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# Influence of lid conformation on lipase enantioselectivity

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**Abstract**

The enantioselectivity of porcine pancreatic lipase (PPL) in the hydrolysis reaction of racemic glycidyl butyrate has been observed to increase substantially upon interfacial activation of the enzyme. The enantioselectivity of *Candida antarctica* lipase B (*Ca*lB), a lipase that does not display interfacial activation, does not change when the substrate concentration exceeds the solubility limit. A hypothesis, based on a kinetic model, is presented that relates the change of enantioselectivity to the conformational changes that accompany movements of the lid upon interfacial activation. The hypothesis was investigated using various forms of the *C. rugosa* lipase (*Cr*l). For several substrates, the enantioselectivities of hydrolysis reactions catalyzed by crude, purified, and crystalline (CLEC®) preparations of *Cr*l in open and closed conformations were measured. As anticipated, the enantioselectivity of open-lid *Cr*l-CLECs in the hydrolysis of racemic ibuprofen methyl ester exceeded that of the closed-lid form. For other esters, however, correlations were less straightforward. It was concluded that apart from affecting the activation barrier leading to the Michaelis–Menten complex, modifications of the lid (open, closed, or modified lid) also induce additional conformational changes in the active site affecting enantioselectivity. 

*Keywords:* Lipase; CLEC; Enantioselectivity; Lid conformation; Free-energy diagram

## 1. Introduction

Lipases hydrolyze insoluble long chain acyl glycerols. Their activity is greatly enhanced in the presence of a water/lipid interface, a phenomenon known as interfacial activation [1,2]. Interfacial activation has been related to the presence of an amphiphilic peptide loop (lid) that can cover the active site. When dissolved in water the lid is closed and the hydrophilic side is turned towards the solvent, possibly to prevent aggregation of the enzyme. Upon adsorption of the lipase onto a water/lipid interface, the lid opens and the hydrophobic side is exposed to the interface. Lipases from *Candida rugosa* [3,4], *Mucor miehei* [5,6], *Geotrichum candidum* [7,8], *Humicola lanuginosa* [9] and pancreas [10,11] have been crystallized both in the absence and presence of substrate analogues or inhibitors. These studies have confirmed the importance of the lid movement: in the presence of a substrate analogue or inhibitor, the lid undergoes a rearrangement rendering the active site accessible to the substrate.

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Adsorption of a lipase onto an interface not only increases activity, but it may also affect the enantioselectivity. Van Tol et al. [12] studied the porcine pancreas lipase-catalyzed kinetic resolution of glycidyl butyrate in water. Apart from the considerable increase in reaction rate when the concentration of glycidyl butyrate exceeded the solubility limit, the enantioselectivity also increased from $E = 8$ in a monophasic to $E = 16$ in a biphasic water/substrate system.

A possible explanation for the observed change in enantioselectivity is that the opening and closing of the lid influences the *E*-value. In this report, we present a theoretical framework that describes the influence of conformational changes of the lid on the enantioselectivity of lipase-catalyzed reactions: opening and closing of the lid is supposed to affect the activation barrier for the formation of the Michaelis–Menten complex. This hypothesis is investigated measuring the enantioselectivity for a lipase that does not possess a lid, and for lipase preparations with the lid fixed in the open and closed conformation.

Firstly, the activity and enantioselectivity of *C. antarctica* lipase B (*Ca*lB), a lipase not possessing a lid, was measured for the hydrolysis reaction of glycidyl butyrate at various substrate concentrations, similar to the experiments carried out by Van Tol et al. [12]. *Ca*lB has been crystallized both in the presence and absence of a detergent [13,14]. A short α-helix, previously identified as a possible lid, does not shield the active site in the absence of detergent molecules in a manner that has been observed for other lipases containing a lid. The kinetics of *Ca*lB have been investigated by Martinelle and Hult [15]. Interestingly, *Ca*lB does not display interfacial activation. The catalytic activity of *Ca*lB resembles more that of an esterase than that of a lipase. In our experiment, the possible effect of interfacial activation on the enantioselectivity was studied.

