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Department of Heteroorganic Chemistry

Unexpected opening of the glycosylation site in hexagonal form of CAL-B. Is it functionally related?

Paweł Strzelczyk[1], Grzegorz D. Bujacz[1], Piotr Kiełbasiński[2] and Jarosław Błaszczyk[2]

[1] *Institute of Technical Biochemistry, Lodz University of Technology, Stefanowskiego 4/10, 90-924 Łódź, Poland*

[2] *Department of Heteroorganic Chemistry, Centre of Molecular and Macromolecular Studies, Polish Academy of Sciences, Sienkiewicza 112, 90-363 Łódź, Poland. email: blaszcz8@cbmm.lodz.pl*

Summary

- We discovered the new, hexagonal crystal form of lipase B from *Candida antarctica* (CAL–B), and determined its crystal and molecular structure.
- The NAG (N–acetyl–D–glucosamine) chain, which was closing the glycosylation site in the orthorhombic form, in our hexagonal structure makes the glycosylation site opened.
- We do not know yet, whether the opening and closing of the glycosylation site by the 'lid' NAG molecules, could be related to the opening and closing of the active center of the enzyme upon substrate binding and product release.
- The packing of molecules in the hexagonal crystal makes the active center of the enzyme very well accessible for the ligand, which, in our opinion, may help in the enzyme–ligand complex formation.

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WHY LIPASES ?

Lipases (EC 3.1.1.3) catalyze hydrolysis of carboxylic acid esters in aqueous media but also esterification or trans-esterification in organic solvents. The studies on applying commercially available hydrolytic enzymes, including lipase B from *Candida antarctica* (CAL-B), *Candida rugosa* lipase (CRL), lipase *PS Amano*, *AK Amano*, *AH Amano* or lipo-protein lipase (LPL), to the synthesis of chiral non-racemic heteroorganic compounds, have been carried out in our Department for many years. We have decided to make theoretical and experimental attempts to explain the catalytic mechanism of the enzymes and the nature of their promiscuous behavior.

WHY CAL-B ?

Among commercially available lipases, which are nowadays expressed in large quantities, lipase B from *Candida antarctica* (CAL-B) is one of the most extensively used biocatalysts, in both research and industry. This enzyme is highly stereoselective in a wide variety of chemical transformations. A broad range of CAL-B applications includes kinetic resolution of racemic alcohols and amines or desymmetrization of diols and diacetates. Numerous examples pertain to the stereoselective synthesis of chiral intermediates for the production of various pharmaceuticals and plant-protecting agents. There are also many cases of promiscuous reactions catalyzed by CAL-B, including aldol reactions, Michael additions, vinyl polymerization, and even oxidations.

CRYSTAL STRUCTURES: CURRENT STAGE

There is only limited information in literature, regarding crystal structure and active site architecture of these enzymes:

* In the Protein Data Bank (PDB), there are 12 structures available for lipase B from *Candida antarctica* (CAL-B): 1TCA, 1TCB and 1TCC, 1LBS and 1LBT, 3ICV and 3ICW, 4K5Q, 4K6G, 4K6H, and 4K6K, and 3W9B.
* For *Candida rugosa* lipase (CRL), there are 9 structures: 1CRL, 1GZ7, 1LPM and 1LPS, 1LPN, 1LPO, and 1LPP, 1TRH, and 3RAR.
* For lipase *PS Amano*, there is one PDB entry, 1OIL.
* For lipases *AK Amano*, *AH Amano*, and lipoprotein lipase (LPL), there are no X-ray crystal structures reported to date.

KNOWN CAL-B CRYSTAL STRUCTURES

Lipase B from Candida antarctica (CAL-B) is a macromolecule with molecular mass of about 33 kDa, and contains 317 amino acid residues in its polypeptide chain. Crystal structures, determined for the wild-type of this enzyme, in unliganded (PDB entries 1TCA, 1TCB, 1TCC) and the ligand-bound forms (PDB entries 1LBS, and 1LBT), show that the active site is composed of a catalytic triad Ser-His-Asp and an oxyanion hole that stabilizes the transition state of the reaction.

