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Lactone Size Dependent Reactivity in *Candida Antarctica* Lipase B: A Molecular Dynamics and Docking Study

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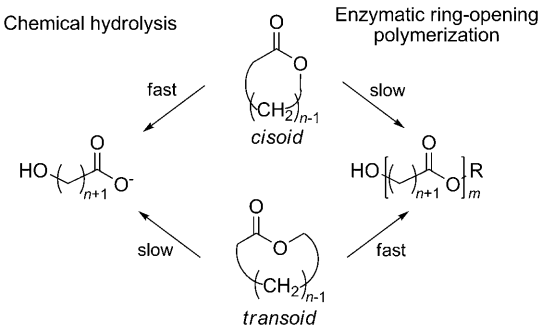
In the past decades, enzymes have emerged as potent catalysts for the preparation of well-defined functional polymers.[1,2] Lipases, for example, readily polymerize a variety of (functional) monomers and lactones.[2] Interestingly, macrolides (lactones with ring size $\geq$12 atoms)—which are notoriously difficult to polymerize chemically—can be readily converted into polyesters of high molecular weight by lipase catalysis.[3] The lipase-catalyzed ring-opening polymerization of lactones of varying ring size was systematically studied by our lab and others.[4,5] Polymerization rate differences of approximately one order of magnitude were found between small and large lactones using *Candida antarctica* lipase B (CALB; E.C. 3.1.1.3) as a catalyst (Scheme 1, right).[4] In contrast, alkaline hydrolysis of the same lactones showed an extremely large reactivity difference of four orders of magnitude with the fastest reaction rates observed for the small lactones (Scheme 1, left).[6] The reaction rates for chemical ring opening polymerization of lactones directly follow the reaction rates as found for the alkaline hydrolysis.[5,7] The ring strain caused by the *cisoid* ester bond in combination with a low activation enthalpy for ring opening is responsible for the high chemical reactivity of the small lactones δ-valerolactone (VL) and ε-caprolactone (CL).[8]



Scheme 1. Overview of reactivity differences for *cisoid* ($n=3$, 4) and *transoid* ($n\geq5$) lactones in alkaline hydrolysis (left) and enzyme catalyzed ring opening polymerization (right).

The considerable difference in chemical and enzymatic reactivity for the small, *cisoid* lactones is intriguing and poorly understood to date. Recently, we proposed that the low reactivity of the small lactones compared to the macrolides in CALB-catalyzed reactions may be due to unfavorable positioning of the *cisoid* ester bond in the enzyme active site; this unfavorable positioning would slow down the rate-determining step in the catalytic cycle (Supporting Information, Figure S1).[9]

We here provide support for this proposal by combining molecular dynamics (MD) simulations, which might reveal possible differences in reaction intermediates, with automatic docking to study substrate binding. The combination of docking and MD studies is extensively used in the field of drug discovery for inhibitor design,[10] but has to our knowledge not been used to elucidate reactivity differences of monomers in enzyme catalyzed polymer synthesis. On the other hand, MD calculations with manually positioned, covalently bound substrates have been used to explain reactivity differences in lipase catalyzed reactions.[11,12] We also experimentally determined the substrate specificity (k_{cat}/K_M) values for CALB for a series of lactones. We selected lactones encompassing a *cisoid* (VL, CL) and predominantly (heptanolactone, HL) or fully *transoid* (dodecanolactone and pentadecanolactone, DDL and PDL, respectively) ester bond conformation.

Accurate data for the substrate specificity (k_{cat}/K_M) values of CALB for the selected lactones VL, CL, HL, DDL, and PDL can be determined directly from competition experiments.[13] Such experiments eliminate differences in reaction conditions and thus directly show intrinsic reactivity differences between multiple substrates. The substrate specificity values of CALB were determined for the various lactones by one-pot ring opening competition experiments using an excess of 1-propanol as nucleophile (SI-S2). Two separate one-pot competition experiments were performed as rate differences between the fastest (PDL) and slowest (CL) lactones were too large to determine accurately from a single experiment (Table 1 and Figure S1). The obtained specificity data are in general agreement with the previously determined kinetic data from the CALB-catalyzed ring opening polymerization.[4] As expected, the substrate specificity (k_{cat}/K_M) values of CALB are larger for the *transoid* lactones (HL, DDL and PDL) than for the *cisoid* VL and CL (Table 1)[14]

In fact, a 16-fold difference was observed for lactones with either a completely *transoid* (PDL) or *cisoid* (CL and VL) ester conformation, which is notably smaller than 9000-fold difference between VL and PDL found for the alkaline hydrolysis (Table 1).

