0.2 mM dNTP mix, 0.2 μg pGPchb DNA template, 0.1 U of Pfu DNA polymerase in 1× reaction buffer at total volume of 25 μl. The duration of synthesis was 14 minutes at 68 °C, followed by the digestion of the parental DNA for two hours at 37 °C with *Dpn*I enzyme. DNA was introduced into *Escherichia coli* XL1B or XL2B cells by electroporation, and colonies were screened with 5-bromo-4-chloro-3-indolyl *N*-acetyl-β-D-glucosaminide (X-NAG) (Sigma) by pouring 2-3 ml of 1.25 mg × ml^{-1} X-NAG in soft agar (0.6% agar dissolved in LB medium). Mutant clones, which were obtained at high frequency (more than 85%), were purified and confirmed by DNA sequencing.

To test the importance of the conserved Asp539-Glu540 pair for the catalytic activity of chitobiase, we subcloned the *chb* gene and generated a number of mutations by site-directed mutagenesis. Mutants were recognized by their inability to convert X-NAG into its insoluble dye, and were further confirmed by DNA sequencing. This system provides an easy tool for genetic analysis of β-*N*-acetylhexosaminidases.

In preliminary screening it was found that alanine replacement mutations D539A and E540A led to a drastic reduction in enzymatic activity (about 3% of wild-type activity). Similarly, E540D was found to be defective in chitobiase activity (0.5% of wild-type activity). Almost no residual activity was observed in the double mutant D539A/E540A (0.1% of wild-type activity).

Sequence corrections

Our electron density maps of both mutants suggested that residues 484 and 566 were incorrectly assigned as Pro and Gly, respectively (Tews *et al.*, 1996). Resequencing the appropriate coding regions showed that these positions code for Gln and Ser, respectively, a conclusion supported by the electron density maps.

Protein purification and crystallization

Induced culture (5 l) was harvested using a low-speed centrifuge. Cells were resuspended in 20 ml of 0.5 M sucrose 20 mM Tris-HCl (pH 8.0) and 0.2 mg × ml^{-1} lysozyme, and incubated for one hour at 4 °C. After centrifugation, NH_4SO_4 was brought to 2 M and the periplasmic proteins were applied on a Phenyl-Sepharose column, pre-equilibrated with 2 M ammonium sulfate and 20 mM Tris-HCl buffer (pH 8.0). After washing with the same buffer, the protein was eluted by a descending gradient of ammonium sulfate (1.2 M to 0 M) and Tris-HCl (pH 8.0) buffer. The volume of the solution containing the protein was reduced using Amicon filter. After dialysis against a large volume of 10 mM sodium phosphate buffer (pH 8.0), the protein was applied on SP-

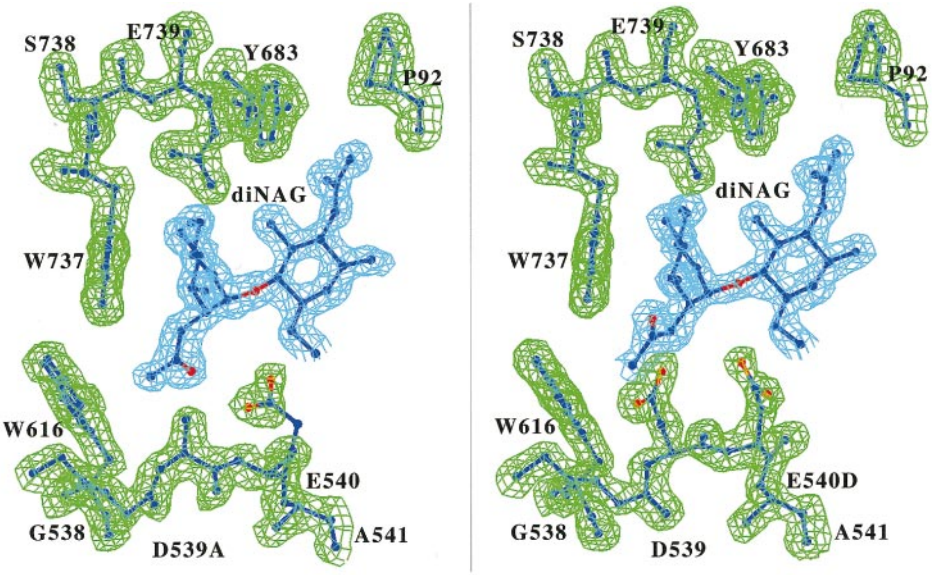

Figure 2. Models of the complexes of *S. marcescens* chitobiase mutants. The Figure focuses on the active sites occupied by the substrate. Part of the calculated electron density maps $2mF_{obs} - DF_{calc}$ (see Materials and Methods) are shown from the final refined models of the mutants D539A at 1.8 Å (left) and E540D at 1.9 Å (right), contoured at 1.5 × σ level. The sugars and amino acid residues are blue. The glycosidic oxygen, O7A of the acetamido group and the carboxylic oxygen atoms of residues 539 and 540 are red. The program O was used to generate this Figure (Jones *et al.*, 1991).

Sepharose column. Bound protein was eluted with 0 M-0.4 M NaCl, 10 mM sodium phosphate buffer (pH 8.0) gradient. The location of the fraction containing the enzyme was determined by assaying for the hydrolysis of pNp-NAG. The fractions with the highest levels of activity were checked by SDS 12.5% PAGE (Laemmli, 1970). The protein was concentrated by ultra filtration and the protein concentration was determined by the Bradford reagent.

