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A combined experimental and computational approach for the rationalization of the catalytic activity of lipase B from *Candida antarctica* in water–organic solvent mixtures

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Abstract

Background: The addition of organic solvents to an aqueous medium for enzymatic reactions offers several advantages, as they can increase the solubility of substrates but can also lead to enzyme inactivation and/or aggregation.

Results: The effect of adding 30% of several water-soluble organic solvents on the catalytic activity of lipase B from *Candida antarctica* (CalB) was studied and the results showed that the highest activity was obtained with the addition of *t*-butanol. *t*-Butanol and acetonitrile were selected and the kinetic parameters, determined to deepen their effect on CalB activity, showed that the addition of acetonitrile improved the enzyme–substrate affinity, while water–*t*-butanol mixtures led to a more than ninefold increase in k_{cat}. To rationalize at a molecular level the kinetic results, molecular dynamic simulations were performed. Analysis of the accessibility of the active-site cavity, solvent occupancy in the site and in the oxyanion hole, and the stability of the catalytic triad in the two solvent mixtures, provided insight into their effects on the catalytic properties of CalB.

Conclusion: The lower occupancy in the oxyanion hole of water molecules and a shorter residence time in the active site of acetonitrile molecules in the acetonitrile–water mixture contribute to the higher enzyme–substrate affinity found experimentally. Conversely, the higher k_{cat} in the *t*-butanol mixture is explained by the higher stability of the catalytic triad and by an increase in the nucleophilicity of the catalytic serine due to the persistent presence of *t*-butanol molecules in the active site.

Keywords: *Candida antarctica* lipase B; organic solvent; enzyme activity; molecular dynamics

INTRODUCTION

Nowadays, most chemical processes tend to be wholly or partially based on the principles of green chemistry; therefore, high reaction yields and reduction of by-products, according to the atomic economy principle, are required.[1] In this perspective, enzymes exhibiting high selectivity and specificity turn out to be optimal green biocatalysts that can be used in a wide range of industrial applications,[2] from the production of pharmaceutically active compounds to energy production, from fine chemistry to polymer science, and so forth.[3] Transformation in organic solvents or in a mixture of water and co-solvent is an emerging research area for the application of biocatalysis in the industrial field.[4] This is because, from the process point of view, the high boiling point and low vapor pressure of the water result in an expensive and time-consuming purification.[5] Moreover, unwanted side reactions such as racemization for chiral compounds, hydrolysis of esters, polymerization and decomposition can occur in water, which limit many of the reactions of interest in enzyme synthesis.[6] If water is used as the reaction medium, it must be considered that it can participate in reactions as an acid/base catalyst or by influencing the stability of the transition state. Furthermore, water can be the reagent or product of the desired reaction, thus modifying its equilibrium.[7] By removing the constraints imposed by water as reaction medium, many potential products or reactants that are insoluble or labile in water can be used in biotransformation.[8] Under non-aqueous conditions, enzymatic stability depends on the nature of the enzymes, whether they are free or immobilized,[9] on the presence of additives,[10] on the water content and on the type of non-aqueous solvent.[11] The presence of organic solvents can lead to irreversible inactivation of the enzymes due to unfolding[12]; furthermore, given the hydrophilic nature of many enzymes, they will tend to aggregate in non-aqueous media causing their mutual deactivation.[13] For these reasons, heterogeneous phase biocatalysts based on lyophilized

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enzyme powders, crosslinked crystals and enzymes immobilized on inert supports are often used. Among the latter, Novozym 435 (N435) is of particular interest. It is a commercially available lipase, produced by Novozymes, based on the immobilization by interfacial activation of the lipase B from *Candida antarctica* (CalB) on the microporous resin Lewatit VP OC 1600, composed of polymethyl methacrylate crosslinked with divinylbenzene. Although this biocatalyst presents some serious problems, such as its release from the support and the mechanical fragility of the support under stirring,[14] it is one of the most widely used commercial biocatalysts in both industry and academia. In fact, Remonatto and coworkers in a recent review[15] reported studies published between 2015 and 2020 regarding the use of immobilized lipases in bioreactor processes, and about 30% of the papers involve the use of N435. Lipases are serine hydrolases (triacylglycerol ester hydrolase, E.C. 3.1.1.3) and are distinguished from esterases by the nature of the substrate and the phenomenon of interfacial activation.[16] To resist the denaturing effect of the interface, lipases have developed remarkably stable structures that can survive even the effect of organic solvents[17]: in the absence of an interface between an organic and aqueous phase, the active site is covered by a polypeptide chain, called a lid, which makes it inaccessible to the substrate.[18] In the presence of a hydrophobic interface, the lipases undergo an important conformational rearrangement, passing to the active state.[19] In some lipases, such as CalB, the lid is small, so the block is only partial, while in the case of other lipases, such as that from *Bacillus stearothermophilus*, there is a double lid.[20] The structure of CalB has been extensively discussed and characterized: it is a globular protein α/β consisting of 317 amino acid residues, with a molecular weight of 33 kDa and a pI of 6.0.[18] Moreover, this enzyme, in homology with the other lipases, has a catalytic triad composed of Ser105-Asp187-His224,[19] where the nucleophilic serine is activated by hydrogen bonds with the histidine and the aspartate, as for many serine proteases.

