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## Molecular mechanism of the hydration of *Candida antarctica* lipase B in gas phase: water adsorption isotherms and molecular dynamics simulations.

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1       **Molecular mechanism of the hydration of *Candida antarctica***  
2       **lipase B in gas phase: water adsorption isotherms and molecular**  
3       **dynamics simulations**

4  
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14       **Dedicated to Professor Karl Hult on the occasion of his 65<sup>th</sup> birthday**

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32 ABSTRACT

33 Hydration is a major determinant of activity and selectivity of enzymes in organic solvents or  
34 in gas phase. The molecular mechanism of the hydration of *Candida antarctica* lipase B  
35 (CALB) and its dependence on the thermodynamic activity of water  $a_w$  was studied by  
36 molecular dynamics simulations and compared to experimentally determined water sorption  
37 isotherms. Hydration occurred in two phases. At low water activity, single water molecules  
38 bound to specific water binding sites at the protein surface. As the water activity increased,  
39 water networks gradually developed. The number of protein-bound water increased linearly  
40 with  $a_w$ , until at  $a_w = 0.5$  a spanning water network was formed consisting of 311 water  
41 molecules which covered the hydrophilic surface of CALB, with the exception of the  
42 hydrophobic substrate binding site. At higher water activity, the thickness of the hydration  
43 shell increased up to 10 Å close to  $a_w = 1$ . Above a limit of 1600 protein-bound water  
44 molecules the hydration shell becomes unstable and the formation of pure water droplets  
45 occurs in this oversaturated simulation conditions. While the structure and the overall  
46 flexibility of CALB was independent of the hydration state, the flexibility of individual loops  
47 was sensitive to hydration: some loops such as part of the substrate binding site became more  
48 flexible, while other parts of the protein became more rigid upon hydration. However, the  
49 molecular mechanism of how flexibility is related to activity and selectivity is still elusive.

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58 **Keywords:** thermodynamic activity of water • hydration mechanism • molecular modeling •  
59 • deglycosylation

60

## 61 INTRODUCTION

62

63 *Candida antarctica* lipase B (CALB) is an efficient catalyst for hydrolysis of ester substrates  
64 in water and esterification in organic solvents. It is used in many industrial applications  
65 because of its high enantioselectivity, wide range of substrates, thermal stability, and stability  
66 in organic solvents.<sup>[1]</sup> CALB belongs to the  $\alpha/\beta$  hydrolase fold family with a conserved  
67 catalytic triad consisting of Ser, His, and Asp/Glu.<sup>[2]</sup> In contrast to most lipases, CALB has no  
68 lid which shields the active site, but the hydrophobic substrate binding site is solvent  
69 exposed.<sup>[3]</sup> It has been shown that like many other enzymes, CALB retains activity in organic  
70 solvents and has interesting catalytic properties such as higher thermostability and altered  
71 stereoselectivity.<sup>[4]</sup> However, it is essential to add small amounts of water to maintain stability  
72 and flexibility of enzymes in organic solvent. Thus protein-bound water is essential for  
73 catalysis and serves as a lubricant for the enzyme.<sup>[5]</sup> In contrast, fully dry enzymes are  
74 inactive and enzymes in organic solvents with high amounts of water show denaturation.<sup>[6]</sup>

75

76 The influence of water on stability, biocatalytic activity, and selectivity of enzymes has been  
77 studied extensively. The best way to quantify the availability of water to the biocatalyst in  
78 non-conventional media is to use the parameter "thermodynamic water activity". Most studies  
79 were performed in organic solvents at varying water activity. A similar dependence of the  
80 biocatalytic activity of enzymes on the thermodynamic activity of water  $a_w$  was found: at very  
81 low water activity, the biocatalytic activity was low, and increased with increasing  $a_w$  to an  
82 optimum. For a higher  $a_w$ , the biocatalytic activity decreased. The optimal  $a_w$  depends on the  
83 enzyme under investigation, the reaction, and the solvent. In contrast, no general trend has  
84 been found for the dependence of selectivity, especially enantioselectivity, on  $a_w$ .<sup>[7, 8]</sup>

85

86 The dependence of flexibility, activity, and selectivity on the protein hydration level has been  
87 extensively investigated in condensed phase, mainly for proteins suspended in co-solvent  
88 mixtures of water and organic solvents.<sup>[5, 9-20]</sup> Water is known to have a crucial role in protein  
89 structure, flexibility, and activity.<sup>[21-25]</sup> Water molecules bind via hydrogen bonds to the side  
90 chain and backbone atoms of proteins,<sup>[26]</sup> to polar atoms of substrates and ligands,<sup>[27]</sup> as well  
91 as to other water molecules. This enables water to mediate protein-protein and protein-ligand  
92 contacts, and to take part in enzyme catalysis. X-ray crystallography has long been used to  
93 analyze water at protein surfaces, since crystal structures determined at high resolution  
94 provide a detailed picture of protein hydration.<sup>[28]</sup> Comparative studies of crystal structures

95 showed that there are water binding sites on the surface and the interior of a protein which are  
96 occupied by water molecules in different crystal structures.<sup>[29-31]</sup>