We have improved the crystallization protocol as follows: co-crystals were grown by the hanging-drop vapor diffusion method. Reservoir buffer contained 2.3 M ammonium sulfate and 100 mM Cacodylate buffer (pH 4.8). The aqueous protein solution 40 mg × ml^{-1}

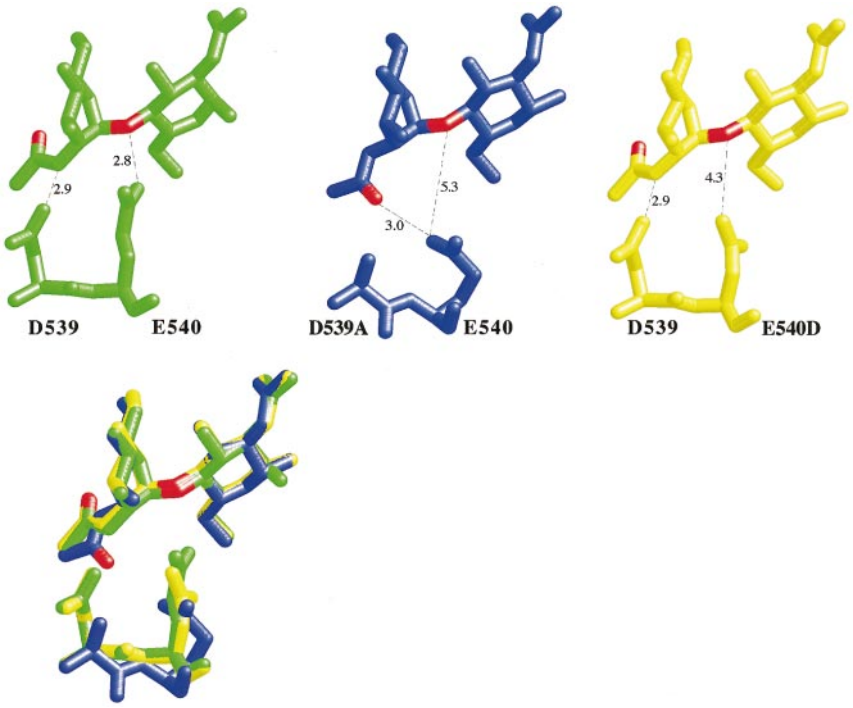

Figure 3. Structural comparisons of the catalytic sites of wild-type and mutant complexes. Wild-type (green), D539A (blue) and E540D (yellow) are shown. Superimposition of the three complexes is shown below. The glycosidic oxygen atom and the proposed acetamido O7 nucleophile are red. Distances are indicated in Å. The wild-type data was taken from Tews *et al.*, 1996 (1qbb). The program RasMol was used to generate this Figure (Sayle *et al.*, 1995).

was mixed with an equal volume of reservoir containing 10 mM diNAG. Crystals about 0.5 mm × 0.2 mm × 0.2 mm in size were formed within two to three days.

Data collection and refinement

Diffraction data were collected from a single crystal of chitobiase D539A mutant complex under cryo-cooling conditions (100 K) at the EMBL X11 synchrotron beamline at the DORISIII storage ring of DESY, Hamburg. The resolution range of the data was 10-1.8 Å (Table 2). The data of a single crystal of the mutant E540D complex were collected at our home facility (Heraklion) using a Rigaku rotating anode (Cu-Kα) X-ray generator ($T = 100$ K) within resolution limits of 10-1.9 Å (Table 2). Data processing was performed using DENZO and equivalent Bragg reflections were merged using SCALEPACK from the HKL package (Otwinowski *et al*., 1997). Molecular replacement was carried out with AmoRe (Navaza, 1994) using as model the native chitobiase structure (PDB code: 1qbb). Prior to refinement, 5% of the data were randomly flagged for cross validation (R_{free}). Refinement was performed under restrained conditions using the programmes REFMAC (Murshudov *et al*., 1997) and ARP (Lamzin *et al*., 1993) from the CCP4 suite (Bailey, 1994) until convergence of the indices *R*-factor and R_{free} was reached (Table 2). Fourier maps with coefficients of $2mF_{obs} - DF_{calc}$ and $mF_{obs} - DF_{calc}$ were calculated, where the m is the figure of merit and D is the error distribution derived from the σ_A function (Read, 1986). Manual interventions were carried out using both electron density maps which were inspected with O (Jones *et al*., 1991), in order to check the agreement of the model with the X-ray data. Finally, the stereochemistry of the model (structural validation) was analyzed using PROCHECK (Laskowski *et al*., 1993) and WHAT_CHECK (Vriend, 1990).

Kinetic analysis

The kinetic constants, K_M and k_{cat}, of wild-type and mutant enzymes were determined with the substrate analogue *p*-nitro-phenyl-NAG (pNp-NAG) at concentrations ranging from 10 μM to 5 mM. The reactions were preformed in 0.1 M potassuim phosphate buffer (pH 7.9) at 42 °C and monitored for the accumulation of *p*-nitrophenol at an absorbance of 405 nm (930 Uvicon spectrophotometer, Kontron Instruments). The kinetic constants were obtained by fitting the measurements, (average of three experiments) of the initial rates of the reactions to the Michaelis-Menten equation using Prism 2.0 software (GraphsPad).

Coordinates and structure factors

Coordinates of *S. marcescens* chitobiase mutants complexed with di-*N*,*N*′-acetyl-glucosamine have been deposited in the RCSB Protein Data Bank. Accession PDB codes for mutants D539A and E540D are 1C7S and 1C7T, respectively. Codes for structure factors are RCSB001441 and RCSB001442, respectively.

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