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CORSO DI LAUREA IN: FOOD AND BEVERAGE INNOVATION AND MANAGEMENT

Evidenze strutturali dell'inibizione da metanolo della lipasi B da *Candida antarctica*

Structural evidence of methanol inhibition of *Candida antarctica* Lipase B

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To the bonds that unit.

INDEX

Abstract

Candida antarctica lipase B (CALB) is one of the most widely studied enzymes, due to its high selectivity and catalytic activity in organic and polymer synthesis.
Because of the great interest in knowing the behaviour of CALB in presence of specific organic solvents, the molecular mechanism of the deactivation of the enzyme by methanol was already investigated by several researchers. In this contest, for having an understanding of the defective activity of the lipase, we addressed the inhibition pathways of CALB by methanol. The structural modifications of CALB in a methanol-water mixture were evaluated in the present work considering that the catalytic activity decreases in such mixture.

The present work reports the crystal structure of the lipase in presence of methanol refined with specific crystallographic software tools, namely WinCoot, REFMAC5 and Phenix able to refine the model every time a correction is made on the enzymatic structure.

WinCoot is the program used for any kind of correction at the atomic level to the crystal structure because capable to provide specific tools for intervening on the enzyme.
Then, the obtained model was refined through with REFMAC5 or Phenix, both used for validating these the modifications.

Having a molecular description of the interaction between solvent and enzyme is prerequisite to a qualitative understanding of enzymatic activity in organic solvents.

Keywords: *Candida antarctica*, lipase B, esterification, methanol inhibition

WORK AIM

Study of the modifications in enzymatic structure by refining the crystallographic model of CALB in a methanol-water mixture for understanding the catalytic activity.

1. INTRODUCTION

Early reports on the production by both bacterial and eukaryotic cells of enzymes able to degrade lipid substrates date to over a century ago. Since then, research on lipolytic enzymes – that includes lipases, phospholipases – has been driven by their central roles in lipid metabolism and in signal transduction.

Lipases are generally versatile enzymes that accept a broad range of substrates. The stability of most lipases in organic solvents paves the way for their exploitation in organic synthesis: in esterification, transesterification, aminolysis and oximolysis reactions (1).

Lipases (triacyl-glycerol hydrolases E.C.3.1.1.3) catalyse the hydrolysis of triglycerides at the oil/water interface, but their ability to form ester bonds under reverse hydrolytic conditions enables them to catalyse various other types of reactions such as esterification, transesterification, polymerisation and lactonization (2) of interest for the food industry.

1.1 Lipases

Lipases are like all enzymes, protein molecules and soluble in water. Their normal function is to hydrolyze triglycerides into diglycerides, monoglycerides, fatty acids and glycerol (Fig.1). A second group of enzymes, esterases were characterized by their ability to hydrolyze carboxylic ester bonds. All known lipases belong to the family of α/β-hydrolases, which share a common fold composed of a central hydrophobic eight stranded β-sheet packed between two layers of amphiphilic αhelices. They also share a common catalytic mechanism. The active sites of lipases are composed of a Ser-Asp/Glu-His motif, which compose a catalytic triad similar in arrangement to serine proteases (3).

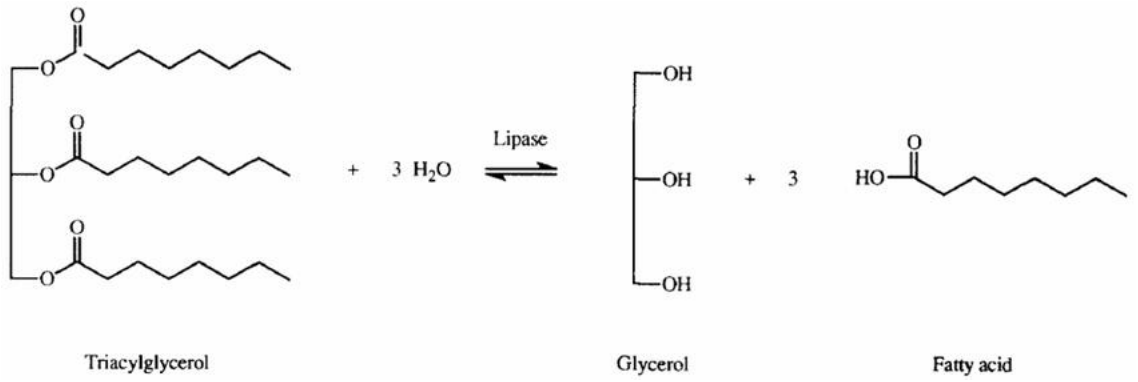

Figure 1. Enzymatic reaction of a lipase catalyzing hydrolysis or synthesis of a triacylglycerol substrate.

Most microbial lipases exhibit maximum activity in the temperature range of 30–40◦C. Thermophilic lipases retaining activities in the temperature range of 50–65˚C have been isolated from both filamentous fungi (*Aspergillus niger, Thermomyces lanuginosus*) and some bacteria (*Pseudomonas* and *Bacillus sp*.).

Maximum activity of most microbial lipases is displayed in the pH range of 5.6–8.5 and maximum stability in the neutral pH range. Some alkaline lipases having pH optima around 9.5 have been discovered, for example from Bacillus and *Pseudomonas* species (3). Lipases are commercially available from different suppliers also in food grade form.

Lipases are used to release short-chain fatty acids from milk fat, and are responsible of the flavour and taste of the final products, as “immobilized biosensors” for the determination of tryacylglycerols concentrations, for the synthesis of aromatic molecules (low molecular weight esters) and they are responsible for the hydrolytic rancidity (3).

The main reason for the use of lipases is the growing interest and demand for products prepared by natural and environmentally compatible means. As a consequence of their versatility in application, lipases are regarded as enzymes of high commercial potential. Lipase catalysed esterification in organic solvents presents challenges, which if dealt with successfully, can result in the generation of a number of useful compounds. Both the range of substrates with which lipases react and the range of reactions catalysed are probably far wider than those of any other enzyme studied to date (2)

Lipases catalyse three types of reactions: i) Hydrolysis: occurs in aqueous media when there is large excess of water, ester hydrolysis is the dominant reaction; ii) Esterification: under low water conditions such as in nearly anhydrous solvents, esterification can be achieved (improved product yields can be obtained if the water content of the medium is controlled); iii) Transesterification: the acid moiety of an ester is exchanged with another one (if the acyl donor is a free acid the reaction is called acidolysis, whereas the reaction is called inter-esterification if the acyl donor is an ester; in alcoholysis, the nucleophile alcohol acts as an acyl acceptor). Lipases are currently used in the production of many commercially important esters (2).

1.2 *Candida antarctica* Lipase B

The anamorphic basidiomyceteous yeast *Candida antarctica* was first isolated from sediment from the bottom of the antarctic lake Vanda, perennially covered with 3–5 m of ice. The original name given to this yeast was *Sporobolomyces antarcticus* and since then the organism has been around under a number of different aliases. When isolates belonging to this species in 1988 were identified as lipase producers, the generally accepted name was *Candida antarctica* (4).

Pseudozyma (Candida) antarctica is then an anamorphic yeast of basidiomycetous affinity. The yeast has attracted industrial attention, primarily as a source of lipase enzymes used in a number of industrial processes and for biodiesel production. *Pseudozyma* has also been considered for production of native surfactant proteins, degradation of plastic wastes, and heterologous proteins (5).

Candida sp. is the most prominent lipase producer among yeast reported in the literature. The species *C. antarctica* produces two different lipases: lipase A and B. CALB is probably the mostly employed hydrolase in the biocatalysis field, meanwhile the use of the lipase A (CALA) has been rather scarce. In 1994 the research group of Uppenberg for the first time described the amino acid and genic sequence of CALB. It was proved that lipase B derived from *C. antarctica* yeast consists of 317 amino acid residues giving molar mass 33 kDa (6) (Fig. 2 – 3).

Candida antarctica lipase B (CALB) is the one that found the widest application in many industrial processes because of its high enantioselectivity, wide range of substrates, thermal stability, and stability in organic solvents. CALB belongs to the α/β hydrolase fold family with a conserved catalytic triad consisting of Ser, His, and Asp/Glu within an acyl binding pocket and a binding pocket for the moiety of secondary alcohols (7).

