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Enhancing the Enantioselectivity of CALB by substrate imprinting: an experimental and molecular dynamics simulation study.

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Abstract

Kinetic resolution of pentan-2-ol by CALB catalyzed enantioselective transesterification, with various alkylpropanoate acyl donors, was studied in a solid-gas reactor. Results show that the leaving alkoxy group influences the enantiomeric ratio of the reaction. Resolution of pentan-2-ol with methyl propanoate gives an enantiomeric ratio of 62. Esters with longer linear alkyl chains, from ethyl to pentyl propanoate give higher enantiomeric ratios, comprised between 103 and 117. Enantiopure ester (*R*)-1-methylpentyl propanoate increases the enantiomeric ratio to 140 compared with $E = 120$ for the racemic mixture. In contrast, enantiopure (*S*)-1-methylpentyl propanoate decreases the enantiomeric ratio to 72. Our data support the notion of an imprinting effect or "ligand-induced enzyme memory" caused by the shape of the leaving alcohol.

To simulate the imprinting effect caused by the alkoxy part of the acyl donor, molecular modeling studies were performed with both (*R*)- and (*S*)- enantiopure 1-methylpentyl propanoate. To investigate how the first step of the reaction, through the first tetrahedral intermediate, affects the enzyme conformation depending on the enantiopure ester substrate used, 20 ns molecular dynamics simulations were carried out. Clustering analysis was done to study relevant conformations of the systems. Differences in the global conformation of the enzyme between systems with *R* or *S* enantiomers were not observed. Interestingly however, orientation of the partially buried side chain for Ile285 was affected. This could explain the increased enantiomeric ratio observed with the substrate ester (*R*)-1-methylpentyl propanoate due to an improved (*R*)-pentan-2-ol/enzyme interaction.

Keywords: enantioselectivity, imprinting effect, kinetics, *Candida antarctica* lipase B, molecular modeling

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1. Introduction

Lipases are α/β hydrolases (EC 3.1.1.3) which catalyze the hydrolysis of triglycerides *in vivo*. They can also form ester bonds under reverse hydrolytic conditions, which enables them to catalyze esterification and transesterification reactions. Furthermore, they are enantioselective catalysts useful in the synthesis of pharmaceutical intermediates and fine chemicals. Lipase B from *Candida antarctica*, CALB, has found widespread applications in the enantioselective synthesis of bioactive molecules and in the resolution of racemic mixtures, due to its high stability in organic media and its large-scale availability [1].

CALB catalyzed resolution of secondary alcohols by transesterification with esters as acyl donors, occurs through a Ping Pong Bi Bi mechanism, which includes two steps. The first is the acylation of the enzyme by the ester substrate, to yield the acyl-enzyme intermediate and the release of the first product, an alcohol formed with the alkoxy group. In the second step, the chiral alcohol interacts with the acyl-enzyme to form a new binary complex and the second product ester.

In the active site of CALB, the acyl and alcohol substrate moieties bind in a hairpin orientation. It is thus not surprising that the acyl chain of the ester interacts with the alcohol and consequently influences the chiral discrimination of alcohols. However it is unexpected that the leaving group, which is the alkoxy part of the ester, also influences the enantiomeric ratio.

The hypothesis of "molecular imprinting effect" has been proposed by several authors to explain such modification of enzyme selectivity or activity [2, 3, 4]. The enzyme molds its active site structure around the imprint molecule and remains "trapped" in this conformation until the substrate enters.

In 2000, Lee *et al.* [5] described a new approach to enhance lipase enantioselectivity by the "substrate matching" strategy. Resolution of three different secondary alcohols with three different acyl donors by lipase-catalyzed transesterification with *Candida antarctica* and *Pseudomonas cepacia* lipases were performed. Results demonstrated that the enantioselectivity of lipases was maximized by using acyl donor and alcohol substrates which matched well. The hypothesis of the "enzyme memory" induced by the acyl donors active site moulding in the first step of the reaction was proposed.

In the present work, we experimentally highlight the significant influence of the alkoxy part of the ester acyl donor on the enantiomeric ratio, for the resolution of pentan-2-ol by CALB. We then established the full kinetic model for a Ping-Pong Bi Bi mechanism with two competing chiral alcohol substrates, in order to verify that the differences in enantiomeric ratio, obtained with different acyl donors, did not simply arise from differences in reaction rates occurring during the acylation step, with the different esters. Our data from both experimental and kinetic studies support the hypothesis of molecular imprinting. We then looked for structural changes using molecular modeling methods.

Molecular modeling is a useful tool to provide a rational explanation of experimental data. In 2010, Lousa *et al.* provided a structural explanation for the imprinting effect [6] observed with pre-treated subtilisin

61 by co-lyophilisation with an inhibitor in the active site, using a molecular modeling approach. Results
62 showed that in the presence of the inhibitor, the active site was maintained in an open conformation which
63 was stable in hexane solvent, in contrast to simulation with "untreated" enzyme. Here, 20 ns molecular
64 dynamics simulations were carried out to study how the first step of the reaction, through the first tetrahedral
65 intermediate, affects the enzyme conformation, depending on the enantiopure ester substrate used.

66 2. Experimental Section

67 2.1. Chemicals

68 Substrates and other chemicals were purchased from SigmaAldrich-Fluka Chemical Co. They were of
69 the highest purity available (98 % minimum) and checked by gas chromatography before use. Substrates
70 were dried by distillation under argon prior to use and stored under argon atmosphere and over molecular
71 sieves. Solvents were purchased from Carlo Erba. Racemic 1-methylpentylpropanoate was synthesized from
72 the corresponding alcohol and propanoic anhydride in pyridine at room temperature [7].