The active site is surrounded by two α-helixes, namely α5 and α10, which have been shown to be very flexible regions.[21] Despite the size of the lid, CalB is still considered an interfacial enzyme that exhibits the phenomenon of interfacial activation by conformational change of the α5 helix: a highly open conformation of this helix is required to bind bulky substrates, and this is favored in a hydrophobic environment.[20,22-24] CalB is suitable for a wide range of applications, having a broad substrate specificity and an extraordinary ability to catalyze different types of reactions (hydrolysis, transesterification, production and degradation of polymers).[25] Considering the wide range of reactions that CalB can catalyze, different solvents will be required to solubilize the various types of substrates. In fact, the effect of organic solvents on structure and reactivity of the enzyme has been extensively studied, both experimentally[26] and computationally.[27] From these works, it is evident that the structural flexibility of CalB generally decreases as the log *P* of the solvent increases. This lower flexibility is a consequence of the interactions of the organic solvent molecules with both the protein and the water bound to the enzyme and its exchange on the surface; these effects can cause a reduction in enzymatic activity.[28] However, it is reported in the literature that CalB in *t*-butanol, despite a high log *P*-value, has a higher catalytic efficiency[29-31] which was suggested to be due to an increase in flexibility.[32] However, this effect is not found with other polar solvents, such as methanol, which influences the network of hydrogen bonds present within the active site, fundamental to the stabilization of the reaction transition state.[33]

In this work, we evaluated the effect of a broad range of organic solvents on the activity of lipase from *Candida antarctica* type B. The hydrolysis reactions were performed in mixtures with 30% organic solvent and, once the most significant ones were identified (*t*-butanol and acetonitrile), the effect of solvent percentages on CalB activity was also evaluated. Molecular dynamics simulations of CalB in the two selected solvents mixtures were then carried out in order to rationalize the kinetic data. In particular, changes in the structural and dynamic properties of the active site, and of the solvent molecules residing within the cavity, were taken into consideration.

EXPERIMENTAL

Materials

Novozym 435 (the commercially immobilized form of *Candida antarctica* lipase B) and *p*-nitrophenyl acetate (*p*-NPA) were purchased from Merck. All the organic solvents were of analytical grade (RPE) and supplied by Merck & Sigma-Aldrich (Milan, IT) and Carlo Erba Reagents (Milan, IT).

Methods

Hydrolytic activity assay of Candida antarctica *lipase type B*

Catalytic activity was monitored spectrophotometrically with a Hewlett Packard HP 8452A instrument, following the hydrolysis reaction of *p*-NPA at 348 nm, which corresponds to the isosbestic point *p*-nitrophenol/*p*-nitrophenoxide. The procedure was adapted from the literature to this case study:[34] a test tube containing the reaction solution (water–organic solvent mixture at different percentages) was thermostated at a temperature of 37 °C. The enzyme at a concentration of 0.5 mg mL^{-1} was added to the reaction solution after 15 min so that it reached thermal equilibrium. To start the hydrolysis reaction, the substrate, solubilized in the same organic solvent present in the reaction mixture, was added to reach a final concentration of 10 mmol L^{-1}. To monitor the evolution of the reaction and determine the reaction rate, 20 µL of the reaction mixture, at specific time intervals, was placed in a 1 mL cuvette in order to carry out the spectrophotometric analyses. Following the same procedure, the kinetic parameters of the enzyme were performed in 5% and 30% acetonitrile and 30% *t*-butanol. It is important to specify that acetonitrile at 5% v/v is the minimum amount required to solubilize the substrate, so under this condition the medium will be considered exclusively aqueous.

Kinetic parameters k_{cat} and K_M in water and in the presence of 30% organic solvents were obtained from the linear regression analysis of the double reciprocal Lineweaver–Burk plots with *p*-NPA concentration varied by 0.5 units from 1 to 3 mmol L^{-1}, and the initial rate was evaluated by carrying out several samplings in the early stages of the reaction. The regression coefficient was always higher than 0.99. All sets of experiments were reproduced at least three times, and the differences between duplicates in each experiment were always below 5%.