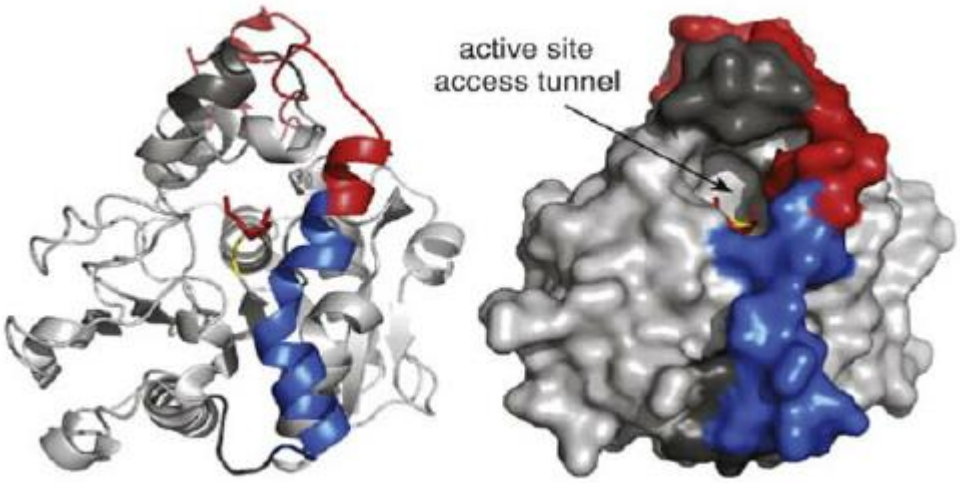

Figure 2. Structure of Candida antarctica lipase B shown as ribbon and surface representation.

The active site is composed of a nucleophilic serine residue activated by a hydrogen bond in relay with histidine and aspartate or glutamate. CALB is highly enantioselective and efficient biocatalyst used in the wide range of organic reactions including kinetic resolutions, aminolysis, esterification conducted in solventless media and in polar organic solvents. Its activity is higher in water than in organic media, therefore numerous studies were performed in order to improve the properties of CALB as a catalyst in other solution such as immobilization. Immobilized enzymes show better catalytic efficiency than the corresponding free enzymes and are able to withstand high temperatures. Furthermore, immobilization allows ease removal of the catalyst from reaction mixture which can be reuse even up to ten times (6).

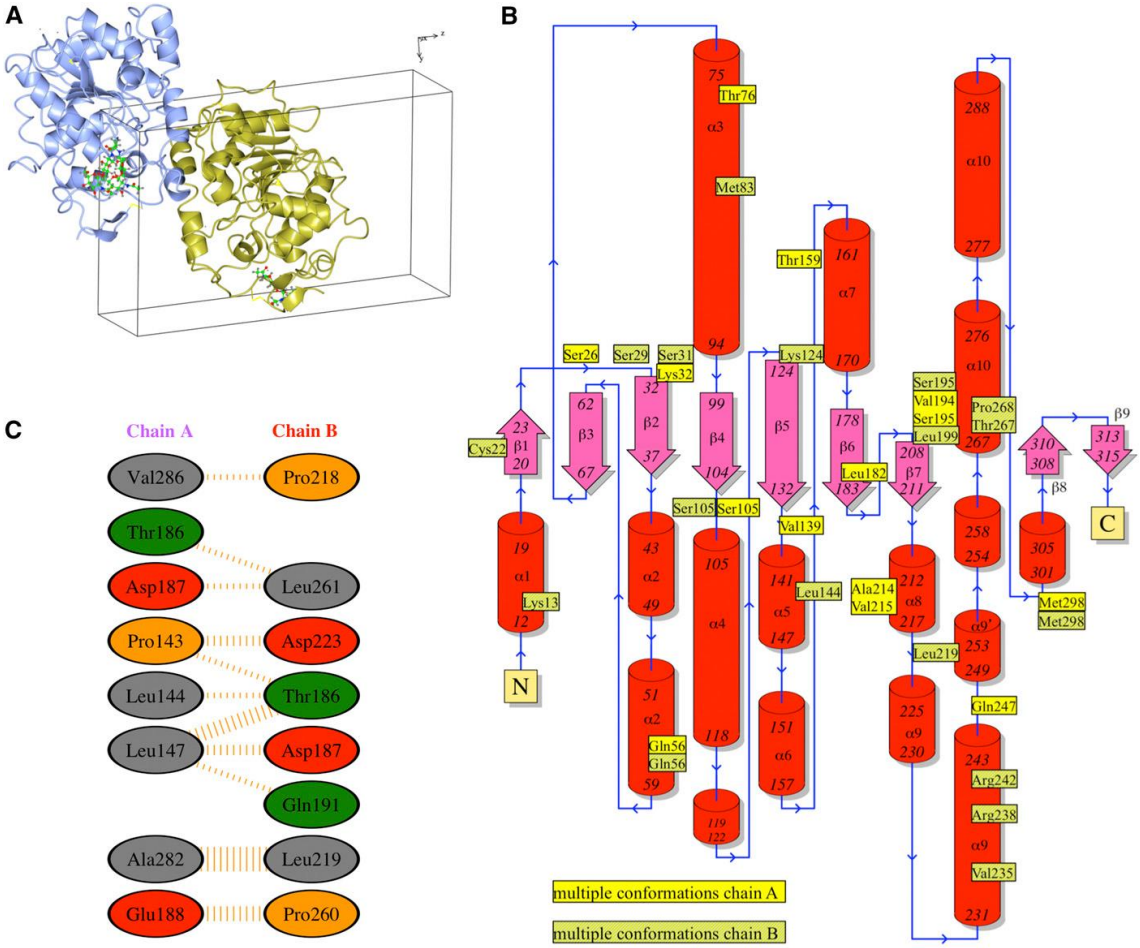

Figure 3. Graphical representation of CALB. A: Asymmetric unit. B: General secondary structure topology with the position of residues showing multiple conformations highlighted. C: Nonbonded contacts between residues, the width of the striped line is proportional to the number of atomic contacts. Figures were generated using PDBsum web server (7).

CALB has two -helixes surrounding the active site, namely 5 and 10, which have been shown to be very flexible regions and could work as a lipase lid by a relative motion between them either by increasing the working temperature or by working in organic solvents.

Such helix motions, in aqueous media, could not be large enough to produce variation in the hydrophobic surface and in the solvent-accessible area or large enough to prevent access to the active site. However, the CALB sequence stretch around the alpha-helix 5 has been shown to significantly influence the catalytic properties of the enzyme, including the enantioselectivity, which, consequently, seems not to be exclusively directed by the fit of the substrate into the enzyme's active site pocket. However, a closed conformation of CALB has never been reported and CALB has never shown any significant interfacial activation. Whether or not CALB lacks a lid structure is matter of debate, according to several molecular dynamics simulation studies (7).

1.3 Organic solvents

The use of enzymes for organic synthesis has become an interesting area for organic and bio-organic chemists. Since many enzymes have been demonstrated to possess activity against non-natural substrates in organic media they have become widely used to carry out synthetic transformations. Hydrolases are the most frequently used enzymes due to their broad substrate spectrum and considerable stability. Additionally, many of them are commercially available and they work under mild reaction conditions and without the necessity for cofactors. Among the hydrolases, lipases (EC 3.1.1.3) are considered the most popular and useful enzymes for asymmetric synthesis.

One of the more serious drawbacks for the use of enzymes in aqueous organic synthesis is the poor water solubility of organic compounds with more of four carbon atoms. Water is also a poor solvent for most applications in industrial chemistry, since many organic compounds are unstable in aqueous solution. Furthermore, its removal is more tedious and expensive than that of organic solvents, the boiling points of which are lower. The use of organic solvents presents several advantages including: (a) easier recovery of products with high yields; (b) the possibility of using non-polar substrates; (c) organic solvents avoid many side reactions, (d) in many cases lipases are thermodynamically more active than in water; (e) shifting the thermodynamic equilibrium to favour synthesis over hydrolysis (8).

So, most of the synthetic reactions on industrial scale are carried out in organic solvents because of the easy solubility of non-polar compounds. The effect of organic system on their stability and activity may determine the biocatalysis pace. Because of worldwide use of lipases, there is a need to understand the mechanisms behind the lipase-catalyzed reactions in organic solvents. Biocatalysis in non-aqueous media has been widely used for the resolution of alcohols, acids or lactones by enzymatic transesterification reactions using different lipases (Klibanov, 2001). Moreover, other processes such as the enzymatic acylation of amines or ammonia have shown themselves to be of great utility for the resolution of amines and the preparation of chiral amides.

1.4 Esterification process

Esterification reaction is an equilibrium reaction and it can be displaced toward the product side by removal of water or by the use of an excess of one of the reactants (Fig. 4). The use of acetone dimethylacetal, which reacts with the water formed to produce methanol and acetone, allows the preparation of methyl esters in high yield. Primary and secondary alcohols are esterified in good yield, but tertiary alcohols give very low yields.