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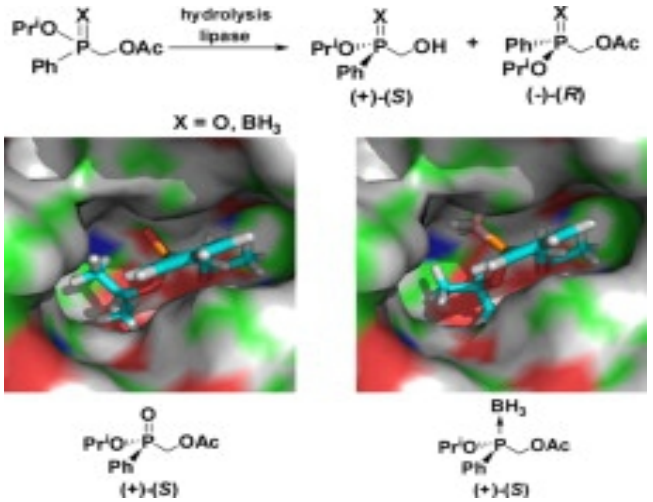
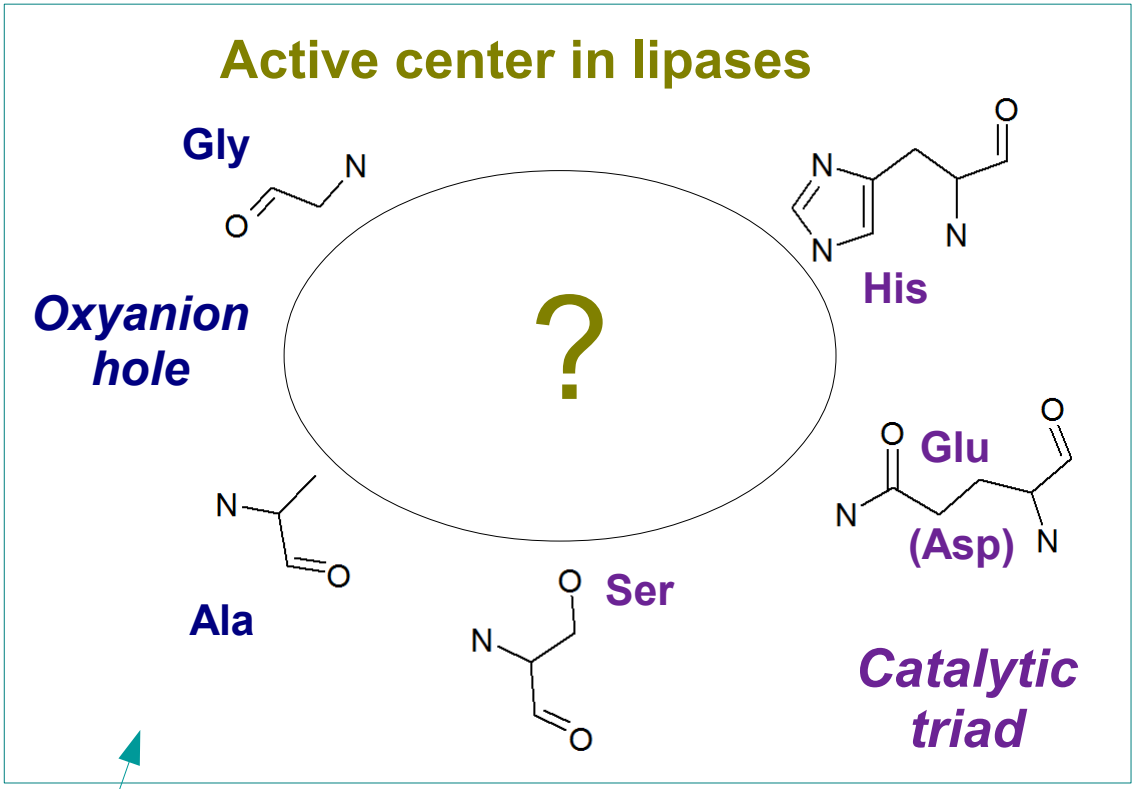
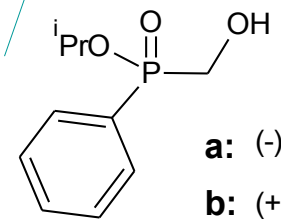