97  
98 Computer simulations of enzymes in different solvents are a valuable tool to investigate the  
99 effect of solvent on the structure and dynamics of proteins.<sup>[21, 32, 33]</sup> While in most simulations  
100 proteins showed no significant structural changes in different solvents, the molecular  
101 flexibility decreased when simulated in organic solvents,<sup>[33, 34]</sup> which has been confirmed by  
102 different experimental techniques such as time-resolved fluorescence anisotropy,<sup>[35]</sup> ESR,<sup>[36]</sup>  
103 and dielectric relaxation spectroscopy.<sup>[37]</sup> It has been suggested that in water polar side chains  
104 orient toward the surface, thus increasing the hydrophilic surface and decreasing the polar  
105 intra-molecular interactions that mediate the rigidity of the protein,<sup>[38]</sup> while in organic  
106 solvents the surface area is reduced which leads to improved packing and increased  
107 stability.<sup>[36]</sup> In addition, a spanning water network was observed in simulations of CALB in a  
108 water/organic solvent mixture.<sup>[33]</sup> The spanning water network was formed in the presence of  
109 a non-polar solvent<sup>[34, 39]</sup> and resulted from a slow exchange of water molecules at the protein  
110 surface.

111  
112 Previously, the catalytic activity of CALB at different water activities was determined in a  
113 continuous solid/gas reactor.<sup>[40, 41]</sup> In this type of reactor, a solid packed enzymatic sample is  
114 percolated by a carrier gas, which simultaneously carries gaseous substrates to the enzyme  
115 and removes gaseous products. The water activity in the reaction medium can be fully  
116 controlled and fixed independently of the other reaction medium components, by  
117 incorporating water in the inlet gas at the correct molar fraction. As a consequence, the sole  
118 role of water on biocatalysis can be precisely assessed.<sup>[42]</sup> In this system, it was shown that  
119 there is an optimal water activity in respect to alcoholysis ( $a_w=0.05-0.1$ ) and enantioselectivity  
120 ( $a_w=0.2$ ).<sup>[40]</sup> The water effect on enantioselectivity has been interpreted by binding of a water  
121 molecule in a well-defined binding site, the "specificity pocket" which is located near the  
122 active site of the enzyme. In this paper, our goal is to study the hydration state of CALB in  
123 gas phase both by measuring water sorption isotherms and by molecular modeling. The  
124 experiments and the molecular modeling studies are performed with a deglycosylated lipase.  
125 Because the commercially available CALB is a glycosylated heterogeneous protein expressed in  
126 *Aspergillus oryzae*, it was deglycosylated and desalted to avoid water binding by salts.

127

## 128 EXPERIMENTAL SECTION AND COMPUTATIONAL METHODS

129

### 130 **Enzymes and chemicals**

131 Chirazyme L-2, lyo (CALB) was purchased from Roche Diagnostics (Penzberg, Germany), as  
132 a crude lyophilized material. Peptide-N-glycosidase F (PNGase F) lyophilized and  
133 endoglycosidases F1, F2, and F3 were obtained from Sigma-Aldrich (Saint-Quentin Fallavier,  
134 France) as “Native Protein Deglycosylation Kit”. All other chemicals used were analytical  
135 grade and obtained from Sigma-Aldrich (Saint-Quentin Fallavier, France).

136

### 137 **Deglycosylation**

138 Deglycosylation of CALB with endoglycosidases F1, F2, and F3 was performed with 3  $\mu$ L of  
139 the Sigma enzymatic kit solution for 0.2 mg of native CALB in 37.5  $\mu$ L de-ionized water in  
140 10  $\mu$ L of 5x reaction buffer. The mixture was then incubated 1 hour at 37°C (see details in  
141 Supporting Information).

142

### 143 **Desalting**

144 Desalting was performed using a PD-10 column (Amersham Biosciences, Pantin, France).  
145 PD-10 desalting columns are pre-packed, disposable columns containing Sephadex™ G-25  
146 medium for group separation of high ( $M_r > 5000$ ) from low molecular weight substances ( $M_r$   
147  $< 1000$ ). After desalting, CALB was freeze dried. It was checked that these different  
148 treatments did not cause loss of enzyme activity.

149

### 150 **Measurement of CALB activity**

151 The CALB enzymatic activity was determined with a pH-stat system which measures the  
152 liberation of butyric acid from tributyrin by titrating with 100 mM sodium hydroxide. 24.25  
153 mL 10 mM potassium phosphate buffer, pH 7.0 are incubated in a thermostated vessel at  
154 25°C, equipped with a magnetic or propeller stirrer (the stirring speed is set to a value that a  
155 further enhancement of the stirrer speed did not increase the base consumption per min). After  
156 addition of 735  $\mu$ L tributyrin, the pH-stat system was started to keep the pH at 7.0. When the  
157 pH stabilized, 5-10 U of enzyme was added. The consumption of 100 mM sodium hydroxide  
158 was monitored for 0 to 15 min. The specific activity was calculated from the base  
159 consumption at the linear part of the graph.

160

### 161 **Electrophoresis analysis**

162 Enzyme purity and deglycosylation was evaluated with sodium dodecylsulfate-  
163 polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% separation gel under the condition  
164 developed by Laemmli.<sup>[43]</sup> A Mini-Protean 3 (Bio-Rad Laboratories, Marnes la Coquette,  
165 France) was used for electrophoresis. As reference proteins molecular weight marker from 6  
166 kDa to 66 kDa was purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). For the  
167 detection of proteins, gels were stained with Coomassie brilliant blue R 250 after the  
168 electrophoresis.