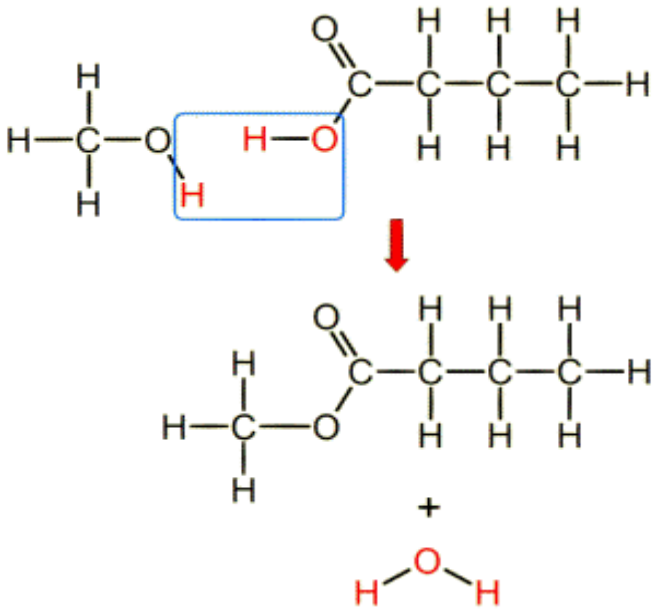

Figure 4. Esterification reaction

In the past two decades, several research groups reported synthesis of cyclic esters in the presence of CALB (Fig. 5). Cyclic esters, in particular lactones, are well known not only for their fragrance properties and a variety of biological effects. Optically active lactones are important building blocks in fine organic synthesis and are applied in the preparation of biodegradable polymers for biomedical applications. In this field enzymes are unique catalysts due to theirs stereoselectivity (8).
The influence of solvent, type of lipase and different alkyl lactates as substrates on the synthesis of (R,R)- lactide were investigated by Jeon. It turned out, that N-435 was the most

effective catalyst in the synthesis of (R,R)-lactide from methyl (R)-lactate in the presence of methyl tert butyl ether. The product was obtained with 59% yield, and ee value equal to 99%.

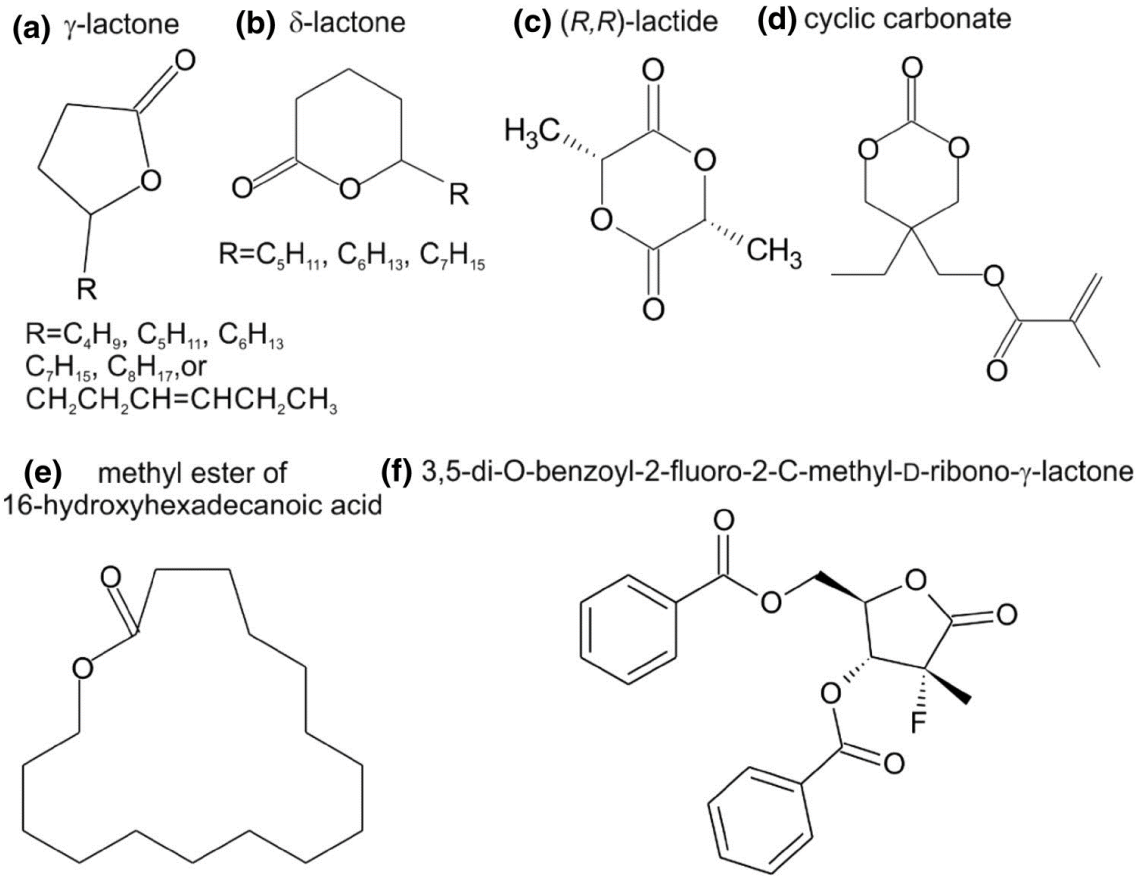

Figure 5. Examples of cyclic esters synthesized in the presence of CALB

Most of the polyesters obtained by enzymatic polymerization in the presence of *C. antarctica* lipase are characterized by a low molar mass and are predominantly designed for biomedical applications (especially in drug delivery systems). Almost all polymerization reactions were conducted in organic solvents, such as: toluene, 1,4-dioxane, isopropyl ether, isooctane, diphenyl ether, cyclohexane. The reaction was also carried out in supercritical carbon dioxide, ionic liquids, and recently, in aqueous dispersion (6).

Many sectors of industry, mainly food, cosmetics and pharmaceutics, have increased their interest in esters due to their flavor property. Flavor esters that possess an aromatic ring in their molecular structure are also known as aromatic esters. These esters are widely found in nature. In this context, from the industrial point of view, enzyme-catalyzed reactions are the most economical approach to reach final green products with no toxicity and no harm to human health.

Lipases play an important role in the aromatic esters production, with several advantages over synthetic route. Lipase-catalyzed reactions usually follows Ping-Pong Bi-Bi or ternary complex mechanism. Aromatic esters can present some biological activities, in addition to their fragrances, which increases the interest in the encapsulation of these compounds. (9)

Moreover, one of the main interests of enzymatic aromatic ester production regards surely the food sector. The extraction of esters and the following utilization in food products as additives, indeed represents one of the most interesting aspect of these molecules.

Furthermore, the kind of ester that could be produced by esterification reaction depends from the reagents available for reacting and consequently the final ester product show differences in the fragrance. In the following figure (Fig. 6) are represented the most important esters and their specific smells.

Figure 6. Table of esters and their smells

2. MATERIAL

2.1 *Candida Antarctica* lipase B

Candida antarctica Lipase B (CALB) was purchased from Hampton Research and crystallized without further purification by Prof. M. Cianci at the European Molecular Biology Laboratory, c/o DESY, Hamburg (Germany).

2.2 WinCOOT

The program Coot (Crystallographic Object-Oriented Toolkit) is used to display and manipulate atomic models of macromolecules, typically of proteins or nucleic acids, using 3D computer graphics. It is primarily focused on building and validation of atomic models into three-dimensional electron density maps obtained by X-ray crystallography methods, although it has also been applied to data from electron microscopy.

Coot displays electron density maps and atomic models and allows model manipulations such as idealization, real space refinement, manual rotation/translation, rigid-body fitting, ligand search, solvation, mutations, rotamers, and Ramachandran idealization.

Coot can be used to read files containing 3D atomic coordinate models of macromolecular structures in a number of formats, including pdb files. The model may then be rotated in 3D and viewed from any viewpoint. The atomic model is represented by default using a stick-model, with vectors representing chemical bonds. The two halves of each bond are coloured according to the element of the atom at that end of the bond, allowing chemical structure and identity to be visualised in a manner familiar to most chemists.

