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# Lipase catalyzed ring-opening polymerization of benzyl malolactonate: an unusual mechanism?

Hubert Casajus<sup>1,†</sup>, Eric Dubreucq<sup>2</sup>, Sylvain Tranchimand<sup>1</sup>, Véronique Perrier<sup>2</sup>, Caroline Nugier-Chauvin<sup>1</sup>, Sandrine Cammas-Marion<sup>1,3\*</sup>

1. Univ Rennes, ENSCR, CNRS, ISCR (Institut des Sciences Chimiques de Rennes) - UMR 6226, F-35000 Rennes, France.

2. L'Institut Agro - Montpellier SupAgro, Univ Montpellier, INRA, CIRAD - UMR IATE, F-34060 Montpellier, France

3. Univ Rennes, INSERM, INRA, Institut NUMECAN (Nutrition Metabolisms and Cancer) UMR\_A 1341, UMR\_S 1241, F-35000 Rennes, France.

**KEYWORDS:** Lipase-Catalyzed Polymerization, Ring-Opening Polymerization, Mechanism, Benzyl Malolactonate, CpLip2.

**ABSTRACT:** The use of safe natural catalyst such as enzymes for ring opening polymerization (ROP) of  $\beta$ -substituted  $\beta$ -lactones such as benzyl malolactonate (MLABe) is an important objective considering the biomedical applications of the resulting (co)polymers. However, the preparation of well-defined polymeric materials using such systems requires an understanding of enzyme-substrate interactions. In this context, we investigated the mechanism of lipase-catalyzed ROP of MLABe, because it appears that it is probably not the same as the one widely described for other lactones such  $\epsilon$ -caprolactone, propiolactone and lactide. Enzymatic-catalyzed ROPs of MLABe in presence of the lipase/acyltransferase CpLip2 and its serine knock-out (serine KO) mutant (CpLip2\_180A) have led to poly(benzyl malate) (PMLABe) terminated by a monobenzyl fumarate group with monomer conversion higher than 70% and weight average molar mass of about 3,600 g/mol ( $\bar{M}_w = 1.42$ ). On the other hand, only less than 7% of MLABe conversion and no polymer formation were observed when the polymerization reaction was conducted in presence of inactivated CpLip2 (heated at 100°C). Moreover, the ROP of MLABe in presence of imidazole, a synthetic mimic of the catalytic histidine, led to a PMLABe terminated by a monobenzyl fumarate group. On the contrary, neither the enzymatic-catalyzed ROP of benzyl dimethylmalolactonate (diMeMLABe), a MLABe with two methyl groups instead of the two "acidic" protons on the lactone's ring, in presence of CpLip2 and CpLip2\_180A nor its chemical ROP in presence of imidazole were successful. Together, all these results suggested that the lipase-catalyzed polymerization of malolactonates occurred through the abstraction of one of the two "acidic" protons of the lactone's ring by the histidine of the catalytic triad leading to the corresponding monobenzyl fumarate responsible for the polymerization of the remaining monomer. Finally, molecular modeling of the positioning of the monomer into the catalytic site of the CpLip2 and DFT quantum-chemical calculations highlighted an interaction of (R) and (S)-MLABe with the

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3 catalytic histidine of the enzyme preferentially to serine, in the form of a strong hydrogen bond  
4 with one of the “acidic” protons of MLABe, thus supporting the important role of the catalytic  
5 histidine in the polymerization of such cyclic lactones.  
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8 INTRODUCTION. Aliphatic polyesters belong to a polymer family with outstanding  
9 biocompatibility and biodegradability properties particularly appreciated when such polymeric  
10 materials are designed for applications in the biomedical field. Such polymers, being in contact  
11 with human bodies, must follow very strict specifications: they must be non-toxic, non-  
12 immunogenic, non-carcinogenic and non-thrombogenic<sup>1</sup>. Moreover, their biodegradation must  
13 lead to low molar mass molecules, which are respectful of the previously cited specifications  
14 and/or eliminated/excreted from the body. These polyesters are usually synthesized by  
15 polymerization reactions using chemical catalysts through either the direct polycondensation of a  
16 diacid with a diol, for example, or ring opening polymerization (ROP) of lactones or lactide<sup>2</sup>. The  
17 ROP technique allows the obtention of polymers having high and controlled molar masses as well  
18 as defined structures<sup>3</sup>. On the other hand, the ROP reactions in presence of cationic or anionic  
19 initiators often requires the addition of organometallic catalysts whose presence even as traces  
20 might lead to unwanted toxicity<sup>4</sup>.

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26 Consequently, alternative synthetic methods are developed to overcome this major drawback,  
27 more particularly when such (co)polymers are synthesized for human health applications.  
28 Therefore, the use of biological catalysts such as lipases is of growing interest. Indeed, enzymes  
29 allow polymerization reactions under mild conditions (temperature, pH and pressures), avoid the  
30 use of organic solvents and most importantly are generally not toxic<sup>5</sup>. The synthesis of polyesters  
31 through ROP of their corresponding cyclic monomers in the presence of lipases was described for  
32 the first time in 1993 by Kobayashi *et al.*<sup>6</sup> and Kohn *et al.*<sup>7</sup>, who synthesized a poly( $\epsilon$ -caprolactone)  
33 thanks to the ROP of  $\epsilon$ -caprolactone in the presence of *Pseudomonas fluorescens* lipase and  
34 porcine pancreatic lipase (PPL), respectively, as catalysts. These preliminary results have opened  
35 the way towards enzymatic-catalyzed polymerization reactions leading to “greener” and more  
36 biocompatible polyesters such as poly( $\epsilon$ -caprolactone)<sup>8,9</sup>.