Effect of solvent and its percentage on CalB activity in water–organic mixture

The effect of some water-miscible organic solvents (Table 1) on the hydrolytic activity of CalB toward the substrate *p*-NPA was studied following the procedure described above. After an initial screening, in which the solvents were present at 30% v/v in the reaction mixture, acetonitrile and *t*-butanol were chosen and their quantities were varied by increasing them to 50% and 70% v/v.

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Table 1. Solvents used for the initial screening, with selected physicochemical properties

Organic solvent	Abbreviation	log P[35]	ε_r[36]
Acetone	ACE	−0.24	20.56
Acetonitrile	MeCN	−0.34	35.94
Dimethyl sulfoxide	DMSO	−1.35	46.45
Ethylene glycol	EG	−1.37[37]	37.70
Isopropanol	i-PrOH	0.05	19.92
N,*N*-Dimethylformamide	DMF	−1.01	36.71
n-Propanol	*n*-PrOH	0.25	20.45
t-Butanol	*t*-BuOH	0.35	12.47

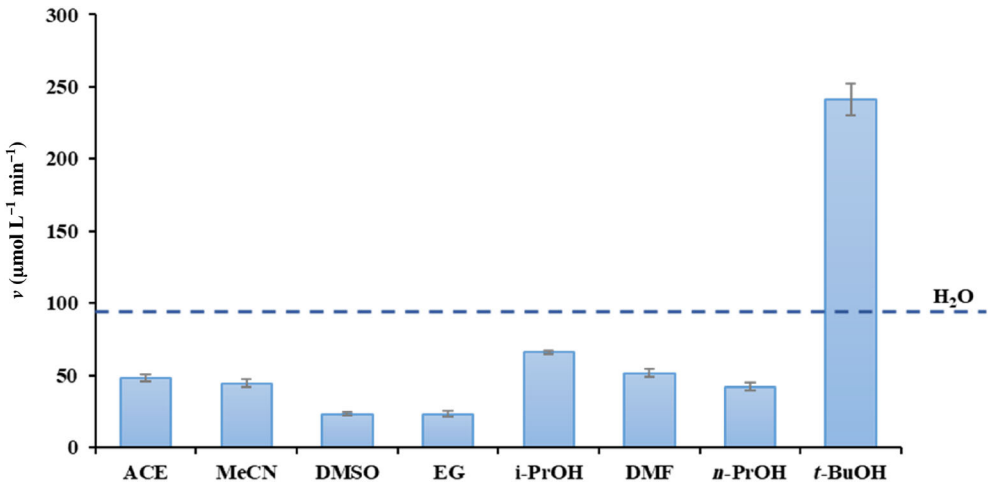

Figure 1. Hydrolysis reaction rate in 30% v/v organic solvent in water at 37 °C.

Computational set-up

The starting configuration of CalB was taken from the structure present in the 1TCA.pdf file.[18] The side chain of the active-site residue Asp134 was protonated on the basis of previous calculations using the Perturbed Matrix Methods,[38-40] which provided a pK_a value of 8.9 ± 1.1, suggesting that Asp134 in CalB should be protonated at neutral, or even at slightly basic, pH.[41] The protein was initially solvated with water using the TIP3P model in a dodecahedral box,[42] large enough to ensure a minimum distance of 1.2 nm between the protein and the box edges. In order to obtain the mixed solvent systems, an appropriate number of water molecules was substituted with *t*-BuOH or MeCN molecules to reach a 30% ratio in volume of the organic solvent. Periodic boundary conditions (PBC) and the particle mesh Ewald (PME) for treating the long-range electrostatic interactions, along with a 1.1 nm cutoff,[43] were used. The bonds involving hydrogen atoms were constrained along the simulations using the LINCS algorithm,[44] allowing the use of a timestep of 2 fs. Each solvated system was relaxed using the steepest descent minimization algorithm. Then, the temperature was increased from 50 to 300 K in 100 ps, and a 200 ns long MD trajectory was generated for each system. The coordinates were saved at each 1 ps. All the simulations were carried on using the GROMACS package[45] and the CHARMM36 force field[46] in the NPT ensemble (constant temperature, pressure and number of molecules), using the velocity rescaling temperature coupling (τ_T = 0.002 ps) and the Berendsen barostat for pressure coupling (τ_P = 1.0 ps).[47]

For the analysis of the occupancy of a given solvent (either water, *t*-BuOH or MeCN) in the active-site cavity, a solvent molecule is considered within the cavity when it is the closest one to the oxygen atom of the side chain of the catalytic serine (Ser105). The corresponding residence time is calculated as the time at which an exchange of the given molecule in the cavity occurs. The mean residence time is then calculated as the average of all the residence times sampled along the whole trajectory. All the analyses were performed using GROMACS tools.