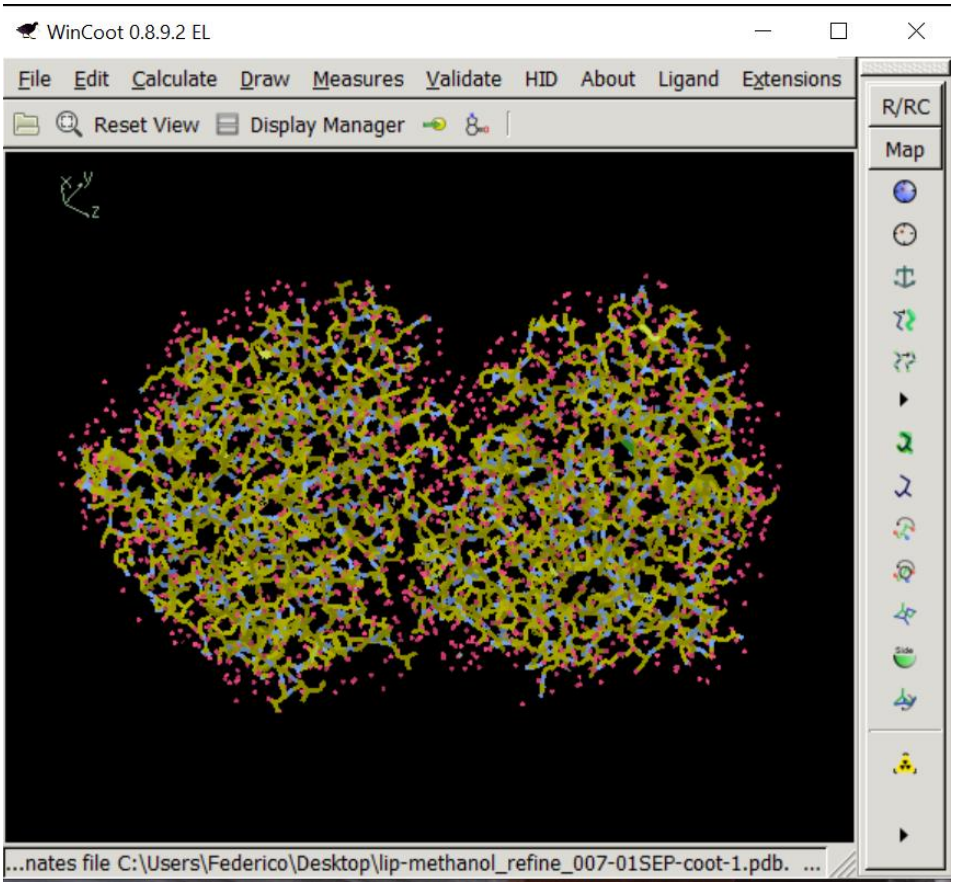

Figure 7. C. antarctica lipase B view in WinCoot software.

Coot can also display electron density, which is the result of structure determination experiments such as X-ray crystallography and EM reconstruction. The density is contoured using a 3D-mesh. The contour level controlled using the mouse wheel for easy manipulation - this provides a simple way for the user to get an idea of the 3D electron density profile without the visual clutter of multiple contour levels. Electron density may be read into the program from CCP4 or PHENIX map formats, though it is more common to calculate an electron density map directly from the X-ray diffraction data, read from an mtz, hkl, fcf or mmcif file.

Coot provides extensive features for model building and refinement (i.e. adjusting the model to better fit the electron density), and for validation (i.e. checking that the atomic model agrees with the experimentally derived electron density and makes chemical sense). The most important of these tools is the real space refinement engine, which will optimize the fit of a section of atomic model to the electron density in real time, with graphical feedback. The user

may also intervene in this process, dragging the atoms into the right places if the initial model is too far away from the corresponding electron density (Fig. 7).

Coot provides tools for the display of three-dimensional data falling into three classes.

(i) Atomic models (generally displayed as vectors connecting bonded atoms).

(ii) Electron-density maps (generally contoured using a wire-frame lattice).

(iii) Generic graphical objects (including the unit-cell box, non-crystallographic rotation axes and similar).

(10)

2.3 CCP4

The Collaborative Computational Project Number 4 (CCP4) in Protein Crystallography was set up in 1979 to support collaboration between researchers working on such software in the UK, and to assemble a comprehensive collection of software to satisfy the computational requirements of the relevant UK groups. The results of this effort gave rise to the CCP4 program suite, which is distributed to academic and commercial users world-wide.

CCP4 was initially supported by the UK Science and Engineering Research Council (SERC), and currently by the Biotechnology and Biological Sciences Research Council (BBSRC). The project is hosted by the Scientific Computing Department of STFC at its Rutherford Laboratory.

The CCP4 software is accessed via a central graphical interface. The CCP4 suite consists of a set of separate programs that communicate via standard format data files. It is hence very easy to add new programs to the suite, or to modify existing ones without disrupting other sections of the suite (Fig. 8). Converting a program to use the standard CCP4 file formats is straightforward.

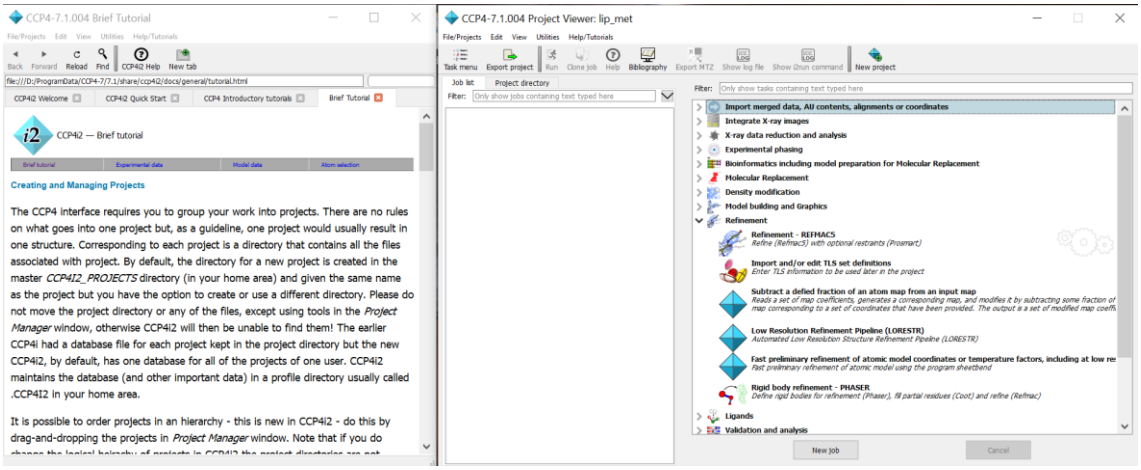

Figure 8. CCP4 starting page for refinement initialization

CCP4 (Collaborative Computational Project, Number 4) exists to produce and support a world-leading integrated suite of programs that allows researchers to determine macromolecular structures by X-ray crystallography and other biophysical techniques. CCP4 aims to develop and support the development of cutting-edge approaches to the experimental determination and analysis of protein structure and to integrate these approaches into the CCP4 software suite. CCP4 is a community-based resource that supports the widest possible researcher community, embracing academic, not-for-profit and for-profit research. CCP4 aims to play a key role in the education and training of scientists in experimental structural biology. It encourages the wide dissemination of new ideas, techniques and practice.

The CCP4 software suite is a collection of programs implementing specific algorithms concerned with macromolecular structure solution from X-ray diffraction data. Significantly, it is a collection of autonomous and independently developed programs. While some have been commissioned by the academic committees overseeing the CCP4 project, the majority originate from the community to address a perceived gap in current functionality or to implement newly developed algorithms. The result is a collection of around 200 programs, ranging from large programs which are effectively packages in themselves to small ‘jiffy’ programs. Over the years the suite has grown continuously, with each major release featuring significant new software

The philosophy of the collection has been to be inclusive, so that several programs may be available to do the same task. The components of CCP4 are thus a collection of programs using a standard software library to access standard format files. There is a policy of continual technical and scientific updates to the suite. Where existing programs have been incorporated, they have often subsequently undergone considerable enhancement above that needed to use the CCP4 file formats (11).

2.4 REFMAC5

REFMAC5 distributed as part of the CCP4 suite, utilizes different likelihood functions depending on the diffraction data employed (amplitudes or intensities), the presence of twinning and the availability of SAD/SIRAS experimental diffraction data. To ensure chemical and structural integrity of the refined model, REFMAC5 offers several classes of restraints and choices of model parameterization (Fig. 9). Reliable models at resolutions at least as low as 4 Å can be achieved thanks to low-resolution refinement tools such as secondary-structure restraints, restraints to known homologous structures, automatic global and local NCS restraints, 'jelly-body' restraints and the use of novel long-range restraints on atomic displacement parameters (ADPs) based on the Kullback–Leibler divergence. REFMAC5 additionally offers TLS parameterization and, when high-resolution data are available, fast refinement of anisotropic ADPs. Refinement in the presence of twinning is performed in a fully automated fashion. REFMAC5 is a flexible and highly optimized refinement package that is ideally suited for refinement across the entire resolution spectrum encountered in macromolecular crystallography (12).