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40 In this context, we have evaluated the possibilities of using lipase-based ROP of a special family  
41 of lactones, *i.e.* the  $\beta$ -substituted  $\beta$ -lactones also called alkyl malolactonates, to synthesize  
42 poly(malic acid) and its derivatives<sup>10,11</sup>. In a first step, we have established, through a Design of  
43 Experiment (DoE) procedure, the optimal experimental conditions to reproducibly synthesize  
44 poly(benzyl malate) with weight average molar masses varying from 5,000 to 13,000 g/mol,  
45 dispersities between 1.2 and 1.6 (depending on the conditions used for the polymerization) and  
46 hydrolysis rates lower than 5%, using PPL as catalyst<sup>10</sup>. PMLABe obtained through lipase-  
47 catalyzed ROP of MLABe were then formulated and the resulting nanoparticles were involved in  
48 several *in vitro* assays whose results have highlighted the influence of the polymer synthesis  
49 method on cell viability in particular<sup>11</sup>. Moreover, physico-chemical characteristics of PMLABe  
50 obtained through lipase-catalyzed ROP of racemic and optically active MLABe led us to question  
51 the mechanism of this enzymatic polymerization<sup>11</sup>. Indeed, we have previously shown that the  
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lipase-catalyzed ROP of R-MLABe and S-MLABe occurred through an O-alkyl bound cleavage with an inversion of configuration of the asymmetric carbon of the lactone's ring, as in the case of anionic ROP of MLABe for which the initiator attacks on the asymmetric carbon of the lactone's ring leading to a ring opening through an O-alkyl bound cleavage and an inversion of configuration of the asymmetric carbon<sup>11</sup>. On the contrary, the mechanism described, and well-accepted, for the lipase-catalyzed ROP of unsubstituted and  $\alpha,\beta$ -alkyl substituted lactones ( $\epsilon$ -caprolactone, butyrolactone, lactide, etc.) involved an O-acyl bound cleavage as the result of the attack of the activated catalytic serine onto the carbonyl of the lactone and the formation of an enzyme-monomer intermediate (canonical mechanism)<sup>12-18</sup>. Therefore, it seems that the lipase-catalyzed ROP of MLABe does not follow the canonical mechanism described for other lactones and lactides, but rather the one described for its anionic ROP<sup>11</sup>.

Consequently, we have realized several experiments involving the use of the lipase/acyltransferase CpLip2 enzyme (a lipase with high affinity for nucleophiles other than water<sup>19</sup>), a modified CpLip2 which catalytic serine was mutated into an alanine residue (serine-KO), an inactivated CpLip2 obtained by heating at 100°C, a MLABe derivative on which the two "acidic" protons of the lactone's ring have been changed by two methyl groups, synthetic mimics of the three amino acids of the lipase's catalytic triad and several molecular modeling experiments as well as DFT quantum-chemical calculations. The obtained results described in the present paper allowed us to propose a mechanism for the lipase-catalyzed ROP of malolactonates.

**MATERIALS AND METHODS.** All chemicals were used as received. Porcine pancreatic lipase (PPL) was purchased from Sigma-Aldrich Co. Dimethyl benzyl malolactonate (diMeMLABe) was a gift from Kymia Nova (Saclay, France). Benzyl malolactonate (MLABe) was synthesized according to literature<sup>20</sup>.

**Nuclear Magnetic Resonance spectroscopy:** The standard temperature was adjusted to 298K. NMR spectra were recorded on a Bruker Avance III 400 spectrometer operating at 400.13 MHz for <sup>1</sup>H, equipped with a BBFO probe with a Z-gradient coil and a GREAT 1/10 gradient unit. The zg30 Bruker pulse program was used for 1D <sup>1</sup>H NMR, with a TD of 64 k, a relaxation delay d1 = 2 s and 8 scans. The spectrum width was set to 18 ppm. Fourier transform of the acquired FID was performed without any apodization in most cases.

**Size Exclusion Chromatography (SEC):** Weight average molar mass ( $M_w$ ) and dispersity ( $\mathcal{D} = M_w/M_n$ ) values were measured by SEC in THF at 40 °C (flow rate = 1.0 mL/min) on a GPC2502 Viscotek apparatus equipped with a refractive index detector Viscotek VE 3580 RI, a guard column Viscotek TGuard, Org 10x4.6mm, a LT5000L gel column (for samples soluble in organic medium) 300 x 7.8 mm and a GPC/SEC OmniSEC Software. The polymer samples were dissolved in THF (2 mg/mL). All elution curves were calibrated with polystyrene standards (weight average molar masses: 1,240; 2,630; 6,220; 13,700; 30,100 and 78,200 g/mol).

**Preparation of the CpLip2 and CpLip2\_S180A (serine KO) lipases:** Recombinant lipase/acyltransferase CpLip2 from *Candida parapsilosis* CBS 604 was produced by heterologous expression in *Komagataella phaffii* (formerly named *Pichia pastoris*) as described previously<sup>21</sup>.

The serine KO mutant CpLip2\_S180A was obtained by site-directed mutagenesis of serine S180 into an alanine residue. The mutation was performed by Life Technologies (Regensburg, Germany) on the gene of CpLip2 in fusion with the signal peptide of the alpha mating factor of *Saccharomyces cerevisiae* within the pPICZ $\alpha$ B plasmid, that was used to transform *K. phaffii* according to the work already described elsewhere<sup>22</sup>. The protein was produced in a 1L bioreactor and purified as described earlier<sup>22</sup>.

**Enzymatic polymerization of MLABe:** The different PMLABe were synthesized as described previously [10] by ring-opening polymerization of the MLABe in presence of the enzyme (PPL, CpLip2 or CpLip2\_S180A) in Tris-HCl buffer pH7. Experiments were realized in a 24-multi reactor Büchi Syncore Line. In a 30 mL-tube, enzyme (8.4 mg) was mixed with pure MLABe (336 mg) and Tris-HCl buffer (125  $\mu$ L, pH7, 105 mM). The mixture was stirred at 390 rpm and 60 °C. After 72 h, reaction was stopped by the addition of 3 mL of THF and 3 mL of chloroform. The mixture was then transferred into a separating funnel. Separations were realized by adding 15 mL of distilled water and 15 mL of chloroform. Organic phases were dried over MgSO<sub>4</sub> and filtered. Finally, solvents were evaporated under reduced pressure. The crude polymers were analyzed by <sup>1</sup>H NMR to determine monomer's conversion (from 70 to 90%) and hydrolysis rate (below 3%). Then, they were dissolved in chloroform and precipitated in a large excess of cold diethyl ether to eliminate unreacted monomer and impurities with a recovering yield of 70 to 90%. After elimination of the supernatant, polymers were dried under vacuum and analyzed by <sup>1</sup>H NMR and SEC.