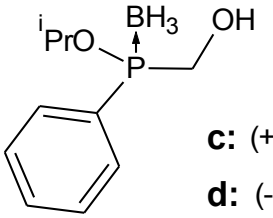


Theory
Experiment



a: (-)-(R) (slow)

b: (+)-(S) (fast)



c: (+)-(?) (fast)

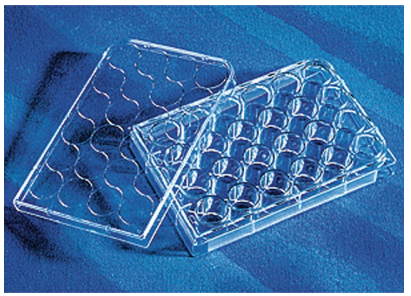
d: (-)-(?) (slow)

PURPOSE OF THIS STUDY

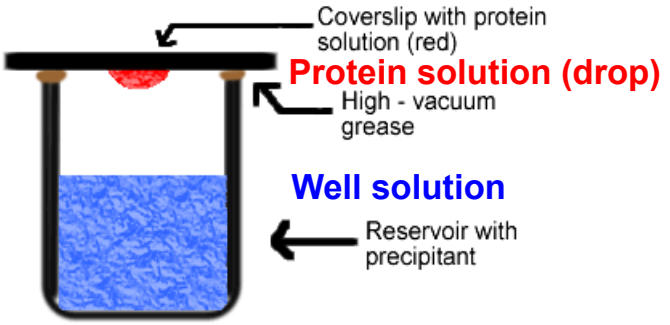
All X-ray structures determined to date are insufficient to explain the mechanism of action of these enzymes towards hetero-organic substrates and their analogs we use in the enzyme-controlled chemical reactions conducted in our lab. Of the particular concern, in one of our ongoing research projects, is the kinetic resolution of racemic P-stereogenic alkoxy(hydroxymethyl)phenylphosphine oxides and P-stereogenic alkoxy(hydroxymethyl)phenylphosphine P-boranes via their enzymatic acetylation. Recently we reported the results of molecular modeling of the hydrolysis reactions of acetoxymethyl(i-propoxy)-phenylphosphine oxide and its P-borane analogue, acetoxymethyl(i-propoxy)-phenylphosphine P-borane, promoted by the CAL-B enzyme. These theoretical calculations suggested a hypothetical explanation of the stereochemistry of the observed hydrolysis reactions. Now, our goal is to confirm our theoretical results experimentally, by crystallization and X-ray crystal structure determination of CAL-B enzyme in the form of a complex with above mentioned heteroorganic ligands and their analogs.

OUR WORK

We would like to present our first result of long-term crystallization screenings of commercially available enzyme candidates, from which most of them were - more likely - of insufficient purity, which prevented these enzymes from producing crystals. While working with crystallization screening of newly purchased, crystallization grade sample of CAL-B enzyme, we discovered new, hexagonal crystal form of this enzyme. This new form is the subject of this work. Due to high symmetry and good diffraction properties, the crystals of this form may be useful to obtain complexes with various ligands.



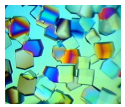
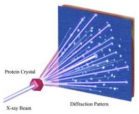
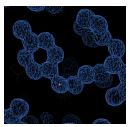
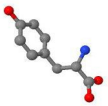
24-well Costar cell culture plate