73 (*R*)-1-methylpentyl propanoate was obtained by enzymatic resolution from vinyl propionate and hexan-
74 2-ol using CALB Novozym[®] 435 in heptane solvent at 35 °C, ee_p enantiomeric excess of ester product
75 was 99.3%. Enriched hexan-2-ol in *S* form taken from the previous reaction was used after purification by
76 chromatography on silica gel (eluent EP/AcOEt: 95/5), then esterification with anhydrid propionic was
77 done to obtain 1-(*S*)-methylpentyl propanoate.

78 2.2. Enzyme used for kinetic studies

79 CALB was produced in the methylotropic yeast *Pichia pastoris* and was expressed extracellularly and pu-
80 rified from the medium by hydrophobic interaction chromatography, followed by gel filtration [8, 9]. Enzyme
81 adsorption was performed onto 60/80 mesh Chromosorb P AW DMCS (acid washed dimethylchlorosilanized)
82 (Varian, France). In a typical adsorption procedure for solid/gas catalysis, enzyme (0.106 mg) was dissolved
83 in sodium phosphate buffer (pH 7.5, 10 mM), and dry Chromosorb P AW DMCS (1 g) was added to the
84 solution. The amount of immobilized enzyme was determined by measuring absorbance at 280 nm, by taking
85 a molar extinction coefficient equal to 40690 M⁻¹.cm⁻¹. After vigorous shaking, the preparation was left
86 for 1 week under vacuum and over P₂O₅ at room temperature.

87 2.3. Enzymatic reactions

88 Initial rate of reaction measurements were performed at 70 °C in a solid-gas reactor as previously de-
89 scribed [10]. Thermodynamic activities for ester and alcohol substrates were respectively $a_{ester}=0.1$ and
90 $a_{alcohol}=0.05$. Reactions were carried out in anhydrous conditions. The amount of enzyme comprised be-
91 tween 20 and 200 mg, depending on the acyl donor used. The total flow was equal to 900 μmol.mol⁻¹.

92 2.4. GC analysis

93 Quantitative analysis of reaction products were conducted using a 7890 GC system from Agilent for the
94 analysis of ester products (*R*)-1- and (*S*)-1-methylbutyl propanoate (55 °C 15 min, 3 °C.min⁻¹, 85°C 5
95 min), at a flow rate of 1.5 ml.min⁻¹ with a Chirasil-Dex CB (25 m, 0.25 mm i.d., 0.25 μm β-cyclodextrin,
96 Chrompack, France) column. Products were detected by FID and quantified using HP Chemstation software.

97 2.5. Enantioselectivity measurements

98 Enantiomeric ratio values for the different kinetics reported in the Results section, were obtained in our
99 laboratory, by measuring the ratio of initial reaction rates for ester products synthesis [11], in a continuous
100 solid-gas reactor with different acyl donors, and immobilized CALB, as previously described [9, 12].

101 3. Computational Methods

102 3.1. Setup of the system

103 The starting CALB enzyme was the R = 1.55 Å crystallographic structure solved by Uppenberg *et al.* [13]
104 (PDB entry 1TCA). To evaluate the effect of the ester substrate on the enzyme structure during the first step
105 of the reaction path, the two tetrahedral intermediates, obtained in the reaction with *R* or *S* 1-methylpentyl
106 propanoate were modelized. The choice of studying the intermediates, instead of free substrates, in the
107 active site was done in order to prevent the substrates from getting out of active site, observed several times
108 in the case of subtilisin by Lousa *et al.* [6]. Furthermore, the formation of the tetrahedral intermediate may
109 have more impact on the structure conformation, because its formation requires the crossing of the energy
110 barrier. Thus, three systems were modelized: free enzyme, enzyme with *R* and *S* tetrahedral intermediates.

111 A transition state analog crystal structure, obtained with phosphonate irreversible inhibitor (PDB entry
112 1LBS) was used to build the tetrahedral part of the reaction intermediate, to allow for the correct location of
113 the central part of the tetrahedral intermediate. The acyl part is a propanoyl group. The negatively charged
114 oxygen was oriented toward the oxyanion hole to establish hydrogen bonds with Thr40 and Gln106.

115 NAMD 2.7 program and the CHARMM22 all-atom force field were used. Calculations were done in
116 an explicit water box (model TIP3P) with boundary conditions (15 Å between the enzyme and the edge
117 of the box). A trajectory of 20 nanoseconds was done for each system. The thermodynamic ensemble
118 is "isotherm-isobar" (NTP). The timestep was 2 fs and the SHAKE algorithm was used to freeze bonds
119 involving hydrogen atoms.

120 Force field parameters for the tetrahedral intermediate were taken from the literature [14]. These pa-
121 rameters were obtained from *ab initio* calculations and were specifically developed for CHARMM22 force
122 field. Other parameters required for modeling the alkyl side chains of alcohols were defined by homology
123 with available CHARMM22 parameters.

124 First, water molecules surrounding the enzyme were minimized by 5000 iterations of the conjugate gra-
125 dient, then the whole system was minimized with 10000 iterations using the same algorithm. The heat steps
126 were carried out in 600 ps, starting from 50 K and going up to 300 K, with a temperature incrementation of
127 1 K every 4 ps. An harmonic constraint of 5 kcal.mol⁻¹ was set up on the enzyme. The equilibration step
128 is the succession of four short dynamics of 200 ps with a decreasing harmonic constraint (5, 3, 0.5 and 0.1
129 kcal.mol⁻¹) followed by one nanosecond without constraint. Then, the production dynamic lasted for 20 ns.