**Polymerization of MLABe in presence of the synthetic catalytic triad:**

- *Synthesis of imidazolium trifluoroacetate salt*<sup>23</sup>: In a round-bottomed flask, 505 mg (7.42 mmol, 1 eq.) of imidazole were dissolved in 50 mL of chloroform. This solution was cooled to 0°C, then, 1.25 mL (16.32 mmol, 2.2 eq.) of trifluoroacetic acid (TFA) was added dropwise. After 24 h stirring, the precipitated salt was filtered, washed with chloroform and dried overnight at 80°C. 578 mg of imidazolium salt were obtained (yield = 43 %). Melting point: P<sub>m, ref</sub> = 127.2 °C [20], P<sub>m, measured</sub> = 128 °C.

- *Polymerization reactions*: Experiments were realized in a 24-multi reactor Büchi Syncore Line. In a 30 mL-tube, imidazolium salt and/or imidazole, and/or TFA, and/or benzyl alcohol (composition depending of the entry as shown by data gathered in Table 1) were mixed with MLABe (200 mg, 0.97 mmol, 1eq.).

**Table 1.** Composition of the reactive mixture.

Entry	Imidazolium salt	Benzyl alcohol	TFA	Imidazole
1	35.3mg/0.194mmol/0.2eq.	4 $\mu$ L/0.039mmol/0.04eq.	0	0
2	35.3mg/0.194mmol/0.2eq.	0	0	0
3	0	4 $\mu$ L/0.039mmol/0.04eq.	0	0
4	0	0	14.8 $\mu$ L/0.194mmol/0.2eq.	0
5	0	0	0	13.2mg/0.194mmol/0.2eq.
6	0	0	0	113 $\mu$ L/0.173 $\mu$ mol/1.07*10 <sup>-4</sup> eq.

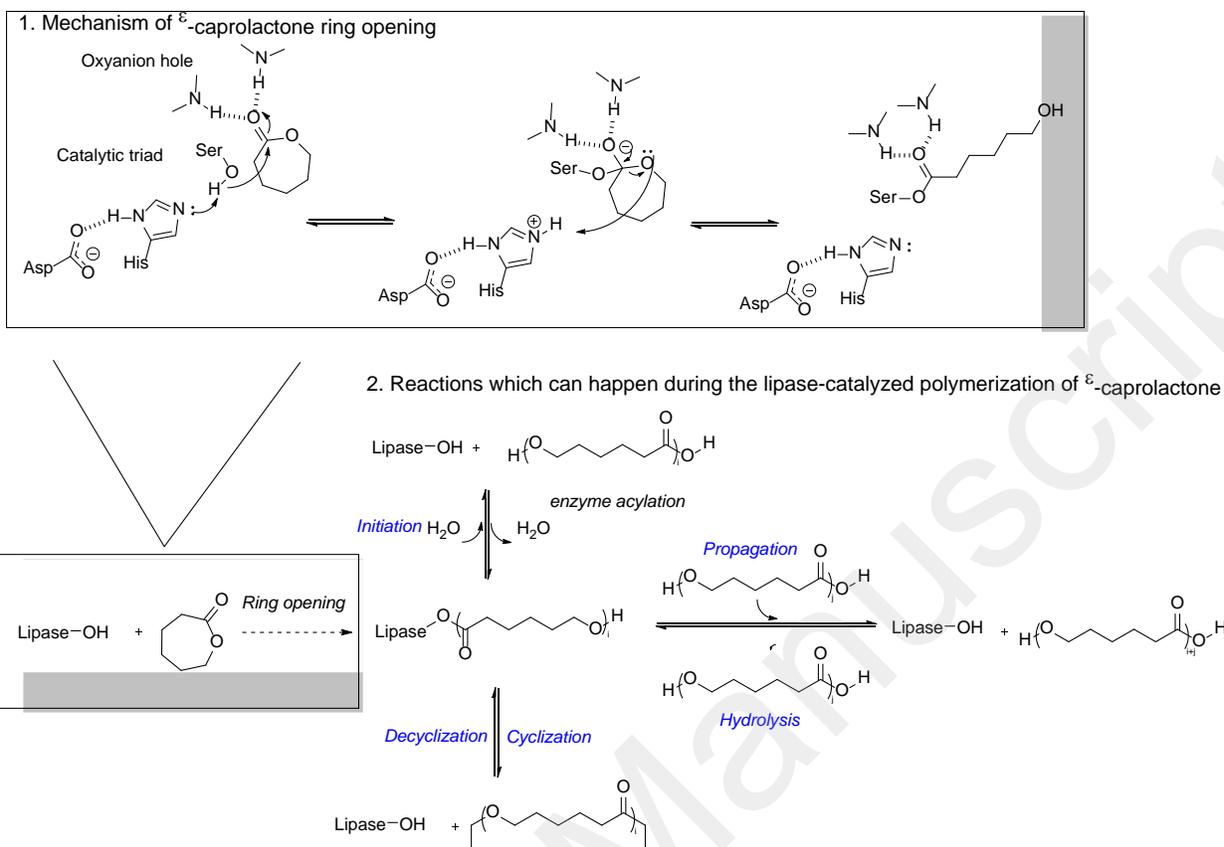
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3 In parallel, ROP of MLABe ( $m = 331$  mg,  $1.6 \cdot 10^{-3}$  mol) in presence of imidazole ( $n = 1.73 \cdot 10^{-7}$   
4 mol, 113  $\mu$ L of a stock solution at a concentration of 0.1g/L) in a catalytic amount, *i.e.* the same  
5 imidazole mole number than the one corresponding to 8.3 mg of PPL (Molar mass = 48,000 g/mol,  
6  $n = 1.73 \cdot 10^{-7}$  mol), was conducted in 172  $\mu$ L of Tris-HCl buffer at pH7. After 72 h at 60 °C and  
7 390 rpm, reactions were stopped by the adding 5 mL of chloroform. The mixture was then filtered  
8 and transferred in a separating funnel. Separation was realized by adding 15 mL of distilled water  
9 and 15 mL of chloroform. The aqueous phase was extracted twice by 7 mL of chloroform. Organic  
10 phases were dried over  $MgSO_4$  and filtered. Finally, chloroform was evaporated under vacuum.  
11 The crude polymers were analyzed by  $^1H$  NMR to determine monomer's conversion and  
12 hydrolysis rate. Then, they were dissolved in chloroform and precipitated in a large excess of cold  
13 diethyl ether. After elimination of the supernatant, polymers were dried under vacuum at room  
14 temperature, and characterized by SEC and  $^1H$  NMR.