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Table 1. Relative reaction rates for the CALB-catalyzed ring opening of lactones in toluene at 45 °C in two separate competition experiments (A/B) and the chemical ring opening in dioxane/water. Substrate specificity values are normalized to DDL.

Substrate, main conformation[a]	Exp[b]	$\frac{(k_{cat}/K_M)_{lactone}}{(k_{cat}/K_M)_{DDL}}$ [c]	$10^4 k_2$, [L s^{-1} mol^{-1}][d]	$\frac{(k_2)_{lactone}}{(k_2)_{DDL}}$
VL (C)	A	0.61 ± 0.01	55 000	9170
CL (C)	A	0.57 ± 0.02	2550	425
HL (C + T)	B	1.8 ± 0.01	3530	588
DDL (T)	A/B	1.0	6.0	1
PDL (T)	B	9.8 ± 0.34	6.5	1.1

[a] (C): *cisoid* ester conformation, (T): *transoid* ester conformation. [b] Two sets of experiments (A/B) with three lactones each were performed: V_{tot} = 8.0 mL, solvent toluene, [lactone] = 0.10 mol L^{-1} per lactone, 0.03 mol L^{-1} 1,3,5-tri-*tert*-butylbenzene (internal standard), 0.60 mol L^{-1} propan-1-ol, 25 mg dry Novozym 435, T = 45 °C. [c] Data obtained by linear regression of initial ring opening rates in competition experiments. [d] Literature data of the second order rate constants for the chemical ring opening of lactones in dioxane/water (60:40 vol%) extrapolated to 0 °C.[6]

Reactivity differences between the small, *cisoid* and large, *transoid* lactones should originate from the rate-determining step—that is, the formation of the acyl-enzyme intermediate in the catalytic mechanism.[7,15] To form the acyl-enzyme complex, nucleophilic attack of the serine 105 residue takes place onto the carbonyl group of the bound substrate. After attack, a tetrahedral intermediate is formed and the distinction between a *cisoid* and *transoid* ester conformation no longer exists. Therefore, reactivity differences should not arise after tetrahedral intermediate formation.

Docking studies were performed to identify the energetically most favorable enzyme-substrate complexes for the various lactones (Details in SI-S3) by using the Lamarckian genetic algorithm (LGA) in Autodock 4.0.[16] As conformational sampling of cyclic structures is impossible in Autodock due to program limitations,[17] all known low-energy conformations of VL, CL and HL[18] were docked as rigid entities into an energy-minimized CALB structure (Table 2). All conceivable conformations were taken into account, as the most stable lactone conformation is not necessarily the catalytically important one. Due to the large number of possible conformations of the macrolides,[6] DDL and PDL had to be omitted from the docking studies.[19]

Each lactone conformation was docked 256 times into the active site and only docking positions with a root mean square deviation (RMSD) of ≤0.50 Å from the solution with lowest energy were taken into account during analysis to avoid the influence of outliers. In most cases, the number of lactone conformations in this cluster of solutions was close to 256 (Table 2). The LGA showed good convergence towards a single solution. Docking of HL conformations 2 and 3, gave a large, second cluster of solutions with almost identical energy (SI-S6.2). Docking results for VL, CL, and HL were first analyzed based on interaction energies (E_{inter}) between the lactone and the enzyme active site (Table 2). For an estimation of the total free energy of the docked state (E_{total}), the ground-state energy of the lactone conformation (E_{intra}) has to be added to the observed docking energy.[20] Here the internal energy was set to 0 kcal mol^{-1} for the lactone conformation with lowest ground state energy. Observed docking energies (E_{inter}) of the investigated lactone conformations with the CALB active site were not significantly different. All values were between −4.3 and −3.3 kcal mol^{-1} (Table 2), showing no preferential interaction of any of the lactone conformations with the enzyme active site. Therefore, E_{total} was largely determined by the ground state energy of the lactone (E_{intra}) (Table 2). All possible states are in thermodynamic equilibrium with each other and their relative importance is determined by the Boltzmann distribution. Both VL, the first CL, and all HL conformations need to be considered as catalytically important since their energy was within 2.7 kcal mol^{-1} from the lowest state, corresponding to 1% existence at 298 K. Secondly, the docking results were analyzed based on geometrical constraints. To form a productively bound first tetrahedral intermediate from the Michaelis–Menten complex, the position of the substrate in the active site needs to be such that: 1) nucleophilic attack of serine 105 onto the lactone carbonyl carbon atom can take place easily, and 2) all catalytically critical hydrogen bonds (Figure 2 A) can be formed. If all these conditions

Table 2. Overview of docking results for rigid lactone conformations into a energy minimized CALB structure. Only docked positions with a RMSD value ≤ 0.50 Å from the best solution were taken into account during analysis.