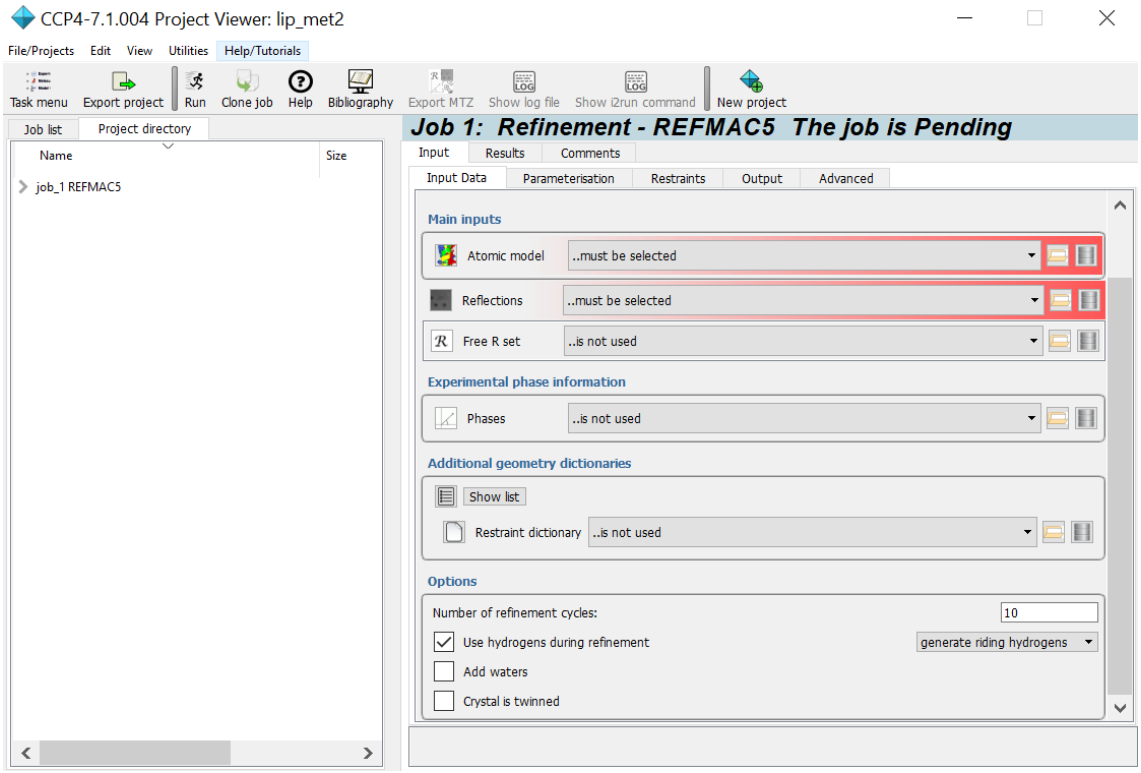

Figure 9. Setting page of REFMAC5 (CCP4 tool)

Crystallographic target functions have two components: one of them describes the fit of the model parameters into the experimental data and the second describes chemical integrity (restraints).

Currently used restraints are: bond lengths, angles, chirals, planes, ncs if available, some torsion angles, reference structures.

The function in crystallographic refinement has a form: L(p)=wLX(p)+LG(p) Where LX(p) is –log-likelihood and LG(p) is -log of prior probability distribution – restraints.

Lx(p) – X-ray part of the function describes fit of the model parameters to the experimental data LG(p) – Geometry part makes sure that the model is consistent with chemistry: bond lengths, angles, torsions, NCS, known structure etc.

After refinement programs usually give coefficients for two type of maps:

1) 2 m F_{obs} - D F_{calc} electron density map. This represents the content of the crystal.

2) m F_{obs} - D F_{calc} electron density maps. This represents the differences between model and map.

“m” is the figure of merit (reliability) of the phase of the current reflection and D is related tomodel error. “m” depends on each reflection and D depends on resolution. If phase information is available then map coefficients correspond to the combined phases. (13).

They represent difference between contents of the crystal and current atomic model. Both these maps must be inspected, and model should be corrected if necessary.

2.5 Phenix

Phenix is a software suite that uses reduced data from X-ray diffraction, electron diffraction, neutron diffraction or cryo-EM to determine macromolecular structures. Each method has a different approach to derive structural information, with Phenix offering specific tools to address the unique properties of the experimental data. Emphasis is put on the automation of all procedures to avoid burdening the user with repetitive, time-consuming and often error-prone tasks.

Macromolecules are essential for biological processes within organisms, engendering the need to understand their behaviour to explain the fundamentals of life. The function of macromolecules correlates with their three-dimensional structure, i.e. how the atoms of the molecule are arranged in space and how they move over time. Two major methods to obtain macromolecular structures are diffraction (usually using X-rays, but also neutrons or electrons) and electron cryo-microscopy, both of which are handled by Phenix (Fig. 10).

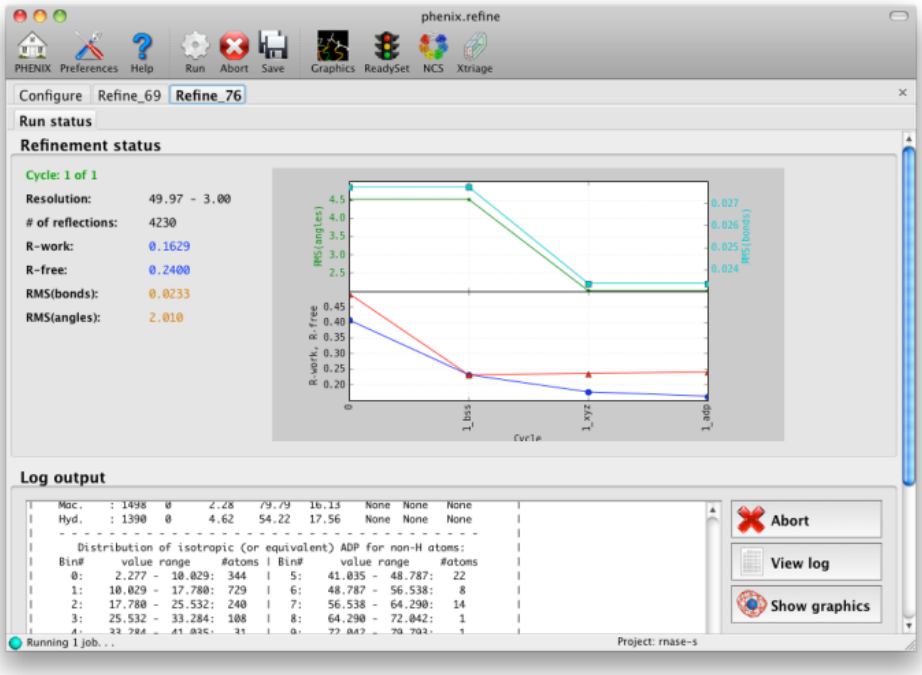

Figure 10. Results page of Phenix

Tasks performed with Phenix include data-quality assessment, map improvement, model building, the validation/rebuilding/refinement cycle and deposition. Each tool caters to the type of experimental data. The design of Phenix emphasizes the automation of procedures, where possible, to minimize repetitive and time-consuming manual tasks, while default parameters are chosen to encourage best practice. A graphical user interface provides access to many command-line features of Phenix and streamlines the transition between programs, project tracking and re-running of previous tasks. (14)

3. METHODS

3.1 Crystallization conditions

Crystallization trials were performed at 293 K using the hanging-drop method using a Quiagen™ EasyXtal 15-well plate. 1 µL of a 15 mg/mL CALB solution in 20mM $Na(CH_3COO)$ pH = 4.8 was diluted with 1 µL of the precipitant solution, made of 200mM $Na(CH_3COO)$ pH = 4.8, 24% (w/v) PEG4000, and 20-25% (v/v) methanol. The drop was equilibrated by vapor diffusion against 500 mL of the precipitant solution. Protein crystals of native CALB appeared within one week and grew as large plates to a size of 0.05 x 0.08 x 0.150 mm^3 (7) .

3.2 X-ray diffraction data collection

Diffraction data were collected at 100 K using synchrotron radiation at the EMBL P13 beam line at the Petra III storage ring, c/o DESY, Hamburg (Germany) (16). The beam line was equipped with a Dectris Pilatus 6M detector and a MD2 goniometer (MAATEL-EMBL) with horizontal spindle axis. Crystals were cooled at 100 K with a cold nitrogen stream. The wave-length was set to 0.826 Å, using a Si(III) crystal monochromator (FMB-Oxford). The data were integrated using the program XDS and scaled with XSCALE (1). Crystals of the native enzyme diffracted to 1.14 Å resolution with unit cell dimensions of a = 39.63 Å, b = 48.77 Å, and c = 71.50 Å, α= 89.01, β=96.79 , γ=108.43 and space group P1.

The structures of the methanol-CALB complex was refined starting from the deposited structure of CALB as search model (Protein Data Bank (PDB): 5A71). The model was subjected to rigid-body minimization and subsequently to refinement steps with REFMAC (2). Manual rebuilding of the models were performed using the COOT graphic interface (3) by inspecting the electron density map, calculated with $2F_{obs}$-F_{calc} or F_{obs}-F_{calc} coefficients and calculated phases from the model. The methanol-CALB complex structure was refined to an R_{factor} and R_{free} (5% of data) of 0.145 (0.229) and 0.169 (0.2513) (7).