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19 **Computational methods:** The 3D structure of CpLip2 lipase/acyltransferase<sup>24</sup> was refined by  
20 molecular dynamics and energy minimization in an explicit TIP3P water box as described earlier<sup>25-</sup>  
21 <sup>27</sup>. The initial geometry of R- and S-MLABe was determined by energy minimization using the  
22 density functional tight binding (DFTB) quantum chemical method with the GFN1-xTB model<sup>28</sup>  
23 and the COSMO<sup>29</sup> water solvation model implemented in the DFTB module of AMS 2019 (SCM,  
24 Amsterdam, NL). Molecular docking was performed with Autodock Vina v1.1.2<sup>30</sup> using the UCSF  
25 Chimera v1.14.1<sup>31</sup> interface. The energy of the structures of the two best poses was minimized  
26 within Chimera using the AMBER ff14SB and the AM1-SCC force fields for atomic partial charge  
27 calculations in the protein and the ligand, respectively, with the protein backbone constrained to  
28 fixed positions, allowing the selection of the conformation with the lowest estimated energy. For  
29 each ligand, sub-models restricted to amino acid residues with at least one atom closer than 5 Å  
30 from the ligand (475-480 atoms) were constructed. The broken amide bonds of the protein  
31 backbone were restored by capping with  $-CO-CH_3$  and  $-NH-CH_3$  groups on the amino and  
32 carboxyl sides, respectively, in line with the backbone of the former neighboring residues. The  
33 geometry of the sub-models was then refined using DFTB (GFN1-xTB and COSMO water implicit  
34 solvation models) for energy minimization, with the position of C and N atoms of the protein  
35 backbone constrained to fixed positions. A final optimization used DFT for energy minimization,  
36 with MLABe and all the atoms (and bound hydrogen atoms) closer than 3 Å from the ligand being  
37 free to move and the other atoms being fixed as a bloc. DFT calculations were performed using  
38 the BP86 GGA functional with D4(EEQ) dispersion correction<sup>32</sup>, the DZ double  $\zeta$  basis set with a  
39 large frozen core and the COSMO water solvation model within the ADF module of AMS. The  
40 interactions between MLABe and the protein sub-model, both defined as closed-shell molecular  
41 fragments, were then analyzed at the same level of DFT by the extended transition state–natural  
42 orbitals for chemical valence (ETS-NOCV) energy decomposition method<sup>33</sup> using ADF. Graphical  
43 representations were obtained with UCSF Chimera v1.14<sup>31</sup>.

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54 **RESULTS AND DISCUSSION.** Lipases are enzymes belonging to the family of hydrolases, class  
55 3 in the EC nomenclature (Enzyme Commission numbers). More specifically, they are serine  
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4  $\alpha/\beta$  hydrolases which have the particularity of being active in presence of an interface between an  
5 aqueous phase (water or buffer) and an organic phase (hydrophobic substrate and/or organic  
6 solvent). Present in plants, insects, animals and microorganisms, lipases play a key role in the  
7 degradation and synthesis of lipids, and catalyze, under physiological conditions, the hydrolysis  
8 of mono-, di- and triglycerides<sup>34</sup>. The Protein Data Bank (PDB) records more than 100 three-  
9 dimensional structures of lipases highlighting the following highly conserved structural elements  
10 specific to these enzymes<sup>35</sup>: *i*). The  $\alpha/\beta$  folding composed by eight  $\beta$  sheets and six  $\alpha$  helices; *ii*).  
11 The catalytic triad composed by a nucleophilic serine, an aspartate/glutamate and a histidine; *iii*).  
12 The oxyanion hole formed by two active amino acids (or more depending on the lipase) of the  
13 active site, stabilizing the enzyme/substrate reaction intermediate through hydrogen bonds  
14 between the substrate and the nitrogen of the amide function; *iv*). The lid formed by one or more  
15  $\alpha$  helices which is opened in presence of the activation interface thus allowing the positioning of  
16 the substrate into the active site; *v*). The active site, mostly composed of hydrophobic amino acids,  
17 and varying from enzyme to another thus explaining the difference in specificity observed in  
18 lipases<sup>35</sup>. The mechanism of ester functions hydrolysis, called canonical mechanism, involves the  
19 three amino acids (aspartate, histidine and serine) of the catalytic triad: briefly, the serine, activated  
20 by the aspartate and histidine, forms a covalent acyl-enzyme intermediate (acylation step) which  
21 is then hydrolyzed thanks to the attack of water molecules activated by the histidine (deacylation  
22 step)<sup>35</sup>. The structure of the acyl-enzyme intermediate has been observed for a deacylation step  
23 slower than the acylation one and, together with detailed kinetic studies, validated and confirmed  
24 the canonical mechanism<sup>35</sup>.