130 *3.2. Clustering analysis*

131 Clustering analysis provides a good overview of enzyme conformations. 2000 structures, extracted from
132 the productive dynamics (one every 10 ps), were used for the dynamic analysis. Using the VMD program [15],
133 RMSD (root-mean-square-deviation) matrices were calculated for the 2000 structures, one diagonal matrix
134 with a size of 2000 by 2000 was obtained for each of the three systems.

135 RMSD was calculated on the backbone for residues 35 to 80, 100 to 240 and 260 to 290. Residues far
136 from the active site were not included, because their mobility is not supposed to influence the active site
137 conformation, which is the region putatively involved in the imprinting effect. In addition, terminal regions
138 are highly mobile (and far from the active site for CALB) and over-influenced the RMSD matrix, therefore,
139 they were not included. In the manner, we aimed to obtain conformational information specific to the rest
140 of the structure and more particularly near the active site.

141 In a second step, RMSD matrix was calculated to focus on the residues of the active site. RMSD were
142 based on every heavy atom, including side chains, of residues 103, 104, 106, 224, 187, 40, 42, 47, 278, 282,
143 285, and the backbone of residue 105 (due to the fact that the side chain changes for each system for this
144 residue). This RMSD matrix was then used to process a hierarchical ascendant classification (HAC). The
145 Ward method [16] was applied to the agglomerative steps used to build the dendrogram. Clustering analysis
146 was carried out using the R statistical software [17]. The average structure of the two most representative
147 structures from productive dynamics was chosen. Then, the closest structures to the average structure were
148 extracted and used for analysis.

149 **4. Results and Discussion**











150 *4.1. Experimental results*

151 Enantiomeric ratios experimentally determined for the resolution of pentan-2-ol by transesterification
152 with various alkoxy propanoates as acyl donors are presented in table 1. Resolution of pentan-2-ol with
153 methyl propanoate displays an enantiomeric ratio of 62. Esters with longer linear alkyl chains, from ethyl
154 to pentyl propanoate give higher enantiomeric ratios up to 117. Enantiomeric ratios equal to 117, 103,
155 115 and 105 were found for ethyl propanoate, propyl propanoate, butyl propanoate and pentyl propanoate,

156 respectively. Enantiomeric ratios for chiral esters with branched alkyl chains were also evaluated. The
 157 racemate *1*-methylpropyl propanoate gives an *E* value of 51, which is quite close to the *E* value (62) for methyl
 158 propanoate. Higher enantiomeric ratios were obtained with the longer racemate *1*-ethylbutyl propanoate
 159 (ratio of 84) and with *1*-methylpentyl propanoate, (ratio of 122).

160 Enantiomeric ratios with the enantiopure (*R*)-*1*-methylpentyl propanoate and (*S*)-*1*-methylpentyl pro-
 161 panoate, were measured and found to be 140 and 72, respectively. Thus, the enantiomeric form of the
 162 chiral ester substrate is essential for determining the enantioselectivity of the reaction. CALB displays
 163 enantioference for the *R* alcohol [9, 18], and using an ester with the *R* chiral form for the alkyl part
 164 increases the enantioselectivity compared to the racemate. In contrast, an ester with the *S* chiral form
 165 for the alkyl part, results in decreased enantioselectivity. Therefore, the more the alkyl part of the chiral
 166 ester resembles the preferred enantiomer, (*R*)-pentan-2-ol, the higher the enantioselectivity attained. A
 167 similar observation was made by González-Sabín *et al.* [19], who obtained higher enantioselectivity when
 168 the alkoxy group of the acyl donor was structurally close to the amine to be resolved. As a consequence
 169 an improved resolution of (\pm)-*cis*-2-phenylcyclopentanamine was obtained with the leaving group (\pm)-*cis*-
 170 phenylcyclopentanol (*E* value = 922), compared to (\pm)-*trans*-phenylcyclopentanol (*E* value = 525).

Table 1: Enantiomeric ratio for CALB catalyzed transesterification involving pentan-2-ol with different alkyl propanoate esters, in solid-gaz reactor at 70 °C.

	Acyl donor ester	<i>E</i>
	methyl propanoate	62
	ethyl propanoate	117
	propyl propanoate	103
	butyl propanoate	115
	pentyl propanoate	105
	<i>1</i> -methylpropyl propanoate	51
	<i>1</i> -(\pm)-ethylbutyl propanoate	84
	<i>1</i> -(\pm)-methylpentyl propanoate	122
	<i>1</i> -(<i>R</i>)-methylpentyl propanoate	140
	<i>1</i> -(<i>S</i>)-methylpentyl propanoate	72

171 4.2. Kinetic equation study

172 To confirm whether the differences in enantioselectivity are due to an imprinting effect caused by the
 173 leaving alcohol, it was necessary to check that these differences, obtained using different acyl donors, do
 174 not simply arise from differences in reaction rates occurring during the acylation step with the different
 175 esters. The complete kinetic model for the Ping Pong Bi Bi mechanism, with two competing chiral alcohol
 176 substrates, was established. The enantiomeric ratio was then expressed as a function of individual catalytic

177 rate constants of the reaction, in order to investigate whether the catalytic rate constants involved in the
 178 acylation step influence the E value.