Secondly, we have measured the enantioselectivities of *C. rugosa* lipase CLECs (*Cr*l-CLECs) in the open and closed form for the hydrolysis of several esters in aqueous solution. Previously, *Cr*l has been crystallized and the three-dimensional structure has been determined using X-ray spectroscopy [3,4]. Through changes in the medium in which the *Cr*l crystals were grown, it was possible to obtain the enzyme in two distinct conformations. Crystals grown in the presence of PEG 8000 yielded a conformation in which the active site was shielded from the solvent by the lid. Crystals grown in the presence of 2-methyl-2,4-pentanediol yielded an enzyme conformation with the active site accessible to the solvent. The former is denoted as the closed form, the latter is denoted the open form. The crystals can be cross-linked to form CLECs [16]. In the *Cr*l-CLECs, we can assume that because of the cross-linking, the open and closed conformations are preserved when the crystals are placed in water and organic solvents. It has been reported that the closed *Cr*l-CLEC displays lower activity and enantioselectivity towards ketoprofen chloroethyl ester than the open *Cr*l-CLEC [17]. From the crystal structure of the closed form of *Cr*l, it was concluded that the lid is not completely closed and small substrate molecules can enter the active site. We believe that in the closed *Cr*l-CLEC, the lid is fixed in the closed conformation and is not opened by the substrate.

## 2. Materials and methods

### 2.1. Materials

#### 2.1.1. Chemicals

Racemic 1-phenylethyl butyrate (Aldrich), ethyl 2-bromo-propionate (Fluka) and 2,2-dimethyl-1,3-dioxolane-4-methanol (Sigma) were obtained from commercial suppliers. Ibuprofen methyl ester was a gift from DSM Anti-Infectives (Delft, The Netherlands) and glycidyl butyrate (2,3-epoxy-1-propyl butyrate) was a gift from DSM (Geleen, The Netherlands). 2,2-Diisopropyl-1,3-dioxolane-4-methanol, 2,2-diisopropyl-1,3-dioxolane-4-methanol butyrate and

2,2-dimethyl-1,3-dioxolane-4-methanol pentanoate were prepared according to standard procedures.

### 2.1.2. Enzymes

Lyophilized *Ca*lB (Chirazyme L-9, lyo) was a gift from Boehringer (Mannheim, Germany). Crude *Cr*l was obtained from Sigma. Purified *Cr*l as used for the production of CLECs, and *Cr*l-CLECs in the open and closed form were generous gifts from Altus Biologics (Cambridge, USA). The lipase was purified and crystallized according to published procedures [17].

## 2.2. Enzyme-catalyzed hydrolysis reactions

Two methods were used to carry out the lipase-catalyzed hydrolysis reactions. In the first method a pH-stat system, consisting of a Metrohm Dosimat (model 655), an Impulsomat (model 614) and a pH-meter (model 632), was used. A thermostated reaction vessel (Metrohm 6.1415.150) was used to control the temperature during the reaction. The reaction mixture consisted of 200 μl substrate in 10 ml, 10 mM phosphate buffer. The reaction was started by adding the lipase to the well-stirred reaction mixture. During the course of the reaction, the pH of the reaction mixture was kept constant through the addition of 1 M NaOH solution from the Dosimat. Samples were prepared by withdrawing 0.3 ml of the reaction mixture at regular intervals, extracting the substrate and the product with 0.3 ml of organic solvent, separation of the organic layer and drying over anhydrous $MgSO_4$. The second method was as described by Lalonde et al. [17]. An emulsion of 100 μl substrate in 1.5 ml, 0.1 M acetate buffer and enzyme was vigorously stirred with a magnetic stirrer. Samples were prepared by periodically withdrawing 20 μl of the reaction mixture, extracting the substrate and the product with 0.2 ml of organic solvent, separation of the organic layer and drying over anhydrous $MgSO_4$. The samples were analyzed using chiral GC or HPLC. At least seven samples were collected during the course of the reaction.