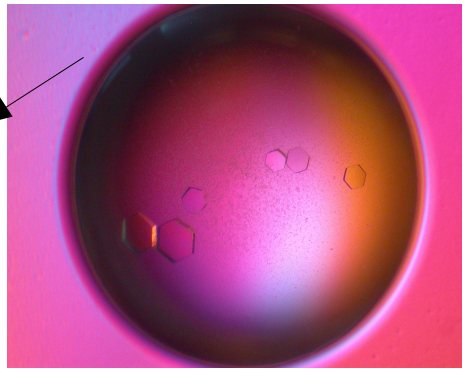


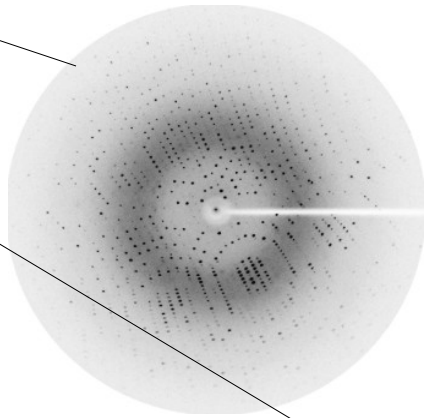
A ”hanging drop” crystallization method

OUR EXPERIMENT

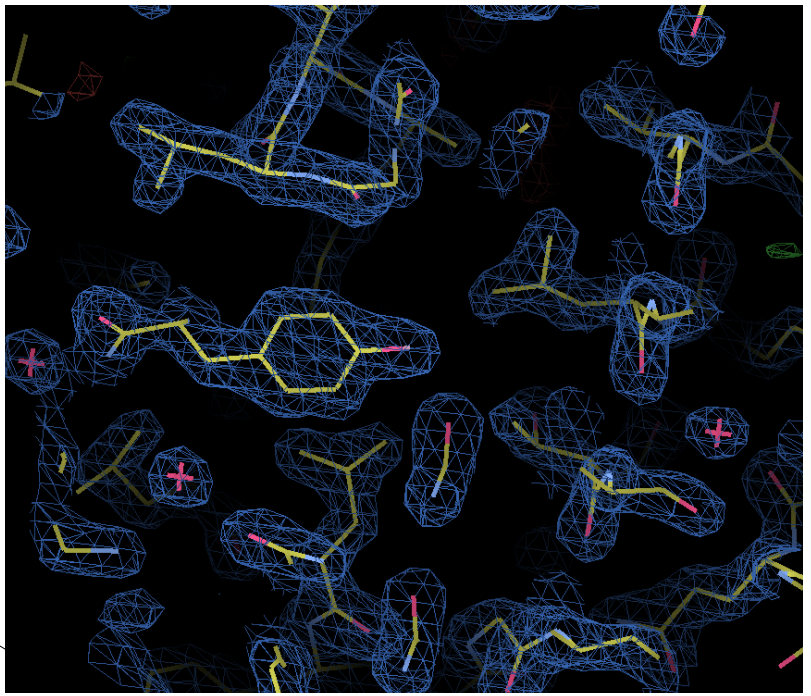
Protein purchase and screening. CAL-B was **purchased** from *Hampton Research* (Aliso Viejo, CA, USA). Prior to crystallization, purchased protein was subjected to **final purification** using size exclusion chromatography. The **crystallization screening** involved the use of the conditions provided by protein manufacturer and also the use of the set of 50 unique solutions from commercially available *Hampton Research Crystal Screen One*. To our surprise, none of these conditions resulted in crystals of suitable quality. Therefore we needed to explore new crystallization conditions. **Crystallization.** We finally got two crystal forms: monoclinic and hexagonal. **Monoclinic crystals** grew from 22% (w/v)PEG 4000, 0.05M sodium acetate (pH 3.6), 10% isopropanol with addition of 5% (w/v) n-octyl-β-D-glucoside. Monoclinic P2(1) form is already known and reported in the literature as PDB entry 1TCB. The hexagonal P6(3)22 form is new. **Hexagonal crystals** grew within two weeks from 24% PEG 3350, 0.1M citric acid, and 0.1M sodium acetate (pH 5.5). **Data collection, structure solution and refinement.** X-ray diffraction data were collected at 100 K using *SuperNova* diffractometer (CuKα radiation). Data were processed using *CrysAlisPro* and merged using *program CCP4*. Structure was solved by molecular replacement using *Phaser*, with PDB 3W9B entry as a starting model which was rebuilt using *Coot* and refined using *REFMAC5*. Electron density allowed to determine the position of all 317 residues of the entire protein sequence without any ambiguity. Structure was deposited in the Protein Data Bank as entry 4ZV7.