RESULTS AND DISCUSSION

As mentioned in the Experimental section, the catalytic activity of CalB was evaluated using *p*-NPA as a model substrate and carrying out its hydrolysis reaction in the presence of organic solvents at 30% v/v in the reaction mixture (Fig. 1). Specifically, some different solvents were chosen, both miscible with water and able to solubilize the substrate.

As shown in Fig. 1, the hydrolytic activity of CalB depended on the organic solvent used in the experiment. The highest activity was obtained with 30% *t*-BuOH, whose reaction rate was approximately 240 μmol L^{-1} min^{-1}. In all other cases, the addition of 30% organic solvents reduced the enzyme activity, compared to pure water, and varied between 20 and 60 μmol min^{-1}, regardless of their physicochemical properties. In particular, solvents with the smallest log *P*-values – that is, dimethyl sulfoxide and ethylene glycol – showed the lowest activity (23 μmol L^{-1} min^{-1}),

but there was no clear correlation between the solvent polarity parameters, such as log *P* or dielectric constant, reported in Table 1, and reaction rate. In fact, solvent not only solubilizes the substrate, but could also establish specific interactions with the enzyme and its active site by inducing denaturation.

As for alcohols, no tests were performed with small alcohols such as methanol and ethanol. In fact, it is well known that methanol exerts a deactivation towards CalB, as it binds in the substrate access channel, with a consequent competitive inhibition.[24,33] The enzyme deactivation caused by ethanol and water–ethanol mixtures is instead due to the dissolution of the polymethyl methacrylate, which constitutes the support of CalB, and to a change in the enzyme secondary structure, producing an increase in β-sheet structure and a decrease in the α-helix content.[48] Despite the solubility of the support, it was also found using other short-chain alcohols, *n*-propanol and *i*-propanol were herein used as this effect is less pronounced.[14] The hydrolysis reaction rate in the presence of 30% *i*-PrOH was 1.4-fold higher compared to *n*-PrOH (62 *vs.* 44 μmol L^{-1} min^{-1}), indicating the slight difference in activity that occurs between linear and branched alcohols, as previously reported by Zieniuk et al.[26] The higher hydrolytic activity when using nonlinear alcohols was even more evident with *t*-BuOH, as previously seen.

Short-chain alcohols, including *t*-BuOH, were recently reported to induce biocatalyst inactivation.[4] This result is not consistent with the beneficial effects reported in the literature, according to which the use of *t*-BuOH as solvent for biodiesel production overcame the negative effects caused by excessive methanol and the by-product glycerol.[29-31]

After this preliminary screening, two solvents were selected, and their percentages were varied by increasing them to 50% and 75% v/v. The best co-solvent in the initial screening, *t*-butanol, and acetonitrile, able to easy solubilize the substrate and simple to parameterize for molecular dynamics studies, were chosen. The trends of the hydrolysis reaction rate of *p*-NPA as a percentage of solvent increases are shown in Fig. 2.

For both solvents, the increase in their percentage resulted in a reduction in hydrolysis rate. In both cases, the reaction rate decreased by about 50% and 80% when the percentage of organic solvents in the mixture was increased from 30% to 50% and 75% respectively. It is important to underline that, despite the significant loss of activity, the hydrolysis rate in the presence of 75% *t*-BuOH is slightly lower than that found in 30% MeCN. Then, considering these results and those of the previous screening, it can be asserted that *t*-BuOH is the best co-solvent among the investigated ones.

Determination of kinetic parameters

To gain more insight into how different reaction conditions may affect the enzyme reaction rate, kinetic parameters of CalB were determined. All data points obeyed Michaelis–Menten kinetics and could be correlated in the Lineweaver–Burk plot for an estimation of the kinetic parameters, reported in Table 2.