The following conditions were used for the various substrates (substrate, *method*, **reaction temperature** (°C), pH, extraction solvent): 1-phenylethyl butyrate, *1*, **35**, 7.5, hexane; 2,2-dimethyl-1,3-dioxolane-4-methyl pentanoate, *1*, **30**, 7.0, 2-butanone; 2,2-diisopropyl-1,3-dioxolane-4-methyl butyrate, *1*, **30**, 7.0, 2-butanone; ethyl 2-bromopropionate, *1*, **30**, 7.5, hexane; ibuprofen methyl ester, *2*, **39**, 6.0, hexane.

## 2.3. Analytical procedure

Chiral GC analyses were carried out using a Hewlett Packard 5890 series II GC with flame ionization detection equipped with a γ-TA chiral column (Astec, trifluoroacetyl-derivatized γ-cyclodextrin), or a Chrompack CP9002 GC with flame ionization detection and autosampler, equipped a β-cyclodextrin column (CP cyclodex B 236 M, Chrompack, The Netherlands). Chiral HPLC analysis were carried out using a Waters HPLC system with autosampler and UV-detection and either a Chiralcel OD-H or Chiralcel OB-H column (Diacel, Germany).

Substrate and product were separated in a single run, except when indicated. The following chiral analysis were used (substrate, *GC* or *HPLC*, **column**, solvent in case of HPLC): glycidyl butyrate, *GC*, **γ-TA**; 1-phenylethyl butyrate, *GC*, **β-cyclodextrin**; 2,2-dimethyl-1,3-dioxolane-4-methyl pentanoate, *GC*, **β-cyclodextrin**; 2,2-diisopropyl-1,3dioxolane-4-methyl butyrate, *GC*, **β-cyclodextrin**; ethyl-2-bromo propionate, *GC*, **β-cyclodextrin**; ibuprofen methyl ester, *GC*, **β-cyclodextrin**; ibuprofen, *HPLC*, **Chiralcel OD-H**.

## 2.4. E-value measurements

Because the enantiomers of the product 2-bromo-propionate could not be separated, the *E*-values for hydrolysis reactions of ethyl 2-

bromo-propionate were calculated from the enantiomeric excess of the substrate $ee_s$ vs. the conversion $\xi$ according to the method described by Chen et al. [18] using Eq. 1:

$$E = \frac{\ln\left[(1-\xi)(1-ee_s)\right]}{\ln\left[(1-\xi)(1+ee_s)\right]}. \quad (1)$$

All other $E$-values were determined from enantiomeric excess values of the substrate and product, $ee_s$ and $ee_p$, respectively, using the method developed by Rakels et al. [19] according to Eq. 2:

$$E = \frac{\ln\left(\frac{1-ee_s}{1+ee_s/ee_p}\right)}{\ln\left(\frac{1+ee_s}{1+ee_s/ee_p}\right)}. \quad (2)$$

The advantages of using the method developed by Rakels have been discussed by Straathof and Jongejan [20]. Nonlinear regression analysis was carried out using the software program E&K calculator [21] on an Apple Macintosh® computer.

### 2.5. Theory

A theoretical explanation for the increase in enantioselectivity observed for the porcine pancreatic lipase (PPL)-catalyzed hydrolysis of glycidyl butyrate was not available at the time of the experiment. Here, we present a thermodynamic model that could explain the observed changes in enantioselectivity upon a change in the lid conformation.

The enantiomeric ratio or $E$-value for simple Michaelis–Menten-type kinetics is defined as the ratio of specificity constants [18]:

$$E_{RS} = \frac{(k_{cat}/K_M)^R}{(k_{cal}/K_M)^S} = \frac{k_2^R k_1^R/(k_{-1}^R + k_2^R)}{k_2^S k_1^S/(k_{-1}^S + k_2^S)}. \quad (3)$$