169

### 170 **Measurement of biocatalyst water isotherms**

171 Water adsorption isotherms of lyophilized lipase were measured at 45°C by using a DVS  
172 advantage automated moisture sorption analyzer (Surface Measurement Systems Ltd.,  
173 London, UK) between 0 and 98% relative humidity. The samples were initially dried for 10 h  
174 at 45°C under totally dried flowing air in the DVS moisture sorption analyzer to obtain the  
175 starting dry sample mass. The required humidities are generated by mixing dry and saturated  
176 vapor gas flows in the correct proportions using two mass flow controllers and one vapor  
177 humidifier. The instrument was run with a  $dm/dt$  ( $m$ , mass;  $t$ , time) set at 0.002%  $\text{min}^{-1}$  to  
178 reach the equilibrium. This means that the relative humidity will remain constant until the  
179 mass variation falls consistently below this threshold. Once this condition has been reached,  
180 the relative humidity of the system will be raised or lowered to the next level.

181 The water adsorption curve was modeled using the Brunauer-Emmet-Teller (BET)<sup>[44]</sup> gas  
182 adsorption equation (eq 1):

$$183 \quad \frac{a_w}{(1 - a_w) \times m} = \frac{1}{m_0 \times C} + \frac{a_w \times (C - 1)}{m_0 \times C} \quad (\text{eq 1})$$

184 where  $m$  is the mass of bound water (in mg per mg of dry enzyme),  $m_0$  is the monolayer water  
185 coverage,  $a_w$  the water activity and  $C$  a constant. From the linear regression of the plot of

186  $\frac{a_w}{(1 - a_w) \times m}$  versus  $a_w$ , the theoretical monolayer water coverage can be calculated:

$$187 \quad m_0 = \frac{1}{S + I}, \text{ where } S \text{ is the slope and } I \text{ the y-intercept.}^{[45]}$$

188

### 189 **Molecular dynamics simulation**

190 The structure of CALB was taken from the Protein Data Bank (entry 1TCA<sup>[2]</sup>), and the  
191 crystallographic water molecules as well as the N-acetyl-D-glucosamine moiety at Asn 74  
192 were removed. The protein was placed in a cubic box with a side length between 170 and 370

193 Å filled with 981-1000 argon atoms as a model carrier gas and different numbers of water  
194 molecules. 8 protein boxes were created with 27, 64, 125, 338, 720, 1708, 2195, and 4091  
195 water molecules. As a reference for the evaluation of the thermodynamic activity of water, a  
196 simulation with 4095 water molecules and 1000 argon atoms was performed.

197  
198 Molecular dynamics (MD) simulations were carried out using the GROMACS 4.0.3 program  
199 package<sup>[46]</sup> and the united-atom force field GROMOS 43a1.<sup>[47]</sup> The system was neutralized,  
200 assuming the catalytic His 224 as positively charged. All simulations were run under  
201 isothermal-isobaric (NPT) ensemble conditions, coupled to the Berendsen barostat (reference  
202 pressure of 1.0 bar and coupling time constant of 0.6 ps) and thermostat (reference  
203 temperature 318.15 K and coupling time constant of 0.1 ps).<sup>[48]</sup> The compressibility was  
204 adjusted to  $1.27 \times 10^{-2} \text{ bar}^{-1}$ <sup>[49]</sup> to reproduce the experimental density of argon in gas phase.  
205 The simulation time step was 2 fs and all H-bonds were constrained using the LINKS  
206 algorithm<sup>[50]</sup> as implemented in GROMACS.

207  
208 After energy minimization of 2000 steps (steepest descent and conjugated gradient) the  
209 system was equilibrated in three subsequent simulations of 100 ps each, applying a decreasing  
210 force constant of 1000, 100, and 10  $\text{kJ mol}^{-1}$  to all protein atoms. Finally, all constraints were  
211 removed and each system was simulated up to 20 ns.

212  
213 The last 5 ns were analyzed to determine for each box the number of water molecules bound  
214 to the protein and the thermodynamic activity of water. The size distribution of water clusters  
215 was determined by the program g-clustsize as implemented in the GROMACS software  
216 package. All protein systems took about 10 ns to equilibrate as confirmed by the maximum  
217 cluster size and the number of clusters in the box reaching a plateau after 10 ns of simulation  
218 (Supporting Figure S3, S5 and S6).

219  
220 In all simulations, one large cluster of  $N_{\text{prot}}$  water molecules attached to CALB was identified,  
221 as well as a number of single water molecules  $N_{\text{gas}}$ . In simulations with less than 1700 water  
222 molecules, no isolated pure water droplets were formed during simulation time. Because the  
223 thermodynamic activity of water is given by the vapor pressure of water above a sample  
224 divided by the vapor pressure of pure water (reference value), it can be derived from  $N_{\text{gas}}$  for  
225 5 systems (27, 64, 125, 338, and 720 water molecules, Supporting Table S2). For the mixture



226 of argon and water, the total pressure is  $p_{\text{tot}} = p_{\text{arg}} + p_{\text{wat}} = 1$  bar. Thus, the partial pressure of  
227 water,  $p_{\text{wat}}$ , can be calculated from the number of water molecules in the gas phase:

228

$$229 \quad p_{\text{wat}} = p_{\text{tot}} * N_{\text{gas}} / N_{\text{tot}} \quad (\text{eq 2})$$

230

231 with the number of water molecules in the gas phase,  $N_{\text{gas}}$ , and the number of particles in the  
232 gas phase (water plus argon),  $N_{\text{tot}}$ . In the pure water simulation with 4095 water molecules, a  
233 gas phase of  $60 \pm 4$  isolated water molecules was in equilibrium with water droplets after 8 ns  
234 of simulation, thus the partial vapor pressure of water in this fully water-saturated system is  
235 taken as a reference, and the water activity of a system with  $N$  water molecules  $a_w(N)$  can be  
236 determined as:

237

$$238 \quad a_w(N) = p_{\text{wat}}(N) / p_{\text{wat}}(\text{reference}) \quad (\text{eq 3})$$

239

240 Inserting (eq 2) into (eq 3) results in (eq 4):

241

$$242 \quad a_w(N) = [N_{\text{gas}}(N) / N_{\text{tot}}(N)] / [(N_{\text{gas}}(\text{reference}) / N_{\text{tot}}(\text{reference}))] \quad (\text{eq 4})$$

243

## 244 RESULTS

245

### 246 **CALB deglycosylation with endoglycosidase F1**

247 Enzymatic methods are much more restrictive than chemical methods for protein  
248 deglycosylation, because the enzymatic activity generally depends on structural elements of  
249 the N-glycans chains. However, enzymes are very effective for deglycosylation of small  
250 glycoprotein quantities or when it is necessary to retain the protein conformation. Complete  
251 deglycosylation of CALB was achieved by using endoglycosidase F1 in high quantity (4.2  $\mu\text{L}$   
252 of commercial preparation for 0.2 mg of native lipase) (Figure 1).

253

### 254 **Water sorption isotherm measurements**

255 It was checked by enzyme activity measurement that neither deglycosylation nor desalting or  
256 freeze drying prior to water sorption isotherm measurement led to a loss of activity. Moreover  
257 enzyme activity was retained after isotherm measurement itself. Therefore it can be assumed  
258 that our results describe water sorption by native CALB.

259

260 The water adsorption and desorption isotherm measurements were carried out for a 0.1  
261 incremental step in  $a_w$ , between 0.00 and 0.98. The experiment was repeated twice, showing  
262 very good reproducibility. Desalted and deglycosylated CALB showed a low adsorption of  
263 water until  $a_w=0.40$ , corresponding to 120 bound water molecules. Then, the isotherm became  
264 exponential until at  $a_w=0.98$  a maximum number of protein-bound water of 1720 and 1790  
265 molecules for two experimental runs was reached (**Figure 2 and Supporting Table S1**). The  
266 isotherm resembles the BET isotherm, whose model assumes a fixed number of independent  
267 binding sites, which accommodate a monolayer of adsorb molecules and allow subsequent  
268 layers to bind more weakly. Using the linear regression of the BET equation in the range of  
269  $a_w$  from 0.1 to 0.4, the water corresponding to the monolayer on the enzyme was found to be  
270 equal to 0.063 mg of water per mg of dry protein. This bound water quantity is obtained for  
271  $a_w$  between 0.3 and 0.4. The amount of water adsorbed when the equilibrium is approached  
272 from the “dry” side was identical to the amount of water adsorbed when the equilibrium is  
273 approached from the “wet” side, corresponding to a very low hysteresis between water  
274 adsorption and desorption (Supporting Figure S8).

275

### 276 **Protein-bound water calculation**

277 For each box the thermodynamic activity of water was calculated from (eq. 3) and the number  
278 of protein-bound water molecules was derived from the size of the largest water cluster  
279 (Supporting Table S2). Upon increasing the water activity, water molecules gradually bind to  
280 the surface of CALB. The analysis of the MD simulations revealed two distinct phases in the  
281 protein hydration: 1) at very low water activity, the water binding sites on the protein surface  
282 were occupied by single water molecules ( **Figure 3a**) corresponding to a molar fraction of 54 mol water/mol protein (3% w/w at  $a_w =$   
283 0.2). The size of these patches increased with water activity and spanning the hydrophilic  
284 surface of the protein, corresponding to a molar fraction of 311 mol water/mol protein (17%  
285 w/w at  $a_w = 0.5$ ). However, the hydrophobic substrate binding site was not covered by the  
286 spanning water network ( **Figure 3b**); 2) Finally, at high water activity  $a_w > 0.5$ , the number of protein-bound water  
287 molecules rapidly increased, and a continuous multilayer of water molecules on the  
288 hydrophilic surface was formed ( **Figure 3c**) with a thickness of up to 10 Å and a molar fraction of 677 mol water/mol protein  
289 (37% w/w at  $a_w = 0.7$ ). Near the active site an isolated water cluster was formed without  
290 hydrogen bonding to the spanning water network or the hydrophobic substrate binding site.  
291 Simulations with 1708, 2195, and 4091 water molecules showed oversaturation and the  
292 formation of pure water droplets. Because the experimental investigations were performed  
293 under non-saturating conditions ( $a_w < 1$ ), these simulations were excluded from analysis.