Data collection and refinement statistics are reported in Table 1.

3.3 Structure analysis

Structural analysis was conducted directly on the electron density maps of CALB searching for each of methanol molecules and checking if these could be considered related to the model or if somehow they should be modified or completely removed from that point.

This analysis was further carried out by taking into consideration specific parameters that suggest the molecule is fitting good to the model. To this regard, WinCoot is perfectly able to provide these tools for the analysis.

The parameters were related to (Fig. 11):

-the structure of the electron density surrounding the molecule;

-the temperature factors of both C and O atoms;

-distances between -OH and -CH_3 groups;

In the first case the shape represents a good indicator of the health of the methanols even if is not fundamental to observe a perfect structure, which is very similar to that of a peanut with two lobes.

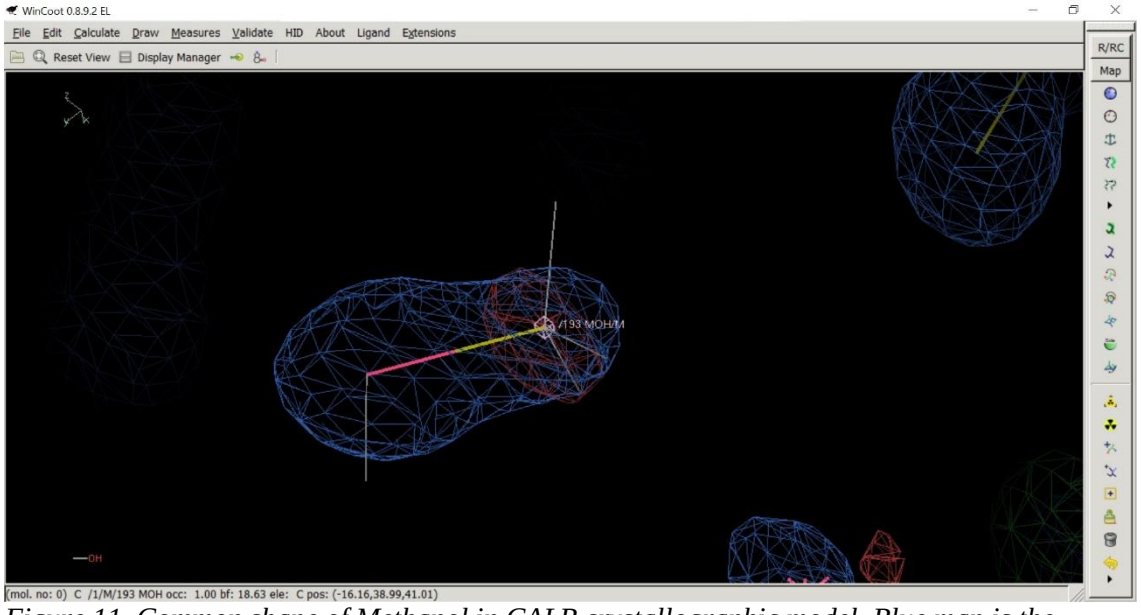

Figure 11. Common shape of Methanol in CALB crystallographic model. Blue map is the $2F_{obs}$-F_{calc} *electron density map at 1.5 sigma countour level, green/red map is the* $2F_{obs}$-F_{calc} *at 3 sigma countour level*

Of further importance the temperature factor, which indicates the kinetics of every molecule and, consequently, gives us an idea of the level of stability that could be attributed to the molecule (Fig. 12).

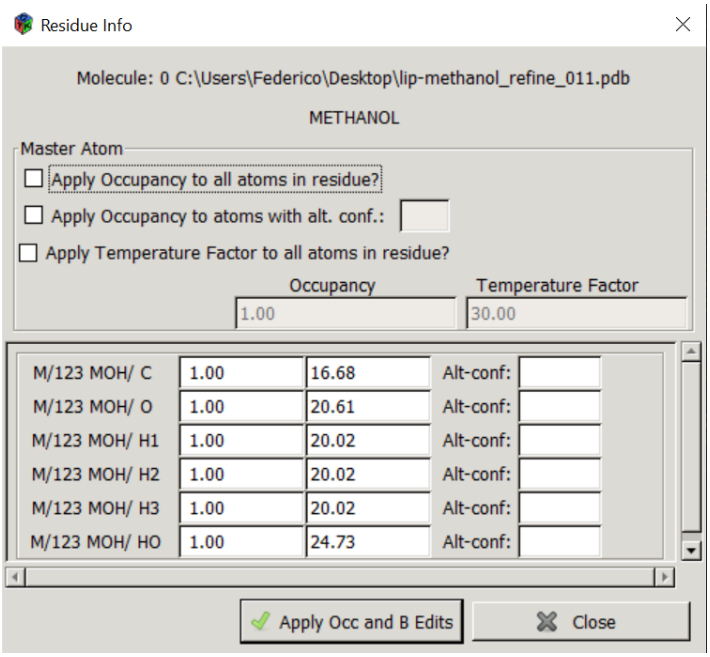

Figure 12. Temperature factors referred to all the atoms of methanol molecule

***Table 1**. Data collection, processing and refinement statistics for the native Candida antarctica and xenon complex.*

Data collection	**Native**
Wavelength (Å)	0.826
Space group	P1
Unit cell (a, b, c, Å)	39.63, 48.77, 71.50
Unit cell (α, β, γ, °)	89.01, 96.79, 108.43
Resolution range (Å)[1]	70.98 - 1.144 (1.185 - 1.144)
Refinement statistics	
Number of monomers	2
R_{factor}[3]	0.145 (0.229)
R_{free}[3]	0.169 (0.251)
Total number of non-hydrogen atoms[3]	5862
Total number of water molecules	914
Total number of methanol molecules	36
Ramachandran plot[6]	
Most favoured region (%)	97.45
Additionally allowed region (%)	2.55
Generously allowed region (%)	0.00

4. RESULTS

4.1 Overall structure of *Candida antarctica* lipase B

In line to what Stauch, Cianci and Fisher showed in their work, even in this case the conformation of the two monomers was resulted different in the sense that for the monomer A it was possible to observe an open conformation while in monomer B a closed one. (Fig. 13)

The residue range Leu140-Ala141-Gly142-Pro143- Leu144-Asp145-Ala146-Leu147, corresponding to alpha-helix 5, undergoes a conformational change from an alpha-helix structure to a loop. The electron density maps at 0.91 Å resolution clearly describe the residue range 140–147 of monomer A as an alpha-helix, corresponding to an open conformation, and of monomer B as an unfolded loop, corresponding to a closed conformation. (7)

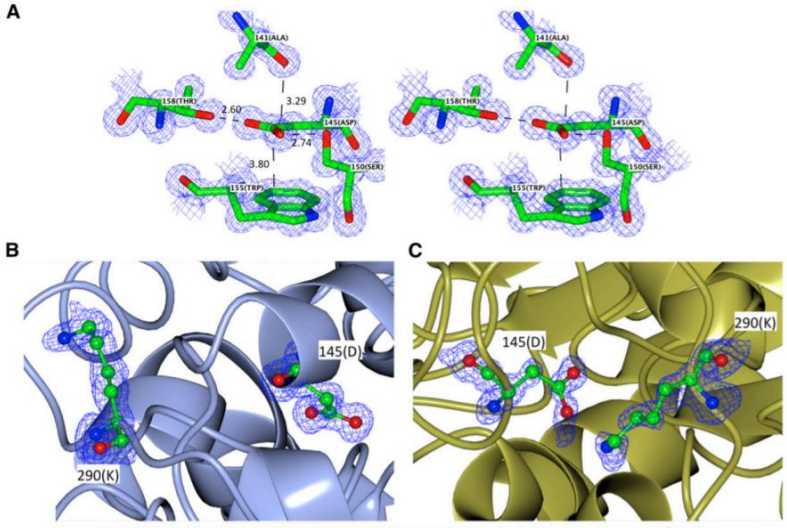

Figure 13. A: Open and closed conformations of CALB. Amino acid residues are colored as carbon, green; oxygen, red; nitrogen, blue.
B: The amino acid residues Asp145 and Lys290 in monomer A (light blue) open conformation C: and monomer B (gold) closed conformation.

It is simple to identify other similarities between CALB of Stauch, Cianci and Fisher study with that of this work. The first one is concerning surely the conformations, as already described or even the number of alpha helixes in the enzymatic structure. Then other similarities are related to the unit cell, Ramachandran plot which show great alignment with those data of Stauch, Cianci and Fisher CALB.