Each microscopic constant can be expressed in terms of the Gibbs free energy of activation, $\Delta G^{\#}$, using the Eyring theory of absolute reaction rates [22,23]:

$$k = \kappa \frac{k_B T}{h} e^{-\Delta G^{\#}/RT}. \quad (4)$$

with $\kappa$ the transmission coefficient, $k_B$ the Boltzmann constant, $h$ the Planck constant, $R$ the gas constant and $T$ the temperature (K). On cancellation of the transmission coefficients on assumption of their equality and introduction of $\beta = 1/RT$, combining Eq. 3 and Eq. 4 leads to:

$$E_{RS} = \frac{e^{-\beta\Delta G^{\#}_{2,R} - \beta\Delta G^{\#}_{1,R}}/\left(e^{-\beta\Delta G^{\#}_{-1,R}} + e^{-\beta\Delta G^{\#}_{2,R}}\right)}{e^{-\beta\Delta G^{\#}_{2,S} - \beta\Delta G^{\#}_{1,S}}/\left(e^{-\beta\Delta G^{\#}_{-1,S}} + e^{-\beta\Delta G^{\#}_{2,S}}\right)}; \quad (5)$$

on substitution of:

$$\Delta G_A = \Delta G_1^{\#}, \quad (6A)$$

$$\Delta G_B = \Delta G_1^{\#} + \Delta G_2^{\#} - \Delta G_{-1}^{\#}, \quad (6B)$$

one arrives at:

$$E_{RS} = \frac{e^{\beta\Delta G_{A,S}} + e^{\beta\Delta G_{B,S}}}{e^{\beta\Delta G_{A,R}} + e^{\beta\Delta G_{B,R}}}. \quad (6C)$$

The enantiomeric ratio is thus comprised of the exponentially averaged energy barriers as seen by the ground state reactants. The larger of the two energy barriers, $\Delta G_{B,S}$ and $\Delta G_{B,R}$ or $\Delta G_{A,S}$ and $\Delta G_{A,R}$, will dominate the sum of the exponentials and thus the enantioselectivity.

Fig. 1 depicts the proposed free energy diagrams for the lipase-catalyzed kinetic resolution in the open (Fig. 1A) and closed (Fig. 1B) conformation. In the open conformation, the activation barrier for the formation of the Michaelis–Menten complex, $\Delta G_{A,S}$ viz. $\Delta G_{A,R}$, is supposed to be small compared to the chemical reaction steps, $\Delta G_{B,S}$ viz. $\Delta G_{B,R}$, and does not affect the enantioselectivity. In the closed conformation, the lid has to open before a substrate can enter the active site and react. Displacement of the lid might raise the activation barriers leading to the Michaelis complexes with equal amounts for both enantiomers. Alterna-

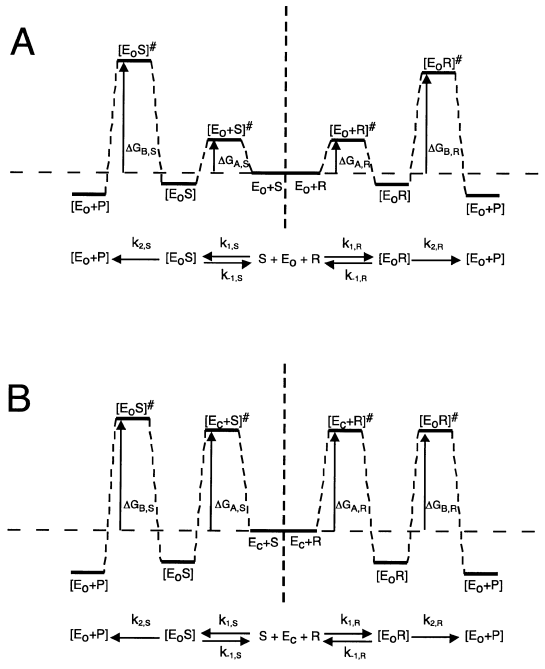



Fig. 1. Free energy diagram and Michaelis–Menten kinetic scheme for lipase-catalyzed kinetic resolution. (A) Lipase in the open conformation; (B) lipase in the closed conformation. The subscript 'c' denotes a lipase species in the closed conformation, the subscript 'o' denotes a lipase species in the open conformation, *S* and *R* denote the two enantiomers of the substrate and *P* denotes the product.

tively, since part of the barrier leading to the Michaelis–Menten complex can be expected to result from diffusion, different availability of substrates at the interface as opposed to free solution could contribute to relative changes in the barrier heights. From Eq. 6C, it can be seen that when the first activation barrier is increased with equal amounts for the two enantiomers, this leads to a decrease in enantioselectivity.