179 4.2.1. Kinetic model determination

180 The enantiomeric ratio is defined as the ratio of specificity constants for R and S enantiomers, according to
 181 the following formula: $E = (k_{cat}^R/K_M^R)/(k_{cat}^S/K_M^S)$. The kinetic parameter determination is straightforward
 182 in the case of a monosubstrate reaction following the classic Michaelis mechanism. The transesterification
 183 studied here corresponds to a much more complex kinetics system. It involves a first substrate ester and
 184 two competing second substrates, R and S forms of the secondary alcohol. It obeys a Ping Pong Bi Bi
 185 mechanism. Classic kinetic experiments provide apparent constants K_M and V_{max} , which are dependent
 186 on the catalytic rate constant of the first step of the mechanism. The Michaelis-Menton constant for the R
 187 alcohol K_M^R is thus equal to $k_2(k_3 + k_4)/k_3(k_4 + k_2)$ (figure 2), where k_2 depends on the leaving alcohol in the
 188 first reaction step. This observation could explain the enantiomeric ratio modification observed when the
 189 leaving alkoxy group changes. Therefore, the relationship between the acylation step rate and enantiomeric
 190 ratio is worth considering. Here, the full kinetic model for a Ping Pong Bi Bi mechanism involving one
 191 ester and two competing alcohols substrates was defined. The kinetic equation was calculated using the
 192 King-Altman method and specificity constants were determined with the Cleland method.

193 We focused on the resolution of a racemic mixture of R and S enantiomeric forms of pentan-2-ol, through
 194 acyl transfer from an ester substrate. In the reaction model a second pathway for the reaction with the
 195 second enantiomer was added, as shown in figures 1 and 2, for Cleland and King-Altman representations.

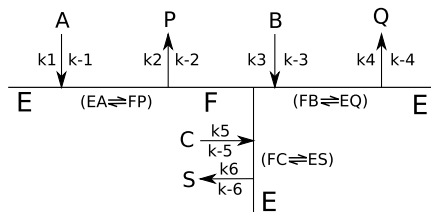


Figure 1: Cleland representation. The enzyme (E), the acyl enzyme (F), the ester (A), the (R) and (S) alcohols (respectively B and C), the leaving alcohol product (P), the (R) and (S) ester products (respectively Q and S).

196 The kinetic profiles of multisubstrate systems can be resolved using the King-Altman method [20, 21],
 197 which, in the present work, has been devised as an interactive web form by BioKin Ltd. (available at:
 198 <http://www.biokin.com/king-altman/>). It was used to obtain the reaction velocity as a function of individual
 199 catalytic rate constants presented above in Cleland and King-Altman representations.

200 The model provides complex equations, whose detailed expression is given in the appendix. Equations
 201 were simplified by considering the system in the absence of products P, Q and S. Indeed, all reaction velocities
 202 were measured under conditions of initial rate of reaction, i.e. with negligible product concentrations. Thus,
 203 the forward velocities for the R and S ester products synthesis (v_{init}^R and v_{init}^S) were obtained:

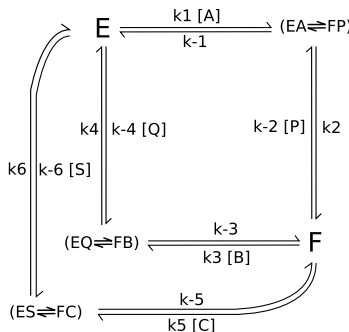


Figure 2: King-Altman representation. The enzyme (E), the acyl enzyme (F), the ester (A), the (R) and (S) alcohols (respectively B and C), the leaving alcohol product (P), the (R) and (S) ester products (respectively Q and S).

$$\frac{v_{init.}^R}{[E]_t} = \frac{n_4[A][B]}{d_8[A][C] + d_9[A][B] + d_{13}[C] + d_{14}[B] + d_{15}[A]} \quad (1)$$

$$\frac{v_{init.}^S}{[E]_t} = \frac{n_8[A][C]}{d_8[A][C] + d_9[A][B] + d_{13}[C] + d_{14}[B] + d_{15}[A]} \quad (2)$$

204 The complete formulas, with detailed values of n_i and d_i are reported in the appendix section.

205 4.2.2. Kinetic parameter determination

206 The Cleland nomenclature [22] allowed the calculation of the Michaelis-Menton constant K_M for (R)
 207 and (S) alcohols (respectively K_M^R and K_M^S) and maximum reaction rates V_{max}^R and V_{max}^S . The parameters
 208 determination was based on coefficients from the global equations 1 and 2.

$$\frac{V_{max}^R}{[E]_t} = \frac{k_2 k_4}{k_4 + k_2} = k_{cat}^R \quad \frac{V_{max}^S}{[E]_t} = \frac{k_2 k_6}{k_6 + k_2} = k_{cat}^S \quad (3)$$

$$K_M^R = \frac{k_2 (k_{-3} + k_4)}{k_3 (k_4 + k_2)} \quad K_M^S = \frac{k_2 (k_{-5} + k_6)}{k_5 (k_6 + k_2)} \quad (4)$$

209 The four parameters K_M^R , K_M^S , V_{max}^R and V_{max}^S enabled the calculation of the enantiomeric ratio E ,
 210 according to the following formula $E = (k_{cat}^R/K_M^R)/(k_{cat}^S/K_M^S)$:

$$E = \frac{k_3 k_4 (k_{-5} + k_6)}{k_5 k_6 (k_{-3} + k_4)} \quad (5)$$

211 Thus, it appears that the E value is not controlled by catalytic rate constants involved in the acylation
 212 step, the first part of the reaction (k_1, k_{-1}, k_2, k_{-2}), (c.f. figures 1 and 2), indicating that the nature of the
 213 leaving alcohol did not influence the enantiomeric ratio E , through kinetic effects.