The K_M value characterizes the affinity between the substrate and the enzyme. A low K_M value means high affinity between enzyme and substrate and greater difficulty in the dissociation of the ES complex. K_M value depends on the characteristics of the reaction mixture catalyzed by the enzyme and the reaction conditions. In fact, as can be seen from the data in the table, two opposite effects were found when the two solvents were added in water. In particular, the addition of MeCN resulted in

Table 2. Effect of organic solvents on CalB kinetic parameters at 37°C

% v/v organic solvent/water	K_M (mmol L^{-1})	10^2 k_{cat} (s^{-1})
—	9.7	9.3
30% MeCN	3.5	3.7
30% *t*-BuOH	32.4	85.5

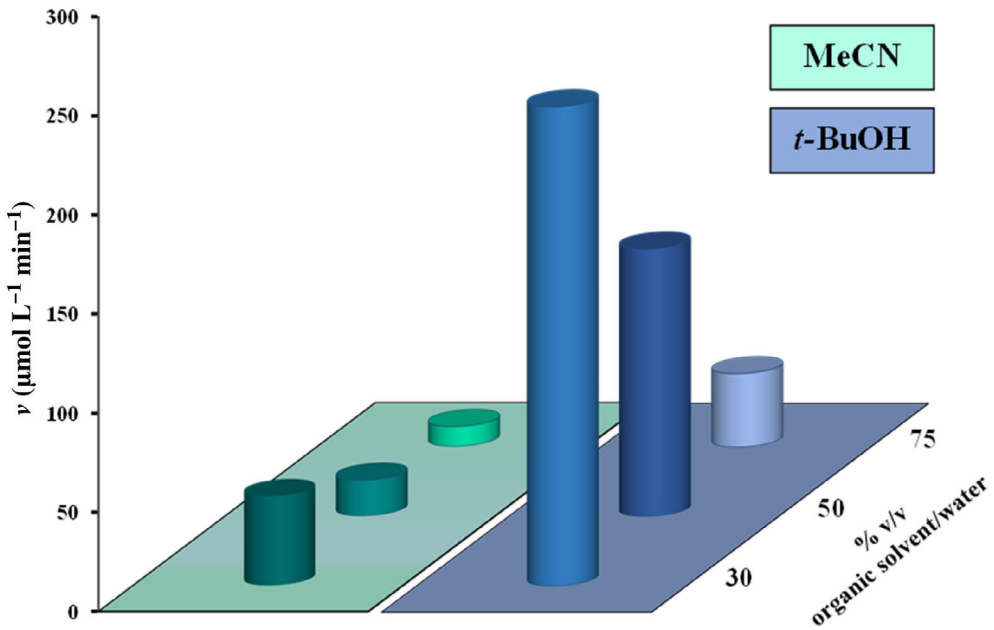

Figure 2. Hydrolysis reaction rate of *p*-NPA at different percentages of MeCN and *t*-BuOH added at 37°C.

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an improvement of the enzyme–substrate affinity (the K_M value was three times lower than in pure water), but at the same time a 2.5-fold decrease in the catalytic constant was also detected. On the other hand, a lower enzyme–substrate affinity was found in the presence of *t*-BuOH, together with a high increase in the catalytic constant (more than ninefold).

Possible explanations for the different K_M and k_{cat} values observed in the different solvents were addressed by means of computational methods described below.

Computational modeling

In order to rationalize at a molecular level the differences in catalytic activity of the CalB enzyme in different solvents, we carried out molecular dynamics (MD) simulations. The set-up of the system was analogous to that employed in previous work[41] and is discussed in detail in the Methods section. It should be noted that in our simulations the enzyme is fully solvated, while in the experiments it is anchored to an inert support. Hence possible effects due to the presence of the resin were not taken into consideration.

To analyze the behavior of the active site of the enzyme in the two solvent mixtures we first examined the distance between the two helices forming the protein lid – that is, helix α5 and α10. In Fig. 3 the distribution of the distance between the centers of mass of the two helices in the two solvents is reported. Two representative configurations are also reported in the top panel of the figure. From the comparison it is evident that the active site is more accessible in the presence of *t*-BuOH than in the mixture with the more polar solvent MeCN. Analysis of the volume of the active-site cavity in the two solvents shows also that the cavity itself is larger in the presence of *t*-BuOH (data not shown). The presence of a wider cavity in *t*-BuOH might not be so crucial in hosting small substrates, as the *p*-NPA used in the present experiments, but might be more relevant in the case of larger substrates.