294  
295  
296  
297  
298 In hydration phase I ( $a_w < 0.5$ ) the number of protein-bound waters was proportional to the  
299 water activity, since hydration occurs at isolated water binding sites and then gradually a  
300 spanning water network was formed which was in agreement with the experimental water  
301 sorption isotherms. In hydration phase II ( $a_w > 0.5$ ) the number of protein-bound water  
302 molecules became exponential, corresponding to the observed multilayer hydration shell  
303 formation.

304

### 305 **Protein structure and flexibility**

306 The root mean square deviation (RMSD) of the CALB backbone atoms from the initial  
307 crystallographic structure ranged between 2.2 Å (simulation with 64 water molecules,  
308 corresponding to  $a_w = 0.23$ ) and 2.7 Å (simulation with 125 water molecules, corresponding to  
309  $a_w = 0.26$ ). No correlation between  $a_w$  and RMSD was found, which indicates that there is no  
310 systematic conformational change upon increasing the hydration level. Interestingly, the

311 simulation with the highest number of water molecules showed one of the lowest RMSD  
312 values (Supporting Figure S3).

313

314 Similarly, the global flexibility measured as root mean square fluctuation (RMSF) averaged  
315 over all residues seems not to be affected by the hydration level (1.1 - 1.2 Å). However, four  
316 regions were identified which were influenced by protein hydration (Supporting Figure S4).

317 For the N-terminus (residues 10-20) and the loop between helices  $\alpha 9$  and  $\alpha 10$  (residues 248-  
318 256) the flexibility decreased with increasing hydration by 1.3 and 0.2 Å, respectively. In  
319 contrast, the loop between sheet  $\beta 5$  and helix  $\alpha 5$  (residues 133-150), and helix  $\alpha 10$  (residue  
320 277-290) showed an increased flexibility with increasing hydration, by 0.7 and 0.6 Å,  
321 respectively. The averaged RMSF in these five regions ranges between 0.9 and 2.2 Å. The  
322 flexibility of the catalytic amino acids Ser 105, Asp 187, and His 224 was low and did not  
323 change upon hydration.

324

325 DISCUSSION

326 The role of water in stability, catalytic activity, and selectivity is still not fully understood.  
327 Though it has been observed that thermodynamic activity is a major determinant of these  
328 properties,<sup>[34, 51-53]</sup> it is difficult to assess its role in mixtures of organic solvent and water,  
329 since the physico-chemical properties of the organic solvent is expected to influence the  
330 result. Therefore, working under solvent-free conditions using a gas-phase reactor has become  
331 a promising method to directly address the effect of water to the properties of enzymes.  
332 CALB is a widely used, robust, and highly selective biocatalyst, which has been shown to be  
333 stable in a wide range of organic solvent – water mixtures, although its enantioselectivity is  
334 sensitive to the solvent and water activity.<sup>[40, 54]</sup> Previously, binding of water to glycosylated,  
335 immobilized CALB was determined, and two phases of protein hydration were observed: up  
336 to a thermodynamics water activity  $a_w$  of 0.5, the amount of water binding to the protein was  
337 proportional to  $a_w$ , while at higher water activities protein hydration increased  
338 exponentially.<sup>[53]</sup> This dependence of CALB hydration on  $a_w$  was confirmed in the present  
339 study on hydration of free, deglycosylated and desalted CALB, although the amount of bound  
340 water was reduced by 30 to 50 %. This decrease shows that salt (from suspension buffer  
341 before enzyme drying) as well as glycosylation strongly influence the water adsorption, as  
342 already observed with other proteins.<sup>[55]</sup> The shape of experimental water sorption and  
343 desorption curves were very similar to water sorption curves previously obtained for other  
344 proteins, following the pattern of the BET model.<sup>[55-57]</sup> Moreover, the value of 0.063 mg of  
345 water per mg of dry protein found for BET monolayer water was in the range 0.05-0.07 found  
346 for other proteins.<sup>[56, 57]</sup> The absence of hysteresis between adsorption and desorption  
347 indicated that there was no irreversibly bound water on the protein. Recently, a very marked  
348 hysteresis between water adsorption and desorption in gas phase, was observed for alcohol  
349 dehydrogenase deposited onto non-porous glass carriers together with the co-factor NADPH,  
350 sucrose as stabilizing agents and salts resulting from the buffers. This hysteresis was mainly  
351 attributed to additives and leaching of biocatalyst away from the carrier surface, instead of  
352 being attributed to the protein itself.<sup>[55]</sup>

353  
354 When trying to find correlation between biocatalyst hydration state and CALB catalytic  
355 activity at different water activity in gas phase, it appears that the optimal water activity in  
356 respect to alcoholysis ( $a_w=0.05-0.1$ ) and enantioselectivity ( $a_w=0.2$ ) both correspond to a  
357 water activity level at which the water is highly structured. The overlaying phenomena of

358 water adsorption and enzyme activity and selectivity will be investigated more precisely in the  
359 future.