As observed the lipase shows 2 monomers (A and B) with several interaction points as described by Stauch, Fisher and Cianci (5) and even disulphide bond that link some cysteine residues in the same monomer (Cys22- Cys64, Cys216-Cys258 and Cys293-Cys311 in both monomer A and B). Two N-acetylglucosamine (NAG) molecules in both monomer A and monomer B. In monomer A, the triad Asn74-NAG-NAG shows two alternative conformations, while in monomer B only a single conformation is observed (Fig. 14) (7).

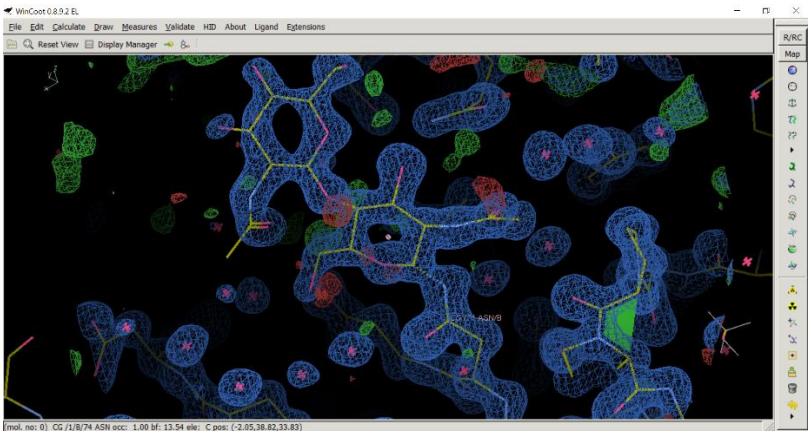

Figure 14. NAG molecules linked to Asn74 (B). Blue map is the $2F_{obs}$-F_{calc} *electron density map at 1.5 sigma countour level, green/red map is the* $2F_{obs}$-F_{calc} *at 3 sigma countour level*

4.2 Identification of methanol molecules in the overall structure of CALB

The crystallographic model of CALB grown in presence of methanol presents 36 methanol molecules which are quite equally distributed around overall lipase structure and highlight the potential inhibition pathway of the entire lipase (Fig. 15).

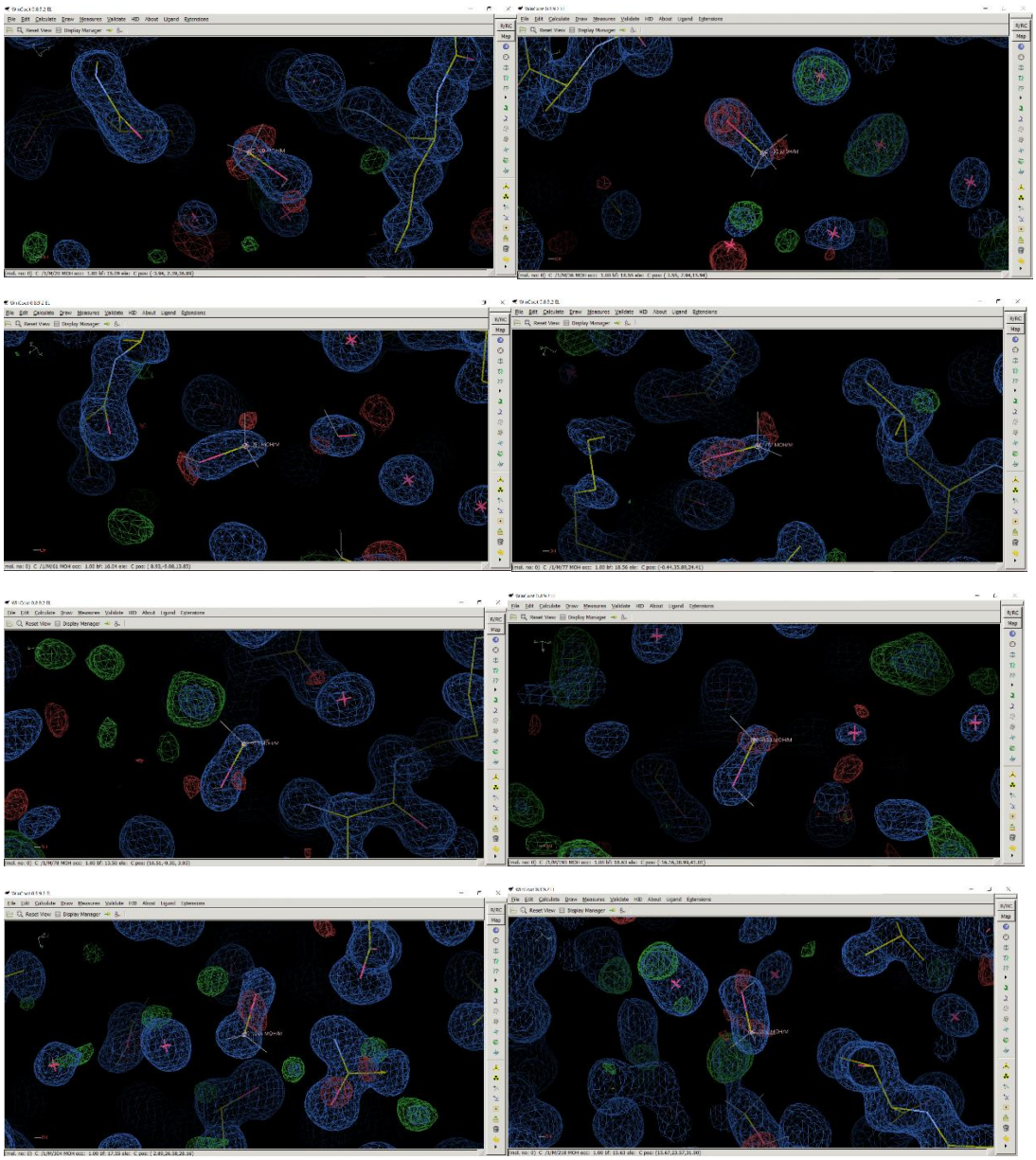

Figure 15. Examples of methanols in CALB. Blue map is the $2F_{obs}$-F_{calc} *electron density map at 1.5 sigma countour level, green/red map is the* $2F_{obs}$-F_{calc} *at 3 sigma countour level*

4.3 The active site of CALB

(Fig. 16) The active site is composed of a nucleophilic serine residue activated by a hydrogen bond in relay with histidine and aspartate or glutamate ("catalytic triad"). Another difference that distinguishes CALB is the fact, that the active site of the enzyme is not covered by a lid, but the binding site for hydrophobic substrate is directly exposed to the solvent. (14)

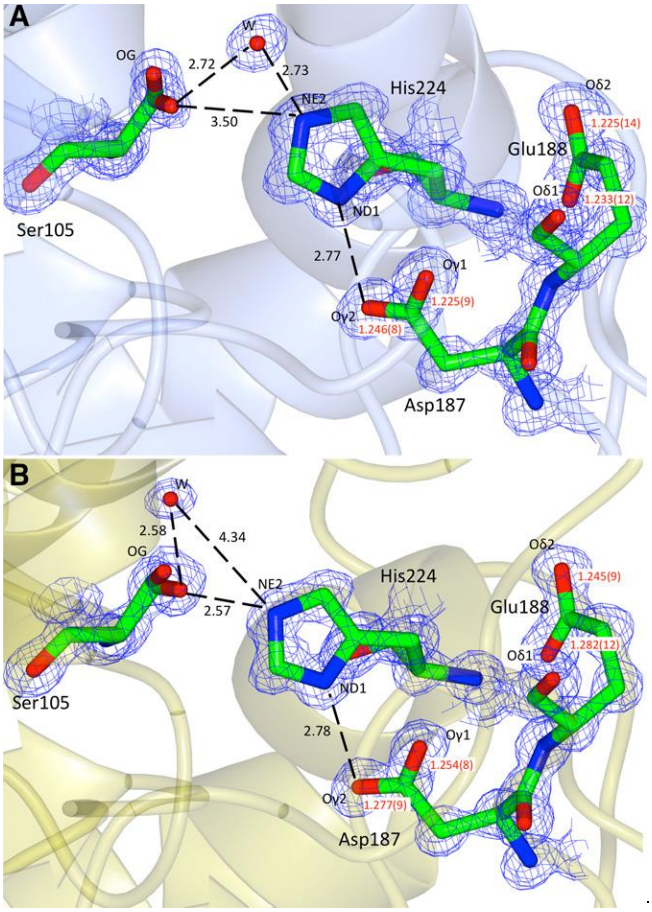

Figure 16. Active site of CALB: open conformation (A); closed conformation (B). (16)

The active site of the lipase monomers is composed in both cases by three different amino acids residues: His 224, Ser 105 and Asp 187 and the main difference between the two monomers is related to the presence of methanols inside the site.