To gain a deeper insight into the accessibility of specific regions of the active-site cavity to the substrate, we analyzed the solvent occupancy in the oxyanion hole, which is the site that hosts the carbonyl group of the substrate and has the role of stabilizing the negative charge localization on the oxygen at the transition state. While the organic solvent molecules cannot access the oxyanion hole because of steric hindrance, water molecules are found to occupy the site, but with different probability in the two solvents. Analysis of the distribution of the minimum distance between the amide N atoms of residues Thr40 and Gln106 of the oxyanion hole and the oxygen atom of the closest water molecule, reported in Fig. 4 (top panel) for both mixtures, shows that while in *t*-BuOH a water molecule is always tightly bound to the oxyanion hole, a water molecule is found in only half of the

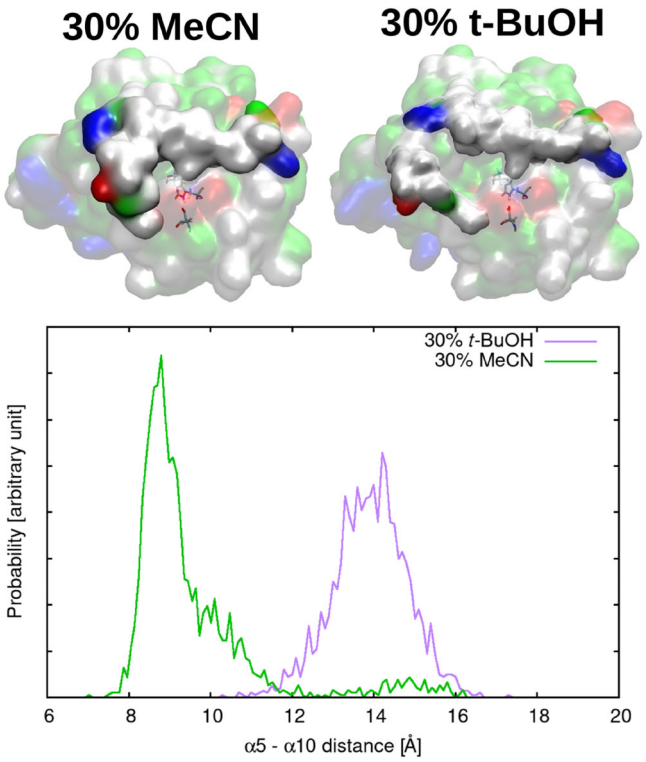

Figure 3. Analysis of the accessibility of the active-site cavity. In the top panel representative configurations of 'open' and 'closed' states of the protein lid are reported. The catalytic triad is highlighted in a stick representation. In the bottom panel the distribution of the distance between the centers of mass of helix α5 and helix α10 along the simulation in 30% *t*-BuOH (purple) and 30% MeCN (green) is reported.

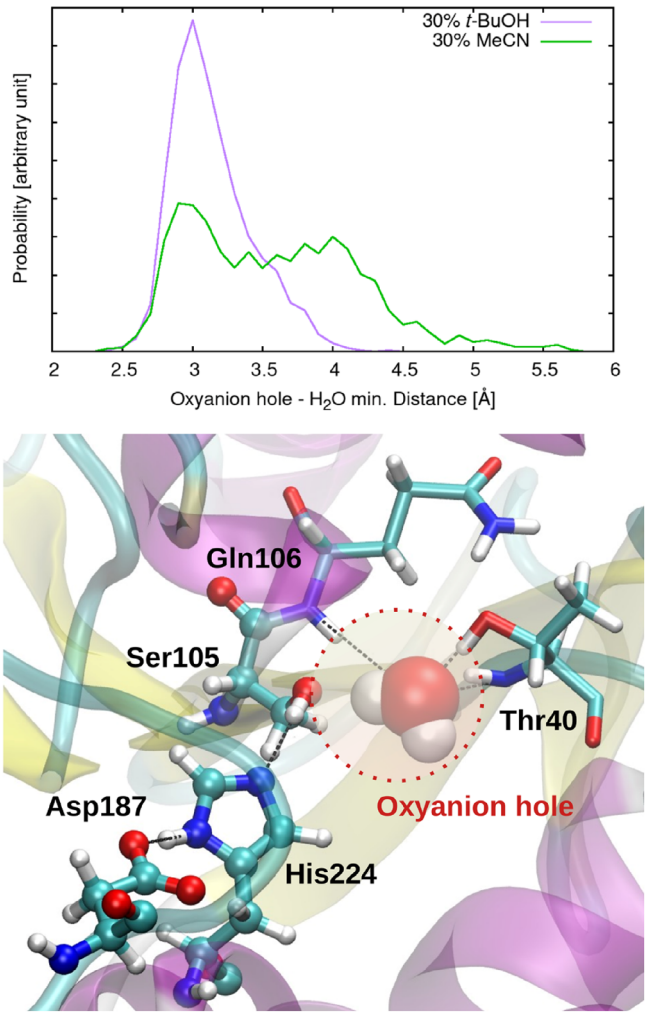

Figure 4. Water occupancy in the active-site cavity. In the top panel the distribution of the minimum distance between the amide N-atoms of the oxyanion hole and the oxygen of the closest water molecule is reported. A representative configuration of the oxyanion hole with a bound water molecule is reported.