360

361 In conclusion to the experimental results, it has to be noticed that the present results  
362 concerning water sorption by CALB in gas phase should also give information about the  
363 water sorption in water - organic solvent mixtures. Indeed, it was shown for several proteins  
364 in different organic solvents that the water sorption isotherms in water - organic solvent  
365 mixtures were approximately superimposable to the water vapor sorption isotherms obtained  
366 in gas phase, provided that data points were plotted both in gas and liquid phase, as the  
367 amount of water bound to protein versus water activity.<sup>[56]</sup>

368

369 MD simulation results help us to understand this similarity between the behavior of the  
370 protein in gas phase and in organic co-solvent mixtures. In both cases, the exchange rate  
371 between the spanning water network at the protein surface and the bulk solvent is much lower  
372 than in liquid water.<sup>[33]</sup> In gas phase and in organic solvent, an increase in the global protein  
373 flexibility is not expected, since water exchange rate is not affected *per se* by the increase of  
374 hydration of the protein surface. This does not exclude that water hydration leads to local  
375 changes of flexibility, either a decrease caused by the spanning water network or an increase  
376 upon formation of water layers, as it was shown by simulation results. Most remarkable in  
377 regard to catalytic activity is the increase of flexibility of helix  $\alpha 10$  located at the entrance of  
378 the active site. Although CALB, in contrast to other lipases, has been shown to have no lid  
379 which opens upon interaction with a hydrophobic substrate interface, this helix might play a  
380 role in interaction with the substrate. In addition, the observed lack of hydration of the  
381 hydrophobic patch contacting the hydrophobic substrate interface seems to be essential for  
382 substrate recognition and protein orientation at the interface.

383

384 To assess the mechanism of CALB hydration in molecular detail, MD simulations at different  
385 water activities were performed which led to a similar dependence of the number of protein-  
386 bound water on  $a_w$ , though the total number of protein-water was substantially higher in the  
387 simulation and similar to the amount of water bound to the immobilized, glycosylated CALB.  
388 Three major conclusions could be drawn from the simulation:

389

390 1. Hydration occurs in two phases: for water activities below a critical water activity ( $a_w = 0.5$   
391 for CALB), water initially binds to well-defined water binding sites at the protein surface,<sup>[25]</sup>

392 <sup>31]</sup> and then gradually a spanning water network is formed.<sup>[33, 39, 58]</sup> The amount of protein-  
393 bound water increased linearly with water activity, depending on the number of water binding  
394 sites on the surface and their affinity toward water. The increase of the number of bound  
395 water molecules with water activity as well as the critical water activity are expected to be  
396 specific for each protein. At water activities above the critical water activity the amount of  
397 protein-bound water molecules increased exponentially.

398

399 2. For oversaturated conditions (systems with more than 1600 water molecules) the multilayer  
400 hydration shell further grew and became unstable which led to the formation of pure water  
401 droplets and a smaller protein hydration shell, with a maximum of 10 Å of thickness at the  
402 hydrophilic side of the protein, while the hydrophobic substrate binding site is not hydrated at  
403 all, but an isolated water cluster attached to the active site residues is formed at the center of  
404 this hydrophobic patch. This observation is also supported by previous studies in liquid phase  
405 for other enzymes,<sup>[9, 11]</sup> where a water monolayer completely covering the protein was never  
406 observed. Thus, the structure and physico-chemical properties of the protein surface not only  
407 lead to the formation of tight water binding sites, but also to a protein-specific shape of the  
408 hydration shell at high water activity.

409

410 3. The effect of hydration to protein flexibility cannot be generalized; while the flexibility of  
411 some parts of the protein surface increased upon hydration, for other parts of the surface the  
412 formation of a water networks led to a decrease of flexibility. It has been shown that catalytic  
413 activity and enantioselectivity of some enzymes are strongly correlated to the thermodynamic  
414 activity of water ( $a_w$ ).<sup>[51]</sup> By carefully measuring the contribution of enthalpy and entropy to  
415 enantioselectivity of CALB,<sup>[59]</sup> the group of Karl Hult pointed to the pivotal role of the  
416 flexibility of a substrate in the binding site which in their model was interpreted as the  
417 contribution of entropy. Although the molecular mechanism of how flexibility is related to  
418 activity and selectivity is still elusive, there are strong indications that flexibility and  
419 dynamics of the enzyme-substrate complex are crucial to understand the effect of substrate  
420 structure, organic solvent, and water activity to selectivity and enzymatic activity.

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430 *discussions and help.*

431



432 LEGENDS TO FIGURES

433

434 **Figure 1:** SDS-PAGE showing CALB deglycosylation with endoglycosidase F1 after 1h30  
435 reaction. Lanes 1 and 2: Chirazyme (200 $\mu$ g/mL) 2 and 1  $\mu$ L, respectively. Lane 3: MW  
436 Markers. Lanes 4 and 5: Chirazyme treated with endoglycosidase F1, 1 and 3  $\mu$ L,  
437 respectively.

438

439 **Figure 2** Water adsorption isotherm at 45 °C of desalted and deglycosylated *Candida*  
440 *antarctica* lipase B (CALB). The open diamonds correspond to the mean value of two  
441 different cycles of water adsorption performed on 0.5mg of the same enzyme sample. The  
442 filled diamonds correspond to the calculation of maximum cluster size of protein-bound water  
443 molecules, derived from MD simulations.

444

445

446 **Figure 3a-c:** Dynamics of protein hydration. MD simulation snapshot after 20 ns of  
447 simulation of the protein with a solvent box with 125 (phase I), 338 (transition between phase  
448 I and II), and 720 water molecules (phase II), corresponding to water activities of 0.26, 0.49  
449 and 0.75, respectively. Each view corresponds to the same orientation of the enzyme, with the  
450 amino acids of the catalytic triad (Ser 105, Asp 187 and His 224) coloured on the surface in  
451 green. Pictures rendered in Pymol visualization software.