Conformation[a]	E_{intra}[b] [kcal mol^{-1}]	Dihedral angle[c]	Struct.[d]	E_{inter}[e] [kcal mol^{-1}]	E_{total}[f] [kcal mol^{-1}]	Docking score
VL 1	0.0	3/C	220	−3.9	−3.9	0.00
VL 2	1.2	−17/C	182	−3.9	−2.6	0.00
CL 1	0.0	8/C	226	−4.0	−4.0	0.00
CL 2	2.6	3/C	256	−3.4	−0.8	0.00
CL 3	4.3	16/C	256	−4.3	0.0	0.00
CL 4	5.0	108/T	249	−3.3	1.7	0.00
HL 1	0.0	−137/T	255	−3.5	−3.5	0.32
HL 2	0.8	−138/T	129	−3.4	−2.6	0.49
HL 3	0.9	128/T	150	−3.6	−2.7	0.06
HL 4	1.3	10/C	256	−3.5	−2.2	0.00

[a] Lactone conformations were numbered according to increasing ground state energy (E_{intra}). [b] Relative energy of the lactone ground state conformation after MM2 energy minimization. [c] Dihedral angle of C_ω-O_{alkyl}-$C_{carbonyl}$-C_1 as an indication for the degree in which this conformation is *cisoid* (C) or *transoid* (T). [d] Number of docked substrate positions out of 256 docking runs taken into account during analysis. RMSD values are ≤0.5 Å with respect to the best solution. [e] Average docking energy as determined by Autodock 4.0. [f] Average total energies: $E_{total} = E_{intra} + E_{inter}$.