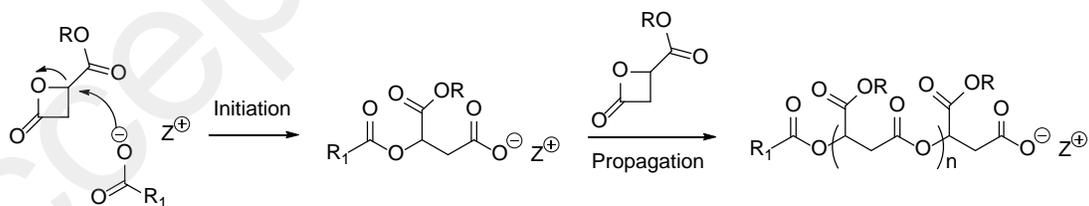
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31 Lipase-catalyzed ROP mechanism of unsubstituted lactones (such as  $\epsilon$ -caprolactone, lactide) and  
32  $\alpha/\beta$ -alkyl substituted  $\beta$ -lactones (such as butyrolactone,  $\alpha$ -methyl propiolactone) has been already  
33 described in the literature<sup>12-18</sup>. It uses the canonical mechanism as a basis, and is widely accepted  
34 by the entire scientific community (Scheme 1). In a first step, the lactone ring is opened by the  
35 lipase (Scheme 1.1): the activated serine attacks the carbonyl of the lactone, thus forming a  
36 tetrahedral intermediate, and the reformation of the carbon-oxygen double bond leads to the  
37 opening of the lactone and to the formation of an acyl-enzyme intermediate. Then, three  
38 possibilities exist for the de-acylation of the enzyme (Scheme 1.2): *i*). Initiation: the initiator (here  
39 water) attacks the enzyme-substrate complex leading to the formation of an activated open  
40 monomer. Conversely, the formation of an enzyme-substrate complex can be observed when the  
41 terminal acid function of the growing monomer or polymer reacts with catalytic serine. *ii*).  
42 Propagation: a growing monomer or polymer attacks the enzyme-substrate complex leading to the  
43 growth of the macromolecular chain. Conversely, lipase can randomly hydrolyze an ester function  
44 of the polymer, thereby forming two polymers with smaller degrees of polymerization. *iii*).  
45 Cyclization: the terminal alcohol function of the polymer reacts with the ester function between  
46 the lipase to the polymer. Conversely, lipase can hydrolyze an ester function of a cyclic polymer  
47 and thus leading to the corresponding linear polymer.  
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**Scheme 1.** Canonical mechanism of lipase-catalyzed ROP of unsubstituted and  $\alpha/\beta$ -alkyl substituted  $\beta$ -lactones<sup>5,12</sup>.

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On the other hand, and contrary to the other lactones mentioned above, the mechanism of enzymatic polymerization of alkyl malolactonates is still not elucidated. The question “Does the lipase-catalyzed ROP of alkyl malolactonate follow the same mechanism as the one described for chemical ROP of alkyl malolactonate (Scheme 2) or the one described by several authors for unsubstituted lactones (Scheme 1)?” is still unanswered right now.

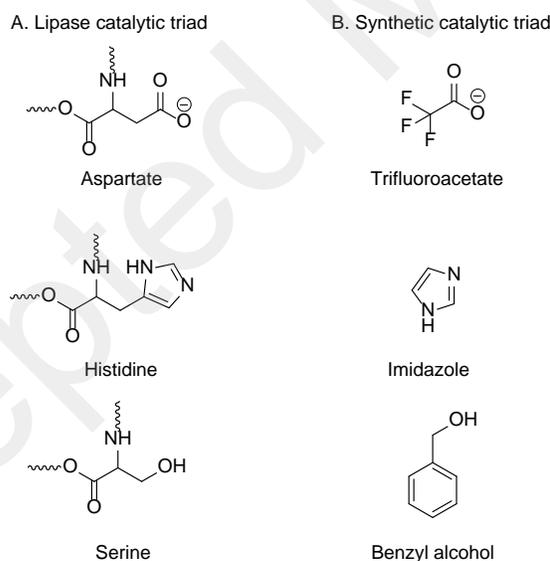


**Scheme 2.** Mechanism of the chemical ROP of alkyl malolactonate<sup>20</sup>.

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The mechanism described in Scheme 1 for the lipase-catalyzed ROP of unsubstituted lactones and lactide is based on a nucleophilic attack of the catalytic serine on the carbonyl of the monomer, leading to the ring opening through an O-acyl bound cleavage. Concerning the lipase-catalyzed ROP of benzyl malolactonate (MLABe), we have recently demonstrated that the lactone ring

opening might occur through an O-alkyl bond cleavage, as for the anionic ROP of MLABe for which the initiator (tetraethylammonium benzoate or other carboxylate salts) attacks on the asymmetric carbon of the alkyl malolactonate thus leading to an O-alkyl ring opening<sup>11,20</sup>. Indeed, the polymers synthesized from R-MLABe using either PPL as catalyst or tetraethylammonium benzoate as initiator have identical rotational powers with the same sign whatever the route of synthesis<sup>11</sup>. If this result seems logical in view of the alkyl malolactonate reactivity, it is much less if the reaction mechanism is considered as a whole. Indeed, according to literature and canonical mechanism, the catalytic serine should react with the carbonyl group of the monomer ring to form an acyl-enzyme intermediate through a hydrolyzable ester bond<sup>12-18</sup>. In the case of lipase catalyzed ROP of alkyl malolactonate, if the attack of the catalytic serine on the asymmetric carbon of the lactone is considered, an alkyl-enzyme intermediate through hardly hydrolyzable ether bond should be obtained. However, we never observed such intermediate in <sup>1</sup>H NMR spectra and SEC chromatograms of both the crude and purified products. Therefore, it would seem that the canonical mechanism cannot be applied in the case of lipase-catalyzed ROP of alkyl malolactonate. In an attempt to understand the mechanism involved in the lipase-catalyzed ROP of this unusual lactones' family, several experiments were conducted. Coulembier *et al.*<sup>23</sup> designed and used a synthetic catalytic triad to study lipase-catalyzed polymerization of D- and L-lactide. As shown by Figure 1, this synthetic catalytic triad is composed by trifluoroacetate mimicking the aspartate with however a lower nucleophilic character, imidazole for the histidine and benzyl alcohol mimicking the catalytic serine.