4.4 Distribution methanol molecules in the active site of CALB

While in most cases enthalpy drives enantioselectivity, sometimes entropy is driving enantioselectivity, such as the experimentally observed preference of *Candida antarctica* lipase B for the (R)-enantiomer of 3-hexanol. Molecular dynamics simulations concluded that the preference toward the (R)-enantiomer is caused by the fact that it accessed a larger volume within the active site than the non-preferred enantiomer, which is in agreement with the higher entropy of (R)-3-hexanol in its transition state (15).

The effect of solvent to structure and dynamics of enzymes have been studied thoroughly. It was suggested that the effect of organic solvents on an enzyme is primarily caused by the interactions between the organic solvent and the enzyme-bound water, and that a low amount of water is required to provide sufficient conformational flexibility to the enzyme (15).

It has been shown that organic solvents affect the flexibility of enzymes, and that that residual water has a major impact on stability, catalytic activity, and selectivity by varying the thermodynamic activity of water in organic solvents (15).

Given these considerations, a very important aspect in the analysis of CALB is so related to the presence and the distribution of methanol molecules in the overall structure of the lipase. Indeed, as already introduced before the total number of methanol is 36 including both monomer A and B.

Of great importance is also the number of methanol considered part of the active sites of the enzyme. To this regard, how is possible to see in the following screenshots, are counted a couple of methanol molecules in both the two monomers (Fig. 17 e Fig. 18).

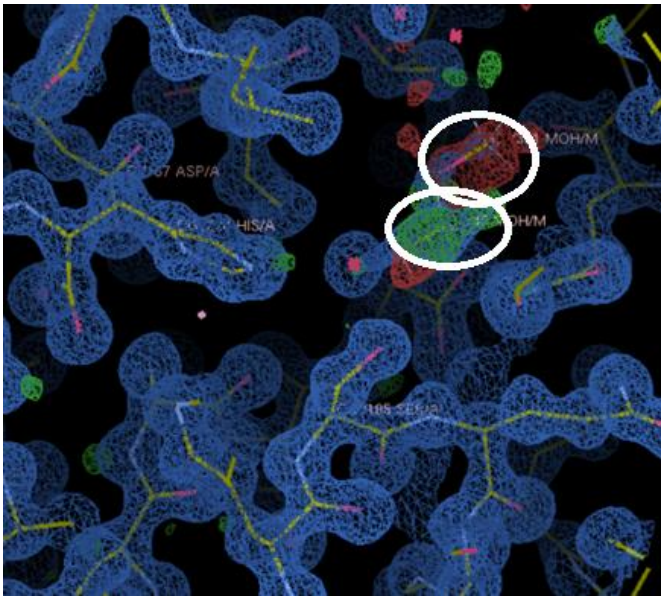

Figure 17. Active site of monomer A of C. antarctica lipase B with two methanols inside. Blue map is the $2F_{obs}$-F_{calc} *electron density map at 1.5 sigma countour level, green/red map is the* $2F_{obs}$-F_{calc} *at 3 sigma countour level*

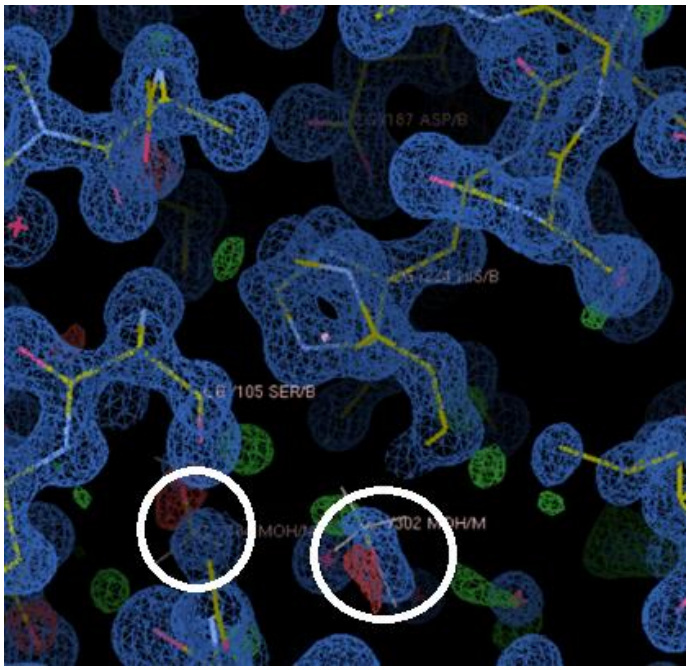

Figure 18. Active site of monomer B of C. antarctica lipase B with two methanols inside. Blue map is the $2F_{obs}$-F_{calc} *electron density map at 1.5 sigma countour level, green/red map is the* $2F_{obs}$-F_{calc} *at 3 sigma countour level*

5. DISCUSSION

5.1 Principles of enzymatic inhibition

Inhibition is a way of controlling or regulation of the enzymatic action.

Catalytic activity of enzymes can be regulated with different types of inhibition:

- reversible: caused by non-covalent bond between ligand – enzyme (Fig.19);

- irreversible: covalent bond between ligand – enzyme (Fig. 20);

-reversible covalent: phosphorylation, ubiquitination.

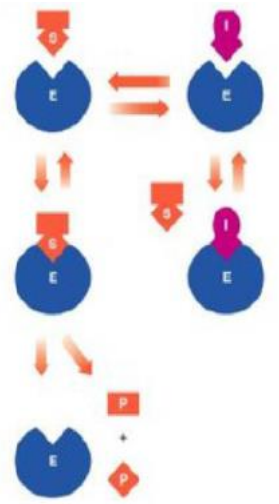

Figure 19. Reversible inhibition

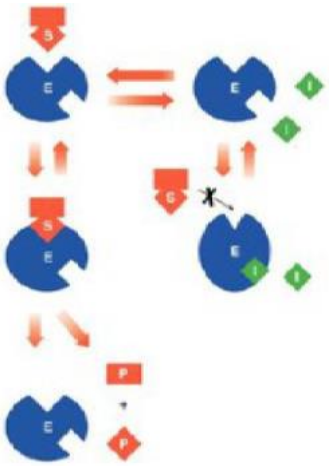

Figure 20. Irreversible inhibition

For reversible kind of inhibition, it is possible to distinguish in: competitive and uncompetitive inhibition. In the competitive one, inhibitor and substrate "compete" for the same site, bonding is not-covalent, so is reversible by dilution and the inhibitor and substrate have common forms of interactions. In this kind of inhibition excess of the substrate moves the equilibrium in favour of the substrate itself. In the uncompetitive inhibition generally are observed two separate sites for substrate and for inhibitor or one single large site to accommodate two substrates and

the bonding is always non-covalent and so reversible. Irreversible inhibitors could be potentially considered an inactivator, which forms covalent bonds with side chains of amino acid present in the active site (3).

5.2 Principles of CALB inhibition by methanol

If in one hand the utility of the organic solvents could give many advantages to the activity of the enzyme because of their capability to increase the solubility of the substrates; in the other hand it should be taken into consideration the concentration of it in the enzymatic environment.

Since the organic solvents are able to modify the thermodynamics of the complex enzyme-substrate it could be seen some side effects potentially negative concerning the catalytic activity. These effects are surely affecting the reaction velocity of the enzyme and so are considered inhibitor effects because reduce the enzymatic activity.

As introduced at the beginning of this paragraph the organic solvent concentration is a potential factor affecting the reaction velocity. To this regard Lotti, Pleiss, Kulschewski, Sasso and Secundoc, in the Journal of Biotechnology 2013, have thoroughly described the behaviour of *C. antarctica* lipase B in toluene–methanol–water mixtures. They concluded that the "two major factors contribute to the observed concentration dependency of the catalytic activity of CALB are the thermodynamic properties of the non-ideal substrate-solvent mixture and the molecular interactions between substrate and enzyme" (15).

Considering all the above-mentioned properties of the methanol-substrate-enzyme complex is possible to evaluate which kind of the inhibition pathways could more probably affecting CALB. As seen in the previous paragraphs (4.2, 5.2) it is possible to talk about a reversible competitive inhibition pathway thanks to the distribution of methanol molecules in the overall structure.
Since methanol is present in the active site and that the site itself is single and not enough big for accommodate two different substrates, it can be assumes that it is reversible because of the non-covalent nature of the bonds and competitive because of the sharing of the same site.

6. Conclusions

The structural analysis on CALB Lipase crystallized in presence of methanol carried out so far, while not yet completed, can lead to the following conclusions.

- The overall structure of the enzyme and its active site is maintained;
- Methanol molecules are found inside the active site interacting with the side chains of the main catalytic residues;
- Because of the point above is possible to talk about a reversible competitive inhibition mechanism of CALB in methanol;
- This implies that other organic solvents with molecules not able to fit inside the active site should be able to provide better reaction yields.

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