From these theoretical considerations, two requirements follow for the lid opening and closing to have an effect on the enantioselectivity. A first prerequisite is that the lid is only opened upon an interaction with the substrate. If the lid opening and closing is an equilibrium reaction between different enzyme conformations that takes place without interaction with the substrate, according to the hypothesis, the enantioselectivity can not be influenced. Lid opening would simply increase the amount of active enzyme and increase activity. We assume that the substrate interacts with the enzyme in the closed conformation at a site distinct from the active site itself since this is shielded from the solvent and substrate. Evidence can be found in the literature that interaction of small molecules with enzymes effects the activity and enantioselectivity [24–27].

A second prerequisite is that the activation barrier for the formation of the Michaelis–Menten complex of the enzyme in the closed conformation is almost as high as for the subsequent enantioselectivity controlling reaction steps. For most enzyme-catalyzed reactions, formation of the Michaelis–Menten complex is assumed to be reversible and fast [28]. The upper rate limit is the diffusion controlled reaction, calculated to be about $10^9$ $s^{-1}$ $M^{-1}$. Most values fall in the range of $10^6$ to $10^9$ $s^{-1}$ $M^{-1}$ [28]. Only when large conformational changes are associated with the formation of the Michaelis–Menten complex, the rate of formation of the Michaelis–Menten complex can become rate-limiting.

Values for reaction rates of lipase-catalyzed reactions have been reported in literature [15,17,29] and an estimate for the activation barrier of the chemical reaction steps can be made. For the calculation of the activation energies, we assume that all lipase molecules are adsorbed onto the interface and in the active open conformation. The measured reaction rates range from 20 to 14,000 $s^{-1}$, equal to an activation barrier for the chemical reaction steps of 49 to 66 kJ $mol^{-1}$ at 300 K. Values for the activation energy of the lid opening are not available, but activation energies for large conformational changes occurring in other enzymes might be used as a guide. Among others, enzymes such as triosephosphate isomerase and lactate dehydrogenase undergo conformational changes during the course of the reaction, associated with dramatic changes in biochemical activity [30–34]. These conformational changes either render the active site accessible to the substrate or

encapsulate the substrate when bound to the active site. The changes appear on the time scale of 10 to $10^4$ $s^{-1}$, equal to an activation barrier of 67 to 50 kJ $mol^{-1}$. These values are in the same range as those observed for the chemical activation barriers.

## 3. Results and discussion

The results for the PPL-catalyzed kinetic resolution of glycidyl butyrate in water, as obtained previously by Van Tol et al. [12], are depicted in Fig. 2A. We have carried out a similar experiment using *Ca*lB, a lipase that does not possess a lid. The reaction rate and enantioselectivity for the *Ca*lB-catalyzed hydrolysis of glycidyl butyrate at various substrate concentrations are plotted in Fig. 2B. As can be seen, *Ca*lB does not display interfacial activation with glycidyl butyrate as a substrate. Also, the enantioselectivity does not change ongoing from a monophasic to a biphasic system. In both cases, an *E*-value of three was measured. The results shown in Fig. 2 suggest that the change in enantioselectivity for the PPL-catalyzed hydrolysis of glycidyl butyrate in a monophasic and biphasic system may be related to the opening and closing of the lid.