**Figure 1.** The lipase (A) and synthetic (B) catalytic triad.

Therefore, such synthetic catalytic triad was synthesized and tested for the polymerization of MLABe. The reaction between the MLABe and the synthetic catalytic triad, complete or partial, was carried out at 60 °C and 390 rpm (conditions similar to those of the enzymatic polymerization). Six polymerization conditions were tested as summarized in Table 1 (“Materials and Methods”).

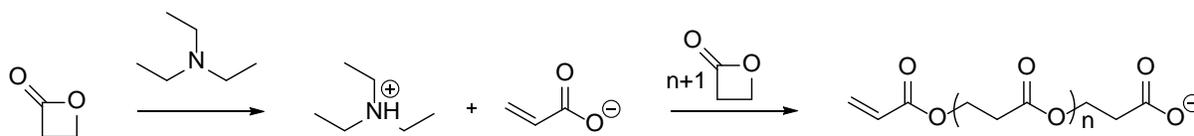
The crude PMLABe were analyzed by proton NMR (conversion rate and hydrolysis rate) and purified PMLABe were analyzed by SEC (dispersity and weight average molar mass, Figure SI.1). As shown by the results gathered in Table 2, the complete synthetic catalytic triad (Table 2, Entry *i*) and the mixture trifluoroacetic acid/imidazole (Table 2, Entry *ii*) are able to polymerize MLABe, but the weight average molar masses of the resulting polymers are very low (600 g/mol and 500 g/mol). Moreover, no polymerization reaction was observed when MLABe was brought together with benzyl alcohol (Table 2, Entry *iii*) or trifluoroacetic acid (Table 2, Entry *iv*) alone. On the other hand, 0.2 equivalent of imidazole (Table 2, Entry *v*) and catalytic amount of imidazole equivalent to the one of histidine into the catalytic triad (Table 2, Entry *vi*) are able to effectively polymerize MLABe with 100% monomer conversion leading to PMLABe with quite high weight average molar masses and low dispersities (3,350 g/mol, dispersity of 1.50 for Entry *v*, and 7,370 g/mol, dispersity of 1.15 for Entry *vi*). It should be noted that no monomer's hydrolysis has been observed whatever the experimental conditions used.

**Table 2.** Characteristics of the PMLABe obtained by ROP of MLABe with the synthetic catalytic triad, complete or partial.

Entry	Conditions	Conversion rate <sup>a</sup> (%)	M <sub>w</sub> <sup>b</sup> (g/mol)	Đ <sup>b</sup>
(i)	Imidazolium salt (0.2 eq.) + benzyl alcohol (0.04 eq.)	100	600	1.40
(ii)	Imidazolium salt (0.2 eq.)	100	500	1.40
(iii)	Benzyl alcohol (0.04 eq.)	0	---	---
(iv)	Trifluoroacetate (0.2 eq.)	0	---	---
(v)	Imidazole (0.2 eq.)	100	3,350	1.50
(vi)	Imidazole (1.07*10 <sup>-4</sup> eq.)	100	7,370	1.15

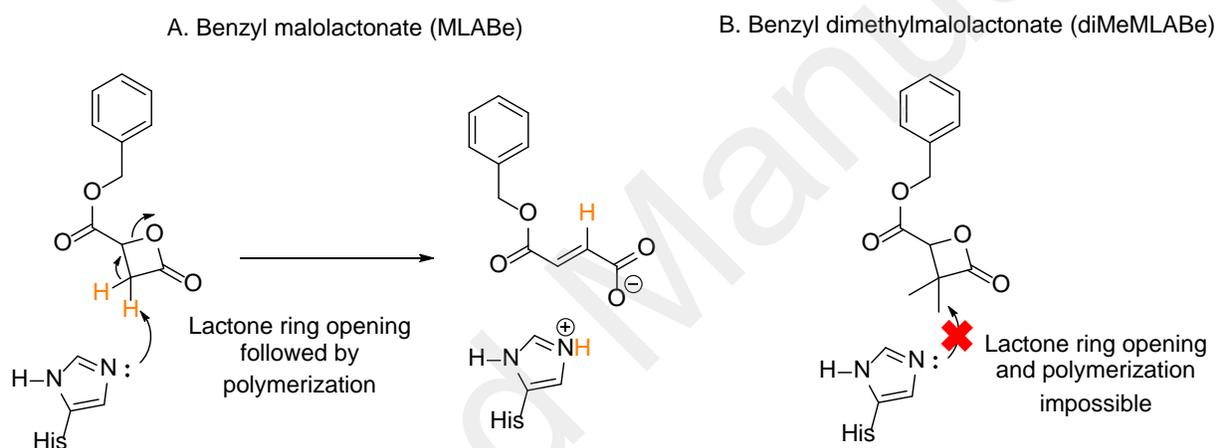
*a.* Determined from <sup>1</sup>H NMR spectra of crude products; *b.* Measured by SEC (THF, Polystyrene standards, 1 mL/min, 40°C; Figure SI-1)

In this experiment, imidazole can be considered as the “synthetic” pendant of histidine of the lipase catalytic triad. From the results obtained, it seems logical to ask whether histidine alone is able to polymerize MLABe, without the intervention of serine and, therefore, of the canonical mechanism. An acid-base mechanism excluding the serine would therefore be possible. Indeed, this kind of mechanism has already been described for PPL-catalyzed aldolization reaction between an aldehyde and a ketone in the presence of water<sup>36</sup>. Moreover, O-alkyl ring-opening has been described for unsubstituted lactones when they are polymerized in the presence of a weak base as initiator: the carboxylate ion thus formed would then be the species responsible for the propagation<sup>37-39</sup>. However, the formation of a carboxylate in this case would come from side reactions, including deprotonations of the monomer or the growing polymer chains<sup>38</sup>. Indeed, Kricheldorf *et al.* have shown that anionic initiators, such as alcoholate, or basic initiators, such as tertiary amine, could abstract one of the two “acidic” protons of the lactone’s ring in  $\alpha$  position of the carbonyl to form the corresponding acrylate (Scheme 3).