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configurations in MeCN. Given that binding of the substrate implies a close interaction of the carbonyl group of the substrate with the oxyanion hole, a lower occupancy of water in the oxyanion hole, as observed for the water–MeCN mixture, should favor substrate binding. This agrees with the different experimental affinity (i.e., K_M) values.

We then analyzed the presence of organic solvent molecules in the active-site cavity. In both mixtures, two solvent molecules are found, on average, in the cavity. Nevertheless, the mean residence time of the organic solvent molecules in the pocket is different in the two mixtures, being 9.2 ± 1.2 ns in *t*-BuOH and 2.5 ± 1.1 ns in MeCN. The mean residence time was calculated as the average

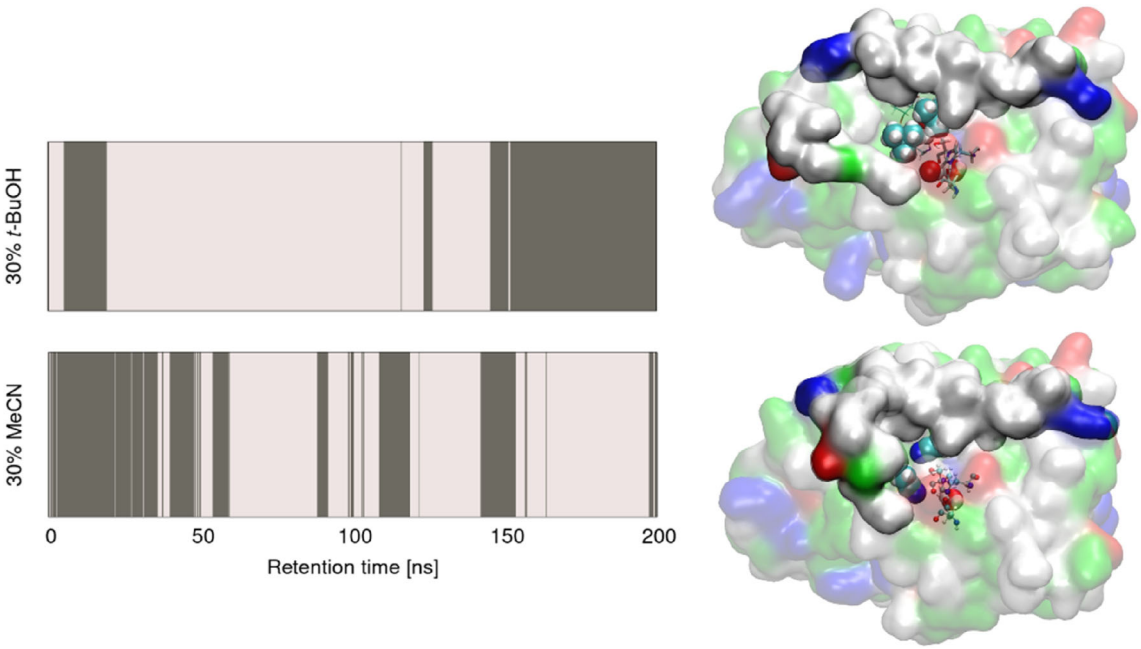

Figure 5. Organic solvent occupancy of the active-site cavity. In the left panel, the residence times of the organic solvent molecules used to calculate their mean residence time in the two water-organic solvent mixtures are reported. Each of the two shades of gray represents the simulation time during which a given solvent molecule is found within the catalytic site. Note that the two colors are not associated with specific molecules but are meant to highlight the exchange between different molecules. In the right panels, representative configurations showing the occupancy of organic molecules in the active-site cavity in the two solvent mixtures are reported.