452

453 FIGURES

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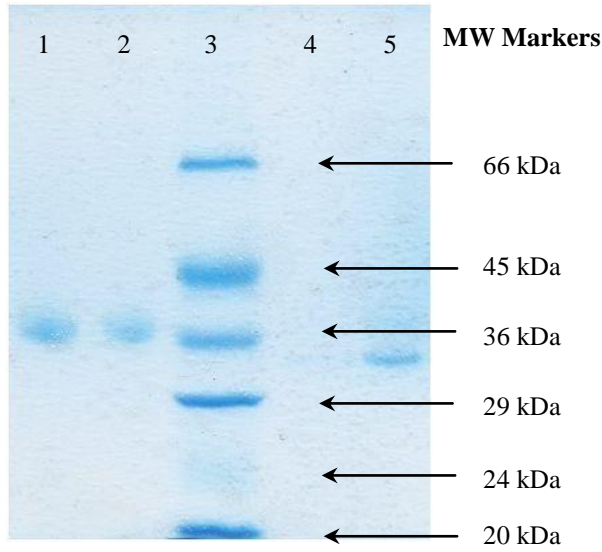
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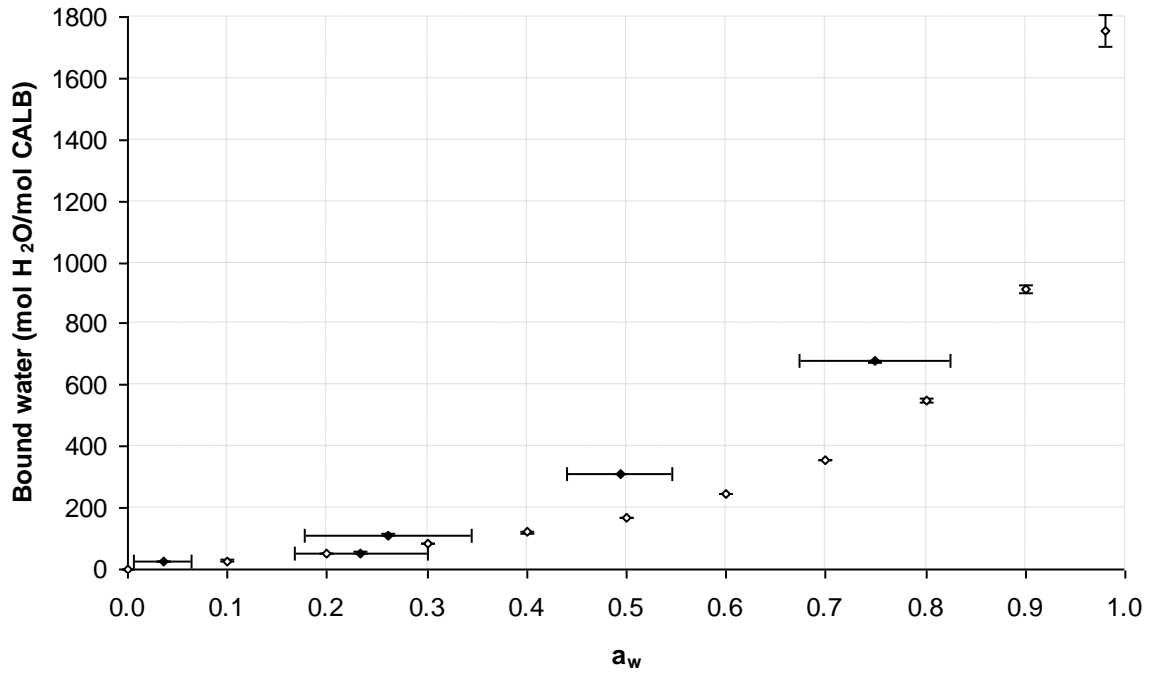
465