**Scheme 3.** Mechanism of the propiolactone ROP in presence of a tertiary amine as initiator<sup>38</sup>.

This acrylate would continue the polymerization of the remaining monomers thus leading to a polymer terminated at one end by a double bond. Furthermore, Guérin *et al.* have shown that these deprotonation reactions also take place during the anionic ROP of alkyl malolactonates<sup>40</sup>. If this deprotonation reaction is considered for the lipase-catalyzed polymerization of MLABe, the first step of the mechanism would consist in the abstraction of an “acidic” proton of the lactone’s ring in  $\alpha$  position of the carbonyl by the basic nitrogen group of imidazole (Figure 2A).



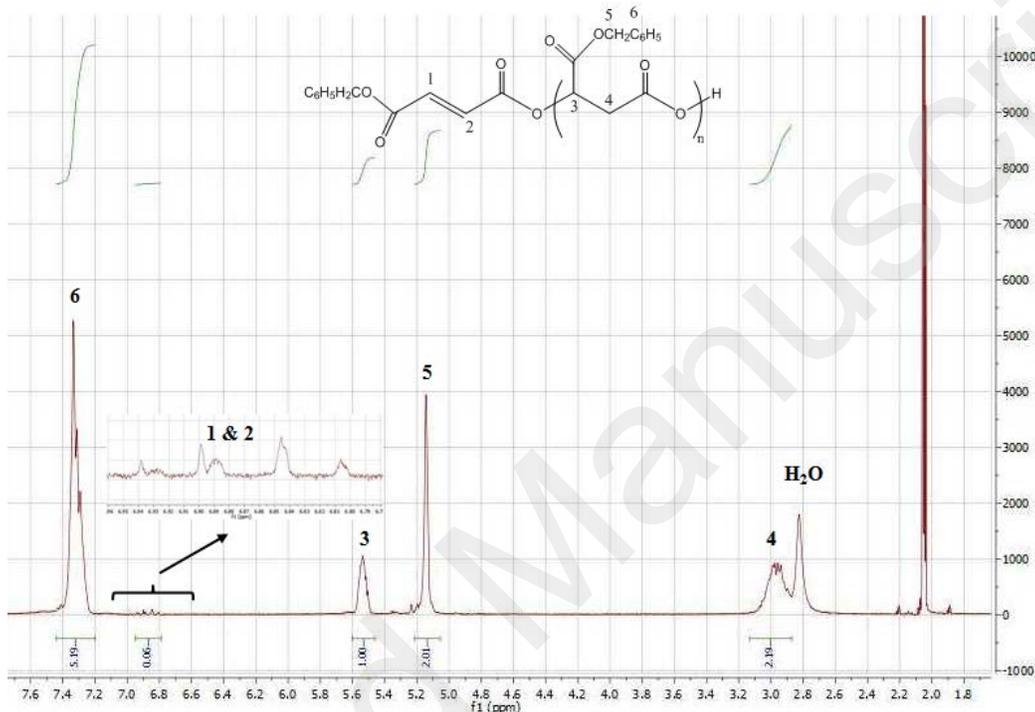
**Figure 2.** Proposed first step for the lipase-catalyzed ROP of: A. MLABe, and B. diMeMLABe.

The carboxylate thus formed would then continue the polymerization of the remaining MLABe. Therefore, if the two “acidic” protons of MLABe’s ring are replaced by methyl groups, which is the case for dimethyl-MLABe, then this first step could no longer take place and the polymerization would not start (Figure 2B).

The enzymatic polymerization of benzyl dimethyl malolactonate (diMeMLABe) was therefore carried out using the optimized protocol established for the polymerization of MLABe. PPL was mixed with diMeMLABe (1/40 PPL/diMeMLABe ratio) in Tris-HCl buffer (105 mM, pH 7), and the mixture was stirred at 390 rpm during 72h at 60°C. The obtained crude product was analyzed by proton NMR and the spectrum showed that no polymerization reaction took place. Indeed, no characteristic peak of a polymer was observed while all the peaks of diMeMLABe (Figure SI.2A) were present thus highlighting both the non-degradation and non-polymerization of this monomer by PPL (Figure SI.2B).

The impossibility to polymerize the diMeMLABe using PPL as catalyst might be in favor of the proposed mechanism (abstraction of one of the two “acidic” protons from the lactone’s ring by the catalytic histidine), even if we cannot ignore that this impossibility might be also due to steric hindrance brought by the presence of the methyl groups on the lactone ring.

Therefore, we carefully analyzed the proton NMR spectra of PMLABe obtained by PPL-catalyzed ROP of MLABe in Tris-HCl buffer pH7 (Figure 3).



**Figure 3.** Proton NMR spectrum ( $\text{CD}_3\text{COCD}_3$ , 400MHz) of the purified PMLABe obtained by PPL-catalyzed ROP of MLABe in Tris-HCl buffer pH7 at  $60^\circ\text{C}$  under 390 rpm stirring speed during 72h.