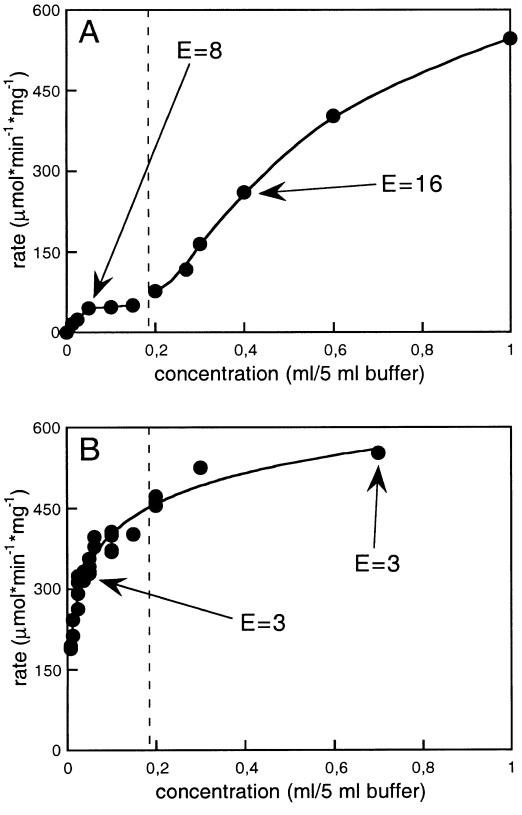



Fig. 2. PPL (2A) and *Ca*lB (2B) activity and enantioselectivity at various concentration of glycidyl butyrate. The dotted line is drawn to mark the solubility limit of glycidyl butyrate in water.

Ideally, we might test the hypothesis that lid opening changes the enantioselectivity would lipase preparations be available in which the lid was fixed in an open or closed conformation. However, for individual lipase molecules dissolved in water, it is not possible to lock the lid in either conformation. This makes it impossible to measure the enantioselectivity of a lipase molecule in the open and closed conformation in both a monophasic and biphasic and thus verify the hypothesis. An alternative is now being offered by CLECs of *Cr*l. The reason to choose *Cr*l as a model lipase is twofold. CLECs of *Cr*l for which the lid was fixed in the open or closed conformation have been prepared before [17]. Furthermore, even when the lid of *Cr*l is not completely opened small substrate molecules can enter the active site and react. For most lipases, activation requires not only the opening of the lid but also the formation of the oxyanion hole [6]. *Cr*l is somewhat different in this respect. Comparing the open and closed crystal structure, it was concluded that the oxyanion-stabilizing residues orient correctly in both conformations [4].

The ideal substrate for the investigations of the effect of lid conformation on enantioselectivity should meet with several criteria: (1) *Cr*l should display reasonable enantioselectivity towards the substrate, (2) chiral analysis of ester and alcohol must be available or alternatively both enantiomers of the substrate, and (3) the substrate should to a reasonable extent dissolve in water. A similar experiment as was carried out with PPL and *Ca*lB could not be repeated with *Cr*l because this enzyme was nonselective

towards the hydrolysis of glycidyl butyrate. It proved to be difficult to find an ester that met all the criteria mentioned above. Substrates such as chloropropionate esters or methyl mandelate were rejected either because of low enantioselectivity or low reactivity. We then turned our attention to esters that were poorly soluble in water but which were hydrolyzed by *Cr*l with reasonable *E*-values.

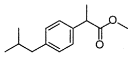
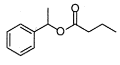
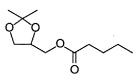
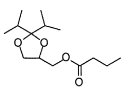
We measured the enantioselectivity of four different preparations of *Cr*l towards the hydrolysis of several esters in water. The four preparations are: crude *Cr*l as obtained from commercial suppliers, purified *Cr*l as used for the production of the CLECs, *Cr*l-CLECs in the open form, and *Cr*l-CLECs in the closed conformation. The results are depicted in Table 1. All esters that were used are poorly soluble in water and throughout the experiment a biphasic water/substrate system is present.

The commercial preparation of *Cr*l (denoted here as crude *Cr*l) is a mixture of several lipase isoenzymes with different biochemical properties and substrate specificity [35,36]. The crude *Cr*l preparation has been fractionated into two isoenzymes, denoted as *Cr*l-A and *Cr*l-B, and it was shown that these isoenzymes display different enantioselectivities [37,38]. The purified *Cr*l most likely resembles *Cr*l-B [17]. As is observed in Table 1 and by Lundell et al. [38], both higher and lower *E*-values have been found for *Cr*l-B compared to the crude preparation.