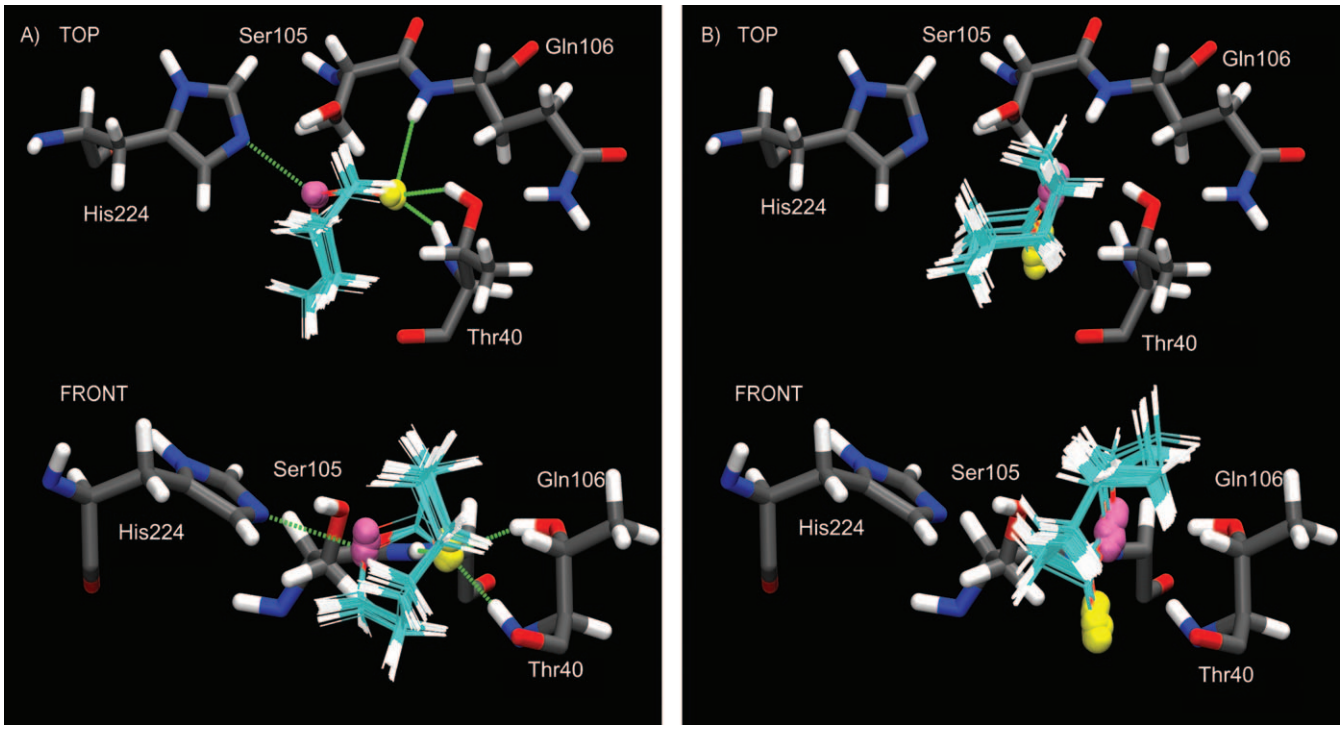

Figure 1. Docking results for: A) productively bound *transoid* HL conformation 1 (average docking score = 0.32, 255 structures); B) unproductively bound *cisoid* HL conformation 4 (average docking score = 0.00, 256 structures). The substrate carbonyl and alkyl oxygen atoms are indicated with a yellow and purple sphere, respectively. Dotted lines for the present hydrogen bond interactions were drawn to guide the eye.

are fulfilled, one can speak of an ideal near attack conformation (NAC).[21]

To compare the correctness of a docked position with respect to the ideal NAC, a normalized, geometry based scoring function was set up using the aforementioned criteria (SI-S3.1.6). It was determined that the higher the docking score (Table 2), the closer the resemblance with the ideal NAC. A graphical representation of all docked positions is given in SI-S6.2.

The docking score of 0.0 (Table 2) found for all VL and CL conformations is indicative of unfavorable positioning of these lactones in the enzyme active site. Generally, hydrogen bonding of the substrate carbonyl oxygen in the oxyanion hole was present and attack of the serine 105 nucleophile onto the substrate carbonyl group was possible. However, the alkyl oxygen of these substrates was positioned incorrectly with respect to the histidine 224 residue. Due to the lack of the catalytically active hydrogen bonds, successful formation of the acyl enzyme intermediate was not expected. On the other hand, two out of three *transoid* HL conformations (HL 1, Figure 1 A, and HL 2) showed higher docking scores (0.32 and 0.49, respectively) indicating highly favorable positioning in the active site. Additionally, the average docking score of the second cluster of solutions for HL conformation 2 (data shown in SI-6.2.1.8) was high as well (0.21, $E_{inter} = -3.4$ kcal mol^{-1}). The more *cisoid* HL conformation (HL 4, Figure 1 B) fitted poorly into the active site.

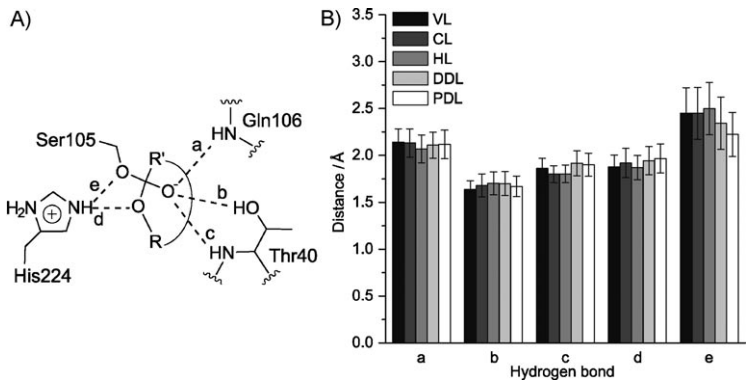

Figure 2. A) Schematic representation of the first tetrahedral intermediate showing the numbering of the catalytically required hydrogen bond towards the oxyanion hole (bonds a–c) and histidine 224 (bonds d and e); B) Overview of average H-bond distances from the 1 ns MD simulations on the first tetrahedral intermediate for various substrates (error bars show 1 standard deviation).

MD simulations were performed to demonstrate that an identical, productively bound first tetrahedral intermediate structure for VL, CL, HL, DDL and PDL exists, thereby excluding reactivity differences from a later stage (SI-S5). Tetrahedral intermediates of all five lactones were manually positioned in the active site of an energy minimized CALB structure such that all catalytically required hydrogen bonds—the three hydrogen bonds in the oxyanion hole and the two hydrogen bonds towards the histidine 224 residue—were present (Figure 2 A).[12] Ring conformations of the substrate were adapted to rule out steric clashes with the enzyme or highly unfavorable dihedral angles in the substrate backbone. After energy mini-

mization, the structures were subjected to 1 ns MD simulations. As the MD simulations could not provide reliable energies, the geometry of the tetrahedral intermediates was analyzed for all simulations from snapshots taken at 200 fs intervals.