The expansion of the proton NMR spectrum between 6.8 and 7.0 ppm has highlighted the presence of peaks corresponding to ethylenic protons, thus seeming to confirm the hypothetic mechanism described in Figure 2A. Moreover, molar masses of PMLABe calculated using the relative integrations of these ethylenic protons and of the macromolecular chain protons (Figure 3) agree with those measured by SEC in THF ( $M_{\text{NMR}} = 6,800 \text{ g/mol}$ ;  $M_w = 7,570 \text{ g/mol}$ ;  $\text{Đ} = 1.12$ ).

Therefore, these results tend to demonstrate the validity of the proposed lipase-catalyzed ROP mechanism of MLABe involving the abstraction of one of the two “acidic” proton of the lactone’s ring probably by the catalytic histidine without the intervention of the catalytic serine.

In order to demonstrate that the lipase-catalyzed ROP of malolactonates does not involve the catalytic serine, we realized the enzymatic ROP of MLABe in presence of a serine knock-out (serine-KO) enzyme. However, PPL mutants being very difficult to obtain, we have selected an easy modifiable recombinant model, the CpLip2 enzyme. Using site-directed mutagenesis of

serine S180 into an alanine residue, we obtained the serine KO mutant CpLip2\_S180A. This mutant was confirmed to show no detectable lipase activity.

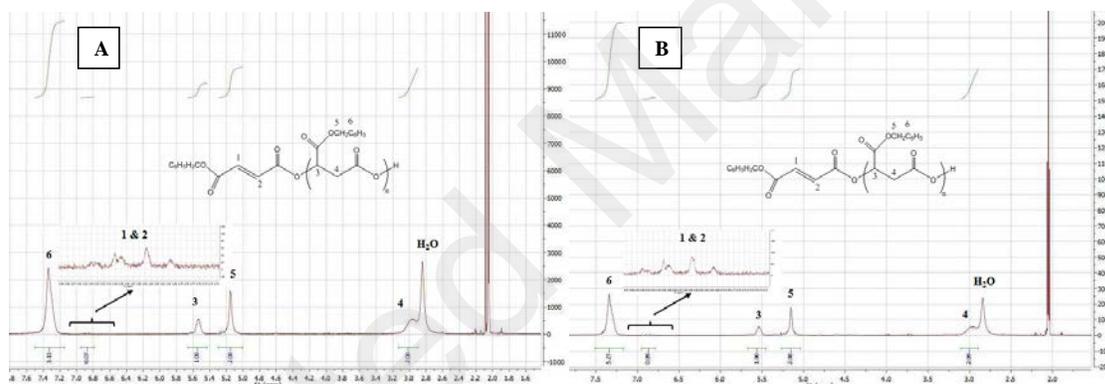
If our hypothesis is valid then the CpLip2-Serine KO should lead to the formation of a polymer. Therefore, the polymerizations of MLABe in the presence of CpLip2 and CpLip2\_S180A in Tris-HCl buffer pH7 (optimized conditions for PPL) have been conducted.

As shown by results gathered in Table 3 and Figure 4, PMLABe were obtained in both cases, with, in addition, the presence of the peaks corresponding to ethylenic protons formed by the abstraction of one of the two “acidic” protons of the lactone’s ring as shown in Figure 2A.

**Table 3.** Characteristics of the PMLABe obtained by ROP of MLABe in presence of CpLip2 and CpLip2\_S180A enzyme in Tris-HCl buffer pH7 at 60°C under 390 rpm stirring during 72 h.

	$M_{RMN}$ (g/mol)	$M_w$ (g/mol) <sup>a</sup>	$\bar{D}^a$
MLABe + CpLip2 + Tris-HCl buffer pH7	4,940	3,640	1.42
MLABe + CpLip2_S180A + Tris-HCl buffer pH7	5,770	3,560	1.42

a. Measured by SEC in THF at 40°C, Standards Polystyrenes, Flow Rate = 1 mL/min.

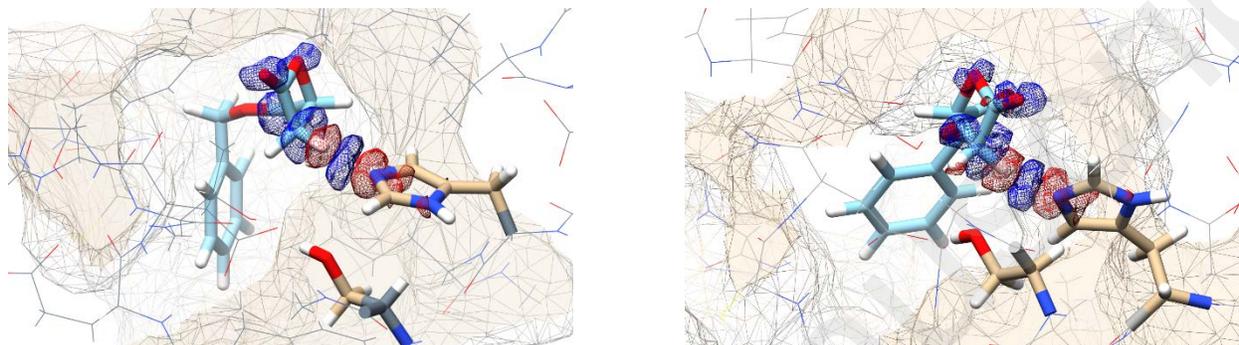


**Figure 4.** Proton NMR spectrum ( $CD_3COCD_3$ , 400 MHz) of purified PMLABe obtained: A. by CpLip2-catalyzed ROP of MLABe in Tris-HCl buffer pH7; and B. CpLip2\_S180A-catalyzed ROP of MLABe in Tris-HCl buffer pH7.

Finally, the CpLip2 has been inactivated by heating at 100°C and enzymatic polymerization of MLABe has been conducted with this inactivated enzyme: the  $^1H$  NMR spectrum of the crude product has highlighted a monomer conversion of less than 7% and the absence of polymer. Such results showed that the catalytic histidine certainly plays an important role in the polymerization of malolactonates