Research steps	Progress
 Crystallization	
 Crystal selecting	
 Diffraction experiment and data processing	
 Structure solution	
 Structure refinement	
RCSB PDB PROTEIN DATA BANK Structure deposition	
ISI Web of Knowledge. Structure publication	



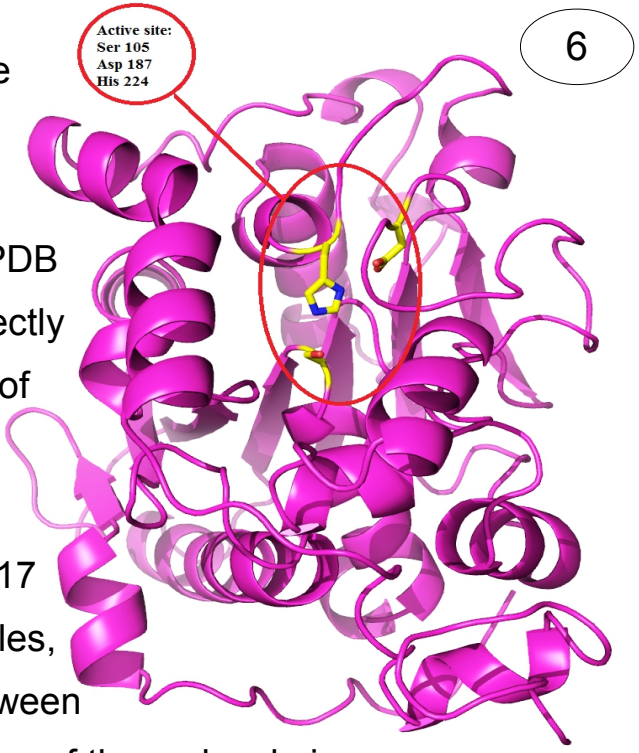


Entry 4ZV7

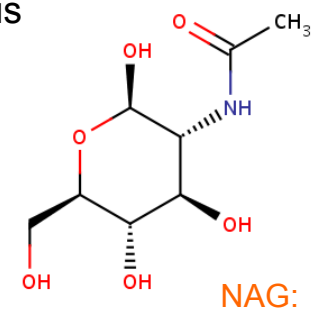


OUR RESULTS

New crystal form of CAL-B discovered. While working with crystallization screening of newly purchased sample of CAL-B, we discovered a new, hexagonal, crystal form. From CAL-B structures which are reported in literature and available in the PDB to date, only 1TCA, 1TCB and 1TCC are ligand-free forms and therefore directly related to our newly obtained hexagonal form (4ZV7). All four structures are of wild-type enzyme, and do not contain a ligand in the active center.



General features of our hexagonal CAL-B. The space group is P6(3)22. A single monomer is present in asymmetric unit. The structure contains all 317 residues of the entire sequence, two NAG (N-acetyl-D-glucosamine) molecules, and 331 waters. Two cis-peptide bonds are present in the structure: one between Pro69 and Pro70, and second between Gln191 and Pro192. The conformation of the molecule is stabilized by presence of three disulfide bridges: Cys22-Cys64, Cys216-Cys258, and Cys293-Cys311. The presence and connectivity of the disulfide bridges, align well with those present in orthorhombic and monoclinic forms (PDB entries 1TCA and 1TCB).