The lengths of the five critical hydrogen bonds (Figure 2A) were identical for all simulated lactone tetrahedral intermediates. In all cases, the oxyanion was kept in place tightly by three strong hydrogen bonds, while the hydrogen bonds towards the histidine 224 residue showed slightly more variation (Figure 2B). The conformational stability of the tetrahedral intermediates was monitored by evaluating the dihedral angles around the substrate backbone. Most flexibility was observed for the backbone of the large lactones (DDL and PDL), while the tetrahedral carbon center was highly rigid in all cases on the time scale of the simulations. The MD simulations showed the existence of a similar and productively bound tetrahedral intermediate in the CALB active site for all lactones. Large reactivity differences can thus be excluded to arise from this stage and should originate from an earlier point in the catalytic cycle as suggested by the docking studies.

The unfavourable distribution between unproductive and productive binding of the substrate results in low enzymatic reactivity for the small, *cisoid* lactones as suggested in our hypothesis. The docking studies showed that the preferred binding modes for all *cisoid* lactone conformations were unproductively bound E•2 complex, zero docking score), whereas the *transoid* conformations were productively bound E•S complex, non-zero docking score). A schematic representation of this situation is given by the energy diagrams given in Figure 3, in which the zero energy level is arbitrarily set to the sum of the energy of the free enzyme and the ground state of the lactone conformation.

The situation for the *cisoid* lactones VL and CL is depicted in Figure 3A: as the modeling shows we could find only unproductive binding of these lactones (E•2 complexes). Since *cisoid* lactones do show reactivity in CALB, a correctly bound E•S complex that is higher in energy must exist. Thus, a direct competition exists between correctly (E•S) and incorrectly (E•2) bound enzyme-substrate complexes, corresponding to competitive inhibition of the enzyme by unproductively bound substrate. This agrees well with the experimentally measured specificity values that are significantly lower (0.6 relative to DDL) for these substrates than expected from their intrinsically high chemical reactivity. Figure 3B shows the situation for the *transoid* HL conformations 1 and 2: here the preferred binding mode is productive. The bound substrate can readily be converted into a catalytically productive first tetrahedral intermediate and competitive inhibition by the substrate only plays a minor role. The good acceptance of this *transoid* lactone is directly observed from the high experimentally determined specificity value of 1.8 relative to DDL. The macrolides DDL and PDL possess a large number of highly flexible, preferentially *transoid* lactone conformations that can be quickly interconverted. Since the active site of the enzyme has been engineered for low-energy *transoid* ester conformations, these compounds can readily react as directly observed from the high specificity value of PDL (9.8 compared to DDL). Competitive inhibition by wrongly bound substrate is not expected to have a major impact on the reactivity in this case.

In conclusion, with the powerful combination of docking and MD studies we acquired a better understanding of apparently low reactivity of the *cisoid* lactones VL and CL in CALB-catalyzed reactions. For these *cisoid* lactones, docking studies revealed competitive inhibition by wrongly bound substrate. An identical catalytically productive first tetrahedral intermediate was observed by MD for all investigated lactones, excluding large reactivity differences from this state in the catalytic pathway. Hence, the reactivity of lactones in CALB-catalyzed reactions is governed by the *cisoid* or *transoid* nature of the ester bond and can therefore not entirely be attributed to hydrophobicity differences as suggested in literature. Moreover, the docking studies showed similar energy values for the interaction between substrate and enzyme of all investigated substrates. Currently, we apply this methodology to understand the remarkable switch in enantioselectivity for the CALB-catalyzed ring opening of ω-methylated lactones when going from a 7-membered (*S*-selective) to an eight-membered (*R*-selective) ring.[9]

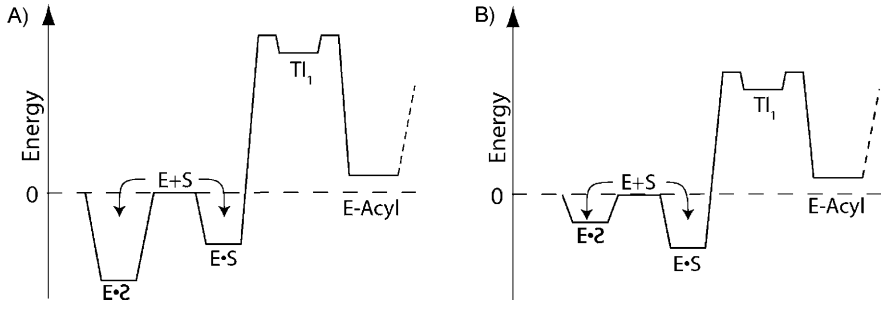

Figure 3. Schematic energy profile for the enzyme catalyzed ring opening of CL, VL and HL. A) VL and CL predominantly bind in an unproductive binding mode, which results in a low catalytic activity; B) HL shows almost only productive binding, and most enzyme molecules with bound substrate can contribute to catalysis.

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Keywords: docking · hydrolases · lactones · molecular dynamics · ring-opening polymerization

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