466



**Figure 1**

467



468

469 **Figure 2**

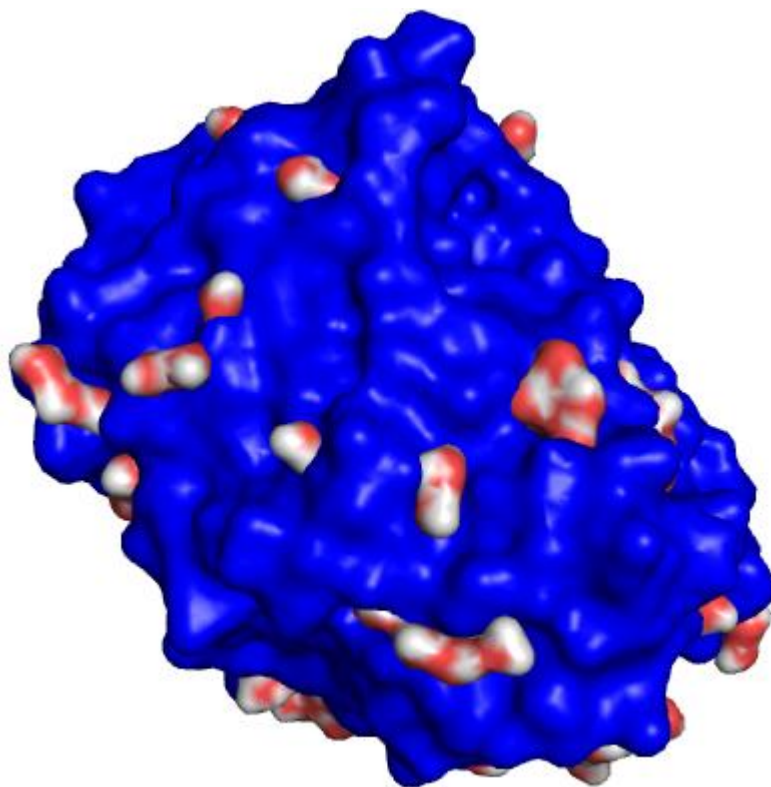
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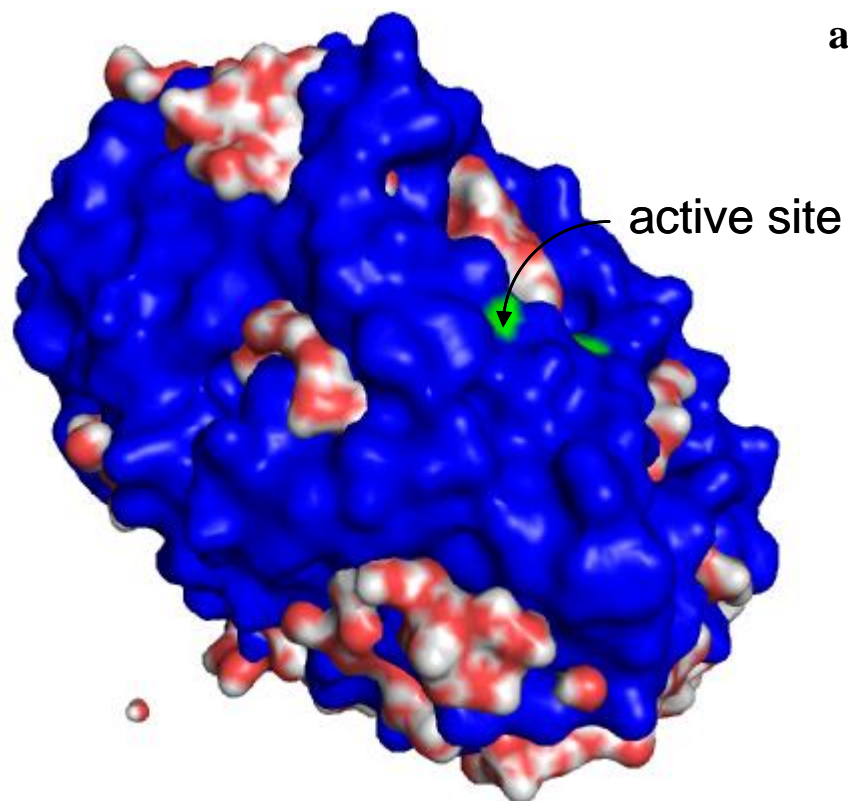
a)

$a_w = 0.26$



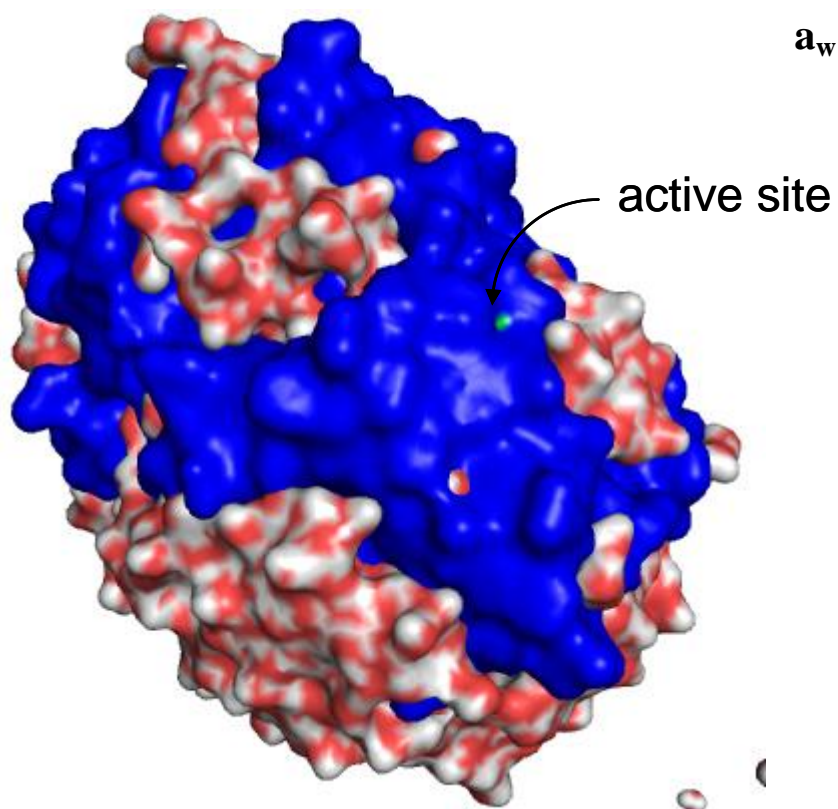
b)

$a_w = 0.49$



c)

$a_w = 0.75$



473

474 **Figure 3a-c**

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