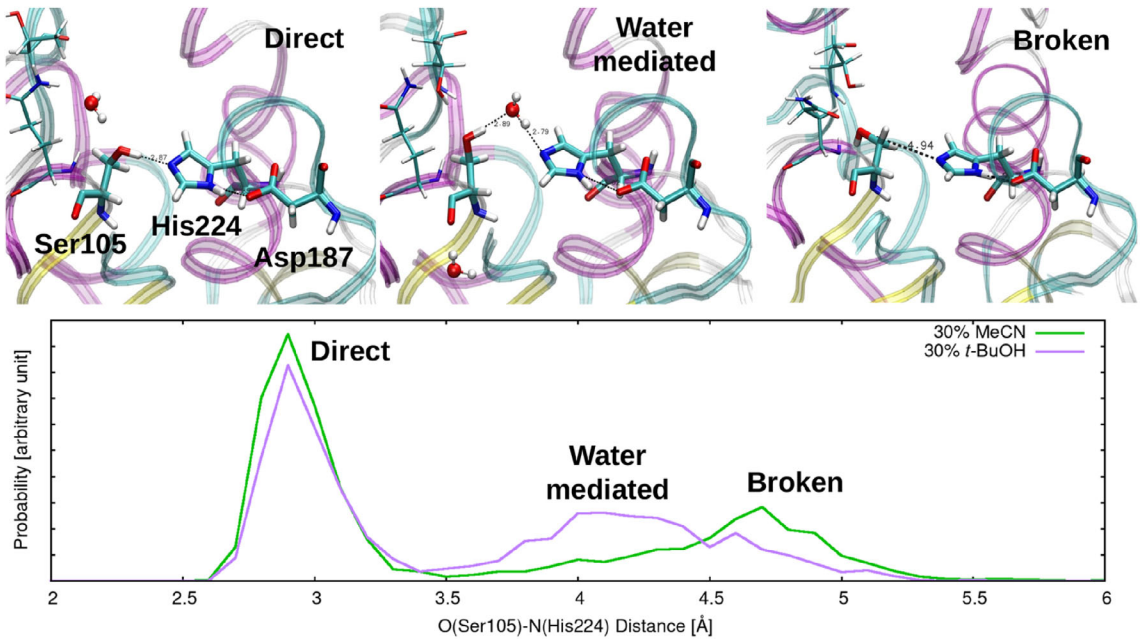

Figure 6. Stability of the catalytic triad. The top panel shows representative configurations of the most populated structures of the catalytic triad. The distribution of the O(Ser105)–N(His224) distance is reported in the bottom panel. The purple and green lines represent the simulations in 30% *t*-BuOH and 30% MeCN, respectively.

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of all the residence times sampled along the full trajectory (see Fig. 5), as described in the Methods section. The lower residence time of MeCN molecules in the active site is an additional factor contributing to the higher substrate affinity found experimentally.

In order to gain insight into the difference in the catalytic rate constants k_{cat} found experimentally in the two solvent mixtures, we analyzed the stability of the catalytic triad over the two simulations. It was previously proposed based on short (few ns long) MD simulations of CalB in different solvents (water, CH_3Cl, *t*-BuOH, MeOH and others) that the polarity of the solvent affects the catalytic triad stability by inducing a change in the length of the Ser105–His224 hydrogen bond (HB) distance.[27] In our simulations, we provide a different explanation for the different catalytic-triad stability observed in the two mixtures of different polarity. The HB distance of the Ser105–His224 couple (namely the O—N distance) was calculated along the two simulations and the corresponding distributions are reported in Fig. 6 (bottom panel). Three main peaks were found: a peak at 3.0 Å corresponding to a stable, 'direct' HB between Ser105 and His224; a peak at ∼4.0 Å corresponding to configurations with a 'water-mediated' HB, through which a proton exchange between the two residues is possible via a Grotthus mechanism;[49] a peak at ∼5.0 Å corresponding to configurations in which the HB is 'broken'. In the water–*t*-BuOH solvent almost 90% of the structures possess 'active' configurations of the triad (i.e., with either a 'direct' or 'water-mediated' HB), whereas in the water–MeCN solvent the 'water-mediated' HB is never observed and only ∼60% of the structures show a 'direct' HB.

Results on the stability of the catalytic triad support the experimental evidence that the CalB catalytic activity is higher in the water–*t*-BuOH mixture than in the water–MeCN one. Additionally, the persistent presence of *t*-BuOH molecules, which are less polar than the MeCN ones, in the active-site cavity should increase the nucleophilicity of the catalytic serine, thus contributing to the higher k_{cat} observed experimentally.

CONCLUSIONS

In this study, the effect of water–organic solvent mixtures on the catalytic activity of lipase B from *Candida antarctica* was evaluated through both kinetic studies and molecular dynamics simulations to understand how the activation/inhibition of the enzyme was related to variations of the active site structure induced by the presence of organic solvents. Acetonitrile and *t*-butanol were selected because of their opposite effect on the catalytic properties. By analyzing the active-site accessibility, solvent residence time in the cavity, water occupancy in the oxyanion hole, stability of the catalytic triad and polarity of the environment, it was possible to gain molecular-level insight into the observed kinetic parameters. Specifically, significant differences in the organic solvent mobility and in the water occupancy of the oxyanion hole play a key role in determining the accessibility of the active site, and thus the enzyme–substrate affinity, while the stability of the catalytic triad and the presence of a less polar environment are crucial to determine the catalytic activity of the lipase B from *Candida antarctica*.

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