The effect of lid opening on the enantioselectivity depends on the activation energy of lid opening steps relative to the activation energy of the chemical reaction steps, as was described in the theoretical section. Upon considering the opening of the lid, a nondiscriminating reaction step (e.g., $\Delta G_{A,R} = \Delta G_{A,S}$, Eq. 6C), the activity and enantioselectivity of the closed *Cr*l-CLEC can only be equal or lower compared to the open *Cr*l-CLEC. For practical reasons, precise measurements of the reaction rates of the open and closed CLECs was difficult. However, the activity of the open *Cr*l-CLEC was higher than that of the closed *Cr*l-CLEC for all substrates (data not shown), a similar result as was found by Lalonde et al. [17] for the hydrolysis of ketoprofen chloroethyl ester. These researchers also observed that the enantioselectivity for the closed *Cr*l-CLEC compared to the open *Cr*l-CLEC was lower, as would be expected from the hypotheses presented in the theoretical section. In the case of ibuprofen methyl ester, we also observed a lower enantioselectivity for the closed CLECs. However, for the other substrates, the *E*-values for the closed *Cr*l-CLECs are higher than those measured for the open *Cr*l-CLECs. Similar results were obtained by Holmquist et al. [39]. Upon modification of the lid covering the active sites of lipases from *Rhizomucor miehei* and *H. lanuginosa*, both an increase and decrease in *E*-values was observed depending on the substrate used.

During the course of the reactions presented in Table 1, a biphasic water/substrate system is present. Purified *Cr*l is expected to be adsorbed onto the interface in the open conformation.

Table 1
Enantioselectivity of four different *C. rugosa* lipase preparations in the hydrolysis reactions of various esters in an aqueous solution See Materials and Methods for experimental details. The crude lipase is a preparation obtained from commercial suppliers. The purified lipase is the same preparation as used for the production of the CLECs.

| | T (°C) | Crude *Cr*l | Purified *Cr*l | *Cr*l-CLEC open | *Cr*l-CLEC closed |
|---|---|---|---|---|---|
|  | 39 | 160 [a] (-13.16) [b] | 37 (-9.37) | 186 (-13.56) | 142 (-12.86) |
|  | 35 | 8 (-5.32) | 14 (-6.76) | 3 (-2.81) | 14 (-6.76) |
|  | 30 | 4.4 (-3.73) | 5.9 (-4.47) | 1.6 (-1.18) | 2.5 (-2.31) |
|  | 30 | 1.3 (-0.66) | 4 (-3.49) | 2.1 (-1.87) | 2.9 (-2.68) |
|  | 30 | 7 (-4.90) | n.d. | 3 (-2.77) | 19 (-7.42) |

[a] *E*-value.
[b] Difference in Gibbs free energy (kJ $mol^{-1}$).

Somewhat surprisingly, the enantioselectivity of purified *Cr*l and the open *Cr*l-CLECs are not equal. A possible explanation for the different enantioselectivities might come from the fact that for the purified *Cr*l, the lid is opened through an interaction with the substrate, while in the open *Cr*l-CLEC, the lid is always open. As stated earlier, the displacement of the lid or differences in diffusion rates might raise the activation barriers leading to the Michaelis complexes. Compared to the closed *Cr*l-CLEC, the open *Cr*l-CLEC reflects the change in the first activation barrier because of different diffusion rates of the substrate. The purified *Cr*l reflects the change in the first activation barrier because of different diffusion rate and/or the conformational rearrangement of the lid. These changes in the activation barrier for the formation of the Michaelis–Menten complex do not have to be equal for the open *Cr*l-CLEC and the purified *Cr*l, resulting in differences in enantioselectivity.

In conclusion, there is a twofold effect of the lid opening. Firstly, the activation barrier leading to Michaelis–Menten complex can be affected. Secondly, lid opening induces small changes in the active site structure influencing the enantioselectivity. This idea is strengthened by observations made by Peters et al. [40] who found that substitution of the active site serine in *H. lanuginosa* lipase by alanine influenced the dynamic properties of the lid covering the active site. Furthermore, comparison of the open and closed structures of *Cr*l reveals differences in the orientations of the side chains in the active site [4]. Changes in the active site are thus coupled to changes in the opening and closing of the lid. Whether subsequently the *E*-values increase or decrease depends, among others, on the substrate used.

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