214 In addition, the ratio of initial reaction rates $v_{init.}^R/v_{init.}^S$ was equal to:

$$\frac{v_{init.}^R}{v_{init.}^S} = \frac{n_4[A][B]}{n_6[A][C]} = \frac{k_3 k_4 (k_{-5} + k_6) [B]}{k_5 k_6 (k_{-3} + k_4) [C]} \quad (6)$$

Therefore, when reaction velocity is measured under conditions of initial rate, where B and C are enantiopure alcohols in racemic mixture, then the ratio of forward velocities $v_{init.}^R/v_{init.}^S$ is equal to:

$$\frac{v_{init.}^R}{v_{init.}^S} = \frac{k_3 k_4 (k_{-5} + k_6)}{k_5 k_6 (k_{-3} + k_4)} = E \quad (7)$$

Thus, the enantiomeric ratio E is equal to the ratio v^R/v^S in conditions of initial rate (insignificant concentration of products) and with a racemic mixture of alcohols at the initial step of the reaction. We can conclude that measuring the ratio of initial reaction rates v^R/v^S is a valid method to determine the enantiomeric ratio E .

Similarly, the relationship between the ratio v^R/v^S in conditions of initial reaction and E was demonstrated by Chen (1982) in the case of the simple Michaelis Menton model [11]. Furthermore, Chen's proposition remains correct in case of the Ping Pong Bi Bi system with R or S as competitive alcohol substrates.

We have confirmed here, then, that 1) E values can be correctly determined by measuring the ratio of initial reaction rates for enantiopure ester synthesis, 2) E values do not depend on catalytic rate constants involved in the acylation step.

4.3. Molecular modeling results

The results presented above suggest that there is an imprinting effect: the first substrate of the reaction and in particular the alkoxy part of the ester causes a conformation change of the enzyme, which is "memorized" by the enzyme and modifies its ability to discriminate between enantiomers of the second alcohol substrates. Interesting results, concerning the sensitivity of the enantioselectivity in relation to the enantiomeric form of the leaving alcohol, indicate that the imprinting effect involves modifications near the active site (table 1). Our attempts to confirm this hypothesis by molecular modeling are presented below.

Two representative structures (Clust1 and Clust2) were obtained from the cluster analysis of each system: free enzyme, enzyme+ R and enzyme+ S tetrahedral intermediates (TI- R and TI- S). RMSD were calculated between them (table 2).

RMSD between two representative structures of the same system is usually lower than other values: 0.588 Å for free enzyme, 0.700 Å for enzyme-TI- R , 0.658 Å for enzyme-TI- S . Highest values were found when the representative structure of the enzyme-TI- R was compared with other structures, RMSD reached 1.347 Å when cluster 2 for the R form and cluster 1 for the S form were compared.

Average structure superposition shows that the most important difference arises from the position of α helix 5, as shown in figure 3. The position of this helix differed between clusters for one examined enzyme structure, and also between R and S structures.

Table 2: RMSD of the backbone between each representative structures of the three systems. Alignment was based on residues 35 to 80, 100 to 240 and 260 to 290

		Enz		Enz+TI- <i>R</i>		Enz+TI- <i>S</i>	
		Clust1	Clust2	Clust1	Clust2	Clust1	Clust2
Enz	Clust1	0	0.588	1.199	0.850	0.666	0.549
	Clust2		0	0.970	1.120	0.724	0.649
Enz+TI- <i>R</i>	Clust1			0	0.700	1.123	0.992
	Clust2				0	1.347	1.233
Enz+TI- <i>S</i>	Clust1					0	0.658
	Clust2						0

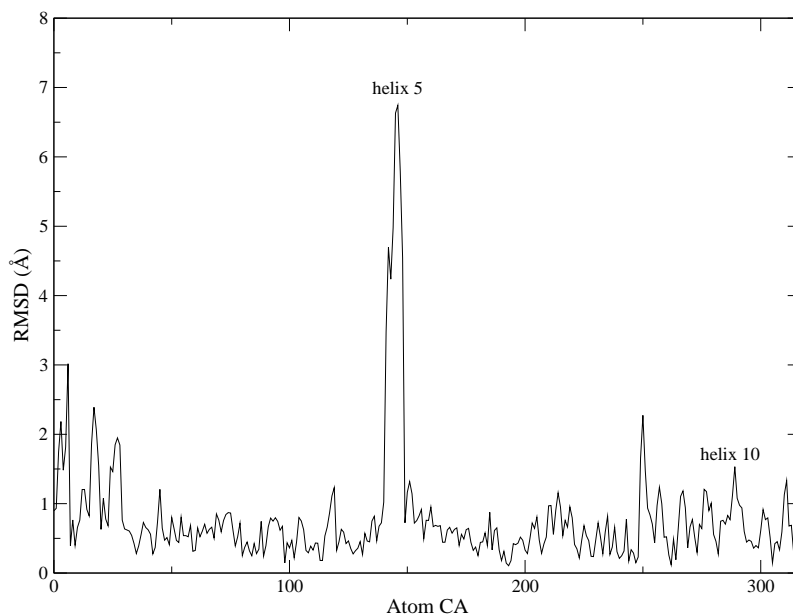


Figure 3: Measure of the deviation (in Å) for the α carbons between the representative structure of the ten last nanoseconds between TI-*R* and TI-*S*.