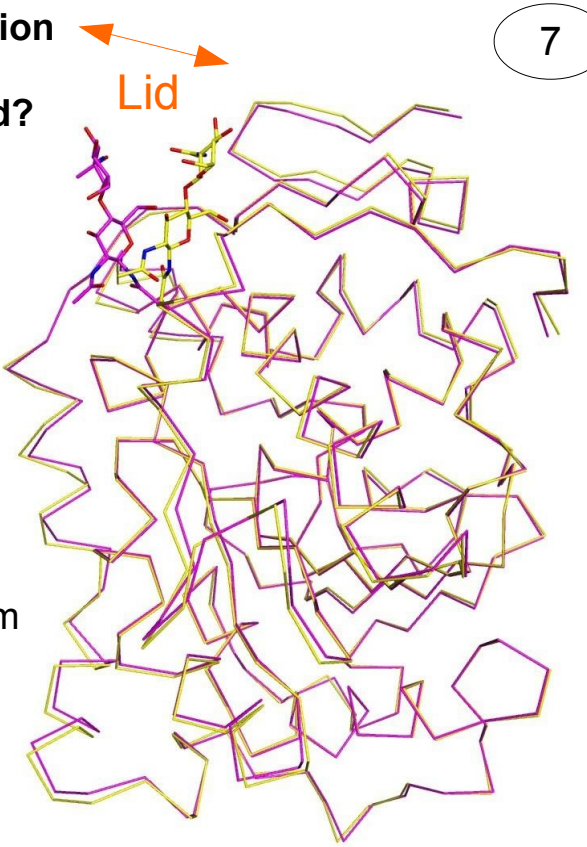


NAG:
N-Acetyl-D-Glucosamine

Orthorhombic *versus* hexagonal form: similarities and differences. Both orthorhombic and hexagonal forms have single monomers in asymmetric units. The fold of hexagonal form 4ZV7 resembles well the fold of orthorhombic form 1TCA. There are more water molecules in the active center in hexagonal than in orthorhombic form. For that reason, the active sites in hexagonal and orthorhombic forms must display some differences in conformation. Catalytic triad residues Ser105, Asp187, and His224, in active site of orthorhombic form, have almost identical position and conformation, except for Cβ-Oγ bond of Ser105, which rotates slightly away from the Nε2 atom of His224. The movement of the Oγ atom of Ser105 is about 0.5 Å.

OUR RESULTS (cont.)

Unexpected opening of glycosylation site in hexagonal CAL-B. Is this opening functionally related?



In both orthorhombic (1TCA) and hexagonal (4ZV7) forms, the NAG molecules are present in the glycosylation site and bound to the side chain Nδ2 atom of Asn74 residue (Figure 3). These NAGs do not align with each other.

In hexagonal form, the side chain of Asn74 and two bound visible NAGs rotate straight away the protein molecule towards the solvent region.

In orthorhombic form, this side chain and two NAG molecules rotate towards the enzyme molecule, and close the glycosylation site. The torsion angle N-Cα-Cβ-Cγ of Asn74, which describes this rotation, in orthorhombic form is -163.4º, but in hexagonal form is only -96.4º (makes rotation of about 70º).

We do not know whether the opening (in hexagonal form) and closing (in orthorhombic form) of the glycosylation site, by the 'lid' NAG molecules, is just an effect of the crystal packing, or, it could be related to the opening and closing of the enzyme active center upon substrate binding and product release.

Conserved, tightly buried water, in all crystal forms. Of special interest is the one water molecule, bound to the Oδ2 atom of the active site residue Asp187 (distance 2.63 Å in hexagonal form) and to Oγ atom of Ser227 residue (distance 3.12 Å in hexagonal form). This water, in orthorhombic form 1TCA is located in same place and has the same connectivity.

The connectivity of this water in monoclinic form 1TCB is almost identical with that in orthorhombic 1TCA.

Hexagonal CAL-B: data collection and refinement statistics.

Crystal system	Hexagonal
Space group	P6322
Unit cell constants a,b,c (Å)	89.03, 89.03, 137.26
No. of molecules in asymmetric unit	1
Solvent content (%)	48.24
Wavelength (Å)	1.54
Temperature (K)	100
Resolution range (Å)	77.11-2.00
Completeness (overall / last shell)	98.1 / 91.1
Redundancy (overall / last shell)	8.8 / 2.9
I / sigma (overall / last shell)	24.58 / 3.34
Unique reflections (overall / last shell)	22034 / 2534
R (merge) (%) (overall / last shell)	0.093 / 0.261
R (work)	0.144
R (free)	0.193
Ramachandran plot statistics:	
favoured regions (%)	97.00
allowed regions (%)	3.00
outliers (%)	0.00

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