244 Other authors have previously shown that the *alpha* helix 5 is highly mobile. Skjøt *et al.* [23] demonstrated
245 that during a 10 ns dynamic trajectory in a water box, α -helix 5 and 10 of CALB displayed significant
246 mobilities. The RMSD matrix provides cluster results mainly based on the orientation of the α helix 5.
247 Here, α helix 5 orientation is not specific to the system studied, but similar mobility was observed for the
248 three studied systems. Our conclusion is that specific differences in the global conformation of the enzyme
249 between TI-*R* and TI-*S* are not observed.

250 In the second part of our study, we focused on the active site. Cluster analysis based on amino acids of
251 the active site was done as described in the Computational Methods section. Amino acids alignment gave
252 good superimposed structures, including side chains orientation (*c.f.* figure 4).

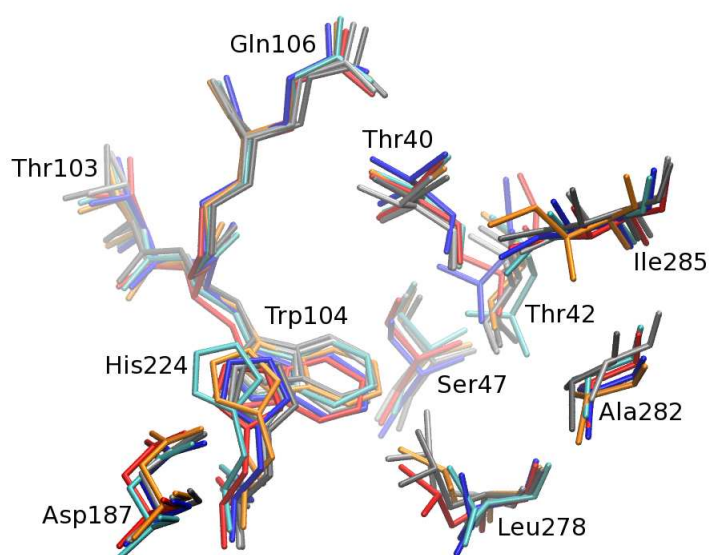


Figure 4: View of the active site for the six clusters obtained for the three studied systems after alignment based on the heavy atoms of residues 103, 104, 106, 224, 187, 40, 42, 47, 278, 282, 285 and the backbone of residues 105. The color code for the clusters is Clust1 for Enz-IT-*S* in blue, Clust2 for Enz-IT-*S* in light blue, Clust1 for Enz-IT-*R* in red, Clust2 for Enz-IT-*R* in orange, Clust1 for enzyme free in gray and Clust2 for enzyme free in dark gray.

253 Interestingly however, the orientation of the side chain of residue Ile285 was different for the cluster 2
254 of TI-*R*. This may be due to the specific constraint generated by the alcohol enantiomer on the side chain
255 orientation which pointed toward Ile285. In the case of the *R* enantiomer, the side chain of Ile285 rotated
256 by 120 degrees in around 10 nanoseconds (figure 5). The cluster analysis was consistent with this fact, and
257 split the trajectory into two dominant clusters, one before the rotation, and the second after it. Side chain
258 orientation in the TI-*S* system is the same as that observed with free enzyme. Residue Ile 285 belongs to α
259 helix 10. Marton *et al.*, demonstrated that mutations of residues Leu282 and Ile282 of the α helix 10 affected
260 enantioselectivity [24]. Thus, side chain rotation of Ile285 may also influence the enantioselectivity of the

261 reaction.

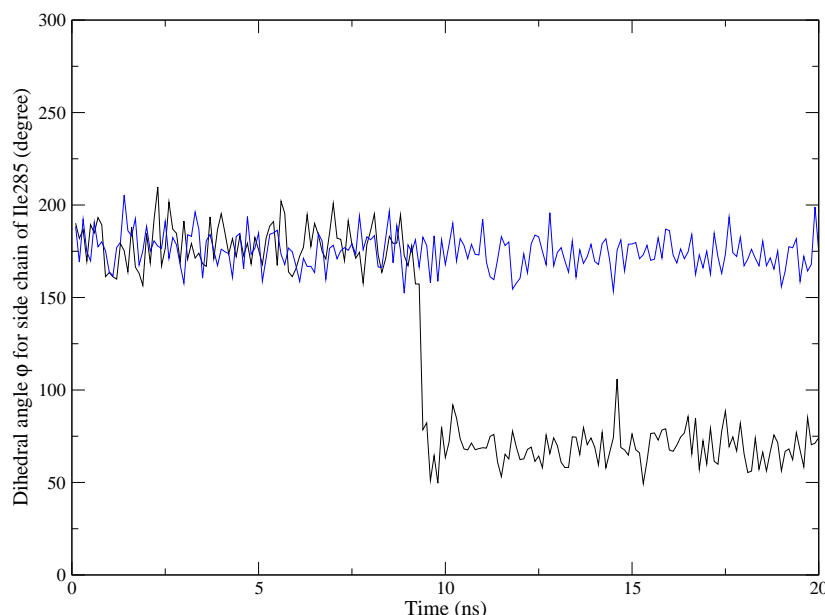


Figure 5: Dihedral angle φ defining the side chain rotation of residue Ile285. In black for the *R* enantiomer and in blue for the *S* enantiomer.

262 A major question concerns the timescale of side chain rotation, such as of the branched-chain of Ile285.
263 The time scale for the rotation of a buried side chain can be very large (10^{-4} to 1 s) [25]. Experimental results
264 show that buried side chains rotate very slowly compared with the time scale of molecular dynamics. NMR
265 was used by Skrynnikov *et al.* [26] to quantitate slow hydrogen-deuterium exchange processes at methyl-
266 containing side chains in proteins. This method was also applied to the study of ms time scale side-chain
267 dynamics of methionine residues in a buried cavity. These authors observed that the methionine residues
268 were sensitive to an exchange event with a rate of the order of 1200 s^{-1} at 20°C and that the corresponding
269 motions may be linked to a process which allows entry and exit of ligands to and from the cavity. Similar
270 NMR studies on a protease, by Ishima *et al.* [27], demonstrated that the hydrogen-deuterium exchange time
271 of buried methyl side chains was above 1 ms.

272 The side chain of residue Ile285 is partially buried, as it is oriented toward the top of the stereospecificity
273 pocket in the active site, and near the side chain of the alcohol. The time scale of Ile285 side chain rotation
274 can thus be considered to be around 1 ms. Previously the same order of magnitude was obtained for
275 pentan-2-ol transesterification in a solid-gas reactor [18]: k_{cat} equal to 800 s^{-1} and 17 s^{-1} for (*R*)- and (*S*)-
276 pentan-2-ol respectively. The similarity of these two time scales namely, side chain rotation and substrate
277 catalysis, is consistent with the hypothesis of "imprinting effect". Ile285 side chain rotation provides a
278 much more suitable active site shape for interacting with the substrate (*R*)- enantiomer alcohol. This is

279 exactly what is experimentally observed: enantioference for the *R* form of pentan-2-ol increases when the
280 (*R*)-1-methylpentyl propanoate is used as acyl donor.

281 Other authors also performed molecular modeling studies to explore enzyme structural changes upon
282 imprinting. Rich and Dordick [28] obtained an increase of subtilisin catalytic rate and also a better control of
283 enzyme substrate specificity, by means of lyophilizing subtilisin in presence of different nucleophile substrates,
284 as "imprinters". By molecular dynamic simulations it was shown that structural changes in the catalytic
285 triad occurred during imprinting, that may contribute to imprinting-induced substrate selectivity.

286 5. Conclusion

287 Experimental results shown here demonstrate that using an ester with an adequate alkoxy group is
288 an efficient method to enhance enantioselectivity. Generally, an alkoxy group larger than ethyl increased
289 enantioselectivity. Furthermore, the resolution of pentan-2-ol was sensitive to the chirality of the alkoxy group
290 of the ester. Thus, (*R*)-1-methylpentyl propanoate increased enantioselectivity compared to the racemic
291 mixture, whereas *S* enantiomer decreased enantioselectivity compared with the racemic mixture.

292 The comprehensive study of the full kinetics for the Ping Pong Bi Bi mechanism, with three substrates,
293 one ester and two competitive *R* and *S* alcohols, allowed us to confirm that the experimental method, based
294 on initial rate measurements employed here for the determination of enantiomeric ratio, is relevant. In
295 particular, it excluded the hypothesis that enantiomeric ratio modifications observed in the experimental
296 results could arise from a kinetic model pitfall.

297 Finally, molecular dynamics simulations were performed to discriminate between conformational changes
298 caused by both (*R*)- and (*S*)- enantiopure 1-methylpropyl propanoate. It appears that the *R* enantiomer
299 causes the rotation of the side chain of residue Ile285, which appears to have an effect on subsequent
300 discrimination between secondary alcohol enantiomers. If this is the general case, molecular imprinting by
301 the first substrate would offer the possibility of controlling enantioselectivity for a second substrate and thus
302 provide a new tool for biocatalyst engineering.

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344 **7. Appendix**

345 Speed rate equation for synthesis of R and S enantiomer products v^R and v^S :

$$v^R = d[Q]/dt = k_{+4}[FB] - k_{-4}[Q][E] \quad (8)$$

$$v^S = d[S]/dt = k_{+6}[FC] - k_{-6}[S][E] \quad (9)$$

$$\frac{v^R}{[E]_t} = \frac{N_R}{D} \quad (10)$$

$$\frac{v^S}{[E]_t} = \frac{N_S}{D} \quad (11)$$

346 Expression of numerators and denominator, N_R , N_S , and D :

$$N_R = +n_1[Q][P] + n_2[C][Q] + n_3[B][S] + n_4[A][B] \quad (12)$$

$$N_S = +n_5[S][P] + n_6[C][Q] + n_7[B][S] + n_8[A][C] \quad (13)$$

$$D = +d_1[P][S] + d_2[Q][P] + d_3[C][S] + d_4[C][Q] + d_5[B][S] + d_6[B][Q] + d_7[A][P] \\ + d_8[A][C] + d_9[A][B] + d_{10}[S] + d_{11}[P] + d_{12}[Q] + d_{13}[C] + d_{14}[B] + d_{15}[A] \quad (14)$$

$$n_1 = -k_{-1}k_{-2}k_{-3}k_{-4}k_{-5} - k_{-1}k_{-2}k_{-3}k_{-4}k_{+6} \quad (15)$$

$$n_2 = -k_{-1}k_{-3}k_{-4}k_{+5}k_{+6} - k_{+2}k_{-3}k_{-4}k_{+5}k_{+6} \quad (16)$$

$$n_3 = +k_{-1}k_{+3}k_{+4}k_{-5}k_{-6} + k_{+2}k_{+3}k_{+4}k_{-5}k_{-6} \quad (17)$$

$$n_4 = +k_{+1}k_{+2}k_{+3}k_{+4}k_{-5} + k_{+1}k_{+2}k_{+3}k_{+4}k_{+6} \quad (18)$$

$$n_5 = -k_{-1}k_{-2}k_{-3}k_{-5}k_{-6} - k_{-1}k_{-2}k_{+4}k_{-5}k_{-6} \quad (19)$$

$$n_6 = +k_{-1}k_{-3}k_{-4}k_{+5}k_{+6} + k_{+2}k_{-3}k_{-4}k_{+5}k_{+6} \quad (20)$$

$$n_7 = -k_{-1}k_{+3}k_{+4}k_{-5}k_{-6} - k_{+2}k_{+3}k_{+4}k_{-5}k_{-6} \quad (21)$$

$$n_8 = +k_{+1}k_{+2}k_{-3}k_{+5}k_{+6} + k_{+1}k_{+2}k_{+4}k_{+5}k_{+6} \quad (22)$$

$$d_1 = k_{-2}k_{+4}k_{-5}k_{-6} + k_{-2}k_{-3}k_{-5}k_{-6} + k_{-1}k_{-2}k_{+4}k_{-6} + k_{-1}k_{-2}k_{-3}k_{-6} \quad (23)$$

$$d_2 = k_{-2}k_{-3}k_{-4}k_{+6} + k_{-2}k_{-3}k_{-4}k_{-5} + k_{-1}k_{-2}k_{-4}k_{+6} + k_{-1}k_{-2}k_{-4}k_{-5} \quad (24)$$

$$d_3 = k_{-1}k_{+4}k_{+5}k_{-6} + k_{-1}k_{-3}k_{+5}k_{-6} + k_{+2}k_{+4}k_{+5}k_{-6} + k_{+2}k_{-3}k_{+5}k_{-6} \quad (25)$$

$$d_4 = k_{-1}k_{-4}k_{+5}k_{+6} + k_{+2}k_{-4}k_{+5}k_{+6} + k_{-1}k_{-3}k_{-4}k_{+5} + k_{+2}k_{-3}k_{-4}k_{+5} \quad (26)$$

$$d_5 = k_{-1}k_{+3}k_{-5}k_{-6} + k_{+2}k_{+3}k_{-5}k_{-6} + k_{-1}k_{+3}k_{+4}k_{-6} + k_{+2}k_{+3}k_{+4}k_{-6} \quad (27)$$

$$d_6 = k_{-1}k_{+3}k_{-4}k_{+6} + k_{-1}k_{+3}k_{-4}k_{-5} + k_{+2}k_{+3}k_{-4}k_{+6} + k_{+2}k_{+3}k_{-4}k_{-5} \quad (28)$$

$$d_7 = k_{+1}k_{-2}k_{+4}k_{+6} + k_{+1}k_{-2}k_{+4}k_{-5} + k_{+1}k_{-2}k_{-3}k_{+6} + k_{+1}k_{-2}k_{-3}k_{-5} \quad (29)$$

$$d_8 = k_{+1}k_{+4}k_{+5}k_{+6} + k_{+1}k_{-3}k_{+5}k_{+6} + k_{+1}k_{+2}k_{+4}k_{+5} + k_{+1}k_{+2}k_{-3}k_{+5} \quad (30)$$

$$d_9 = k_{+1}k_{+3}k_{+4}k_{+6} + k_{+1}k_{+3}k_{+4}k_{-5} + k_{+1}k_{+2}k_{+3}k_{+6} + k_{+1}k_{+2}k_{+3}k_{-5} \quad (31)$$

$$d_{10} = k_{-1}k_{+4}k_{-5}k_{-6} + k_{-1}k_{-3}k_{-5}k_{-6} + k_{+2}k_{+4}k_{-5}k_{-6} + k_{+2}k_{-3}k_{-5}k_{-6} \quad (32)$$

$$d_{11} = k_{-1}k_{-2}k_{+4}k_{+6} + k_{-1}k_{-2}k_{+4}k_{-5} + k_{-1}k_{-2}k_{-3}k_{+6} + k_{-1}k_{-2}k_{-3}k_{-5} \quad (33)$$

$$d_{12} = k_{-1}k_{-3}k_{-4}k_{+6} + k_{-1}k_{-3}k_{-4}k_{-5} + k_{+2}k_{-3}k_{-4}k_{+6} + k_{+2}k_{-3}k_{-4}k_{-5} \quad (34)$$

$$d_{13} = k_{-1}k_{+4}k_{+5}k_{+6} + k_{-1}k_{-3}k_{+5}k_{+6} + k_{+2}k_{+4}k_{+5}k_{+6} + k_{+2}k_{-3}k_{+5}k_{+6} \quad (35)$$

$$d_{14} = k_{-1}k_{+3}k_{+4}k_{+6} + k_{-1}k_{+3}k_{+4}k_{-5} + k_{+2}k_{+3}k_{+4}k_{+6} + k_{+2}k_{+3}k_{+4}k_{-5} \quad (36)$$

$$d_{15} = k_{+1}k_{+2}k_{+4}k_{+6} + k_{+1}k_{+2}k_{+4}k_{-5} + k_{+1}k_{+2}k_{-3}k_{+6} + k_{+1}k_{+2}k_{-3}k_{-5} \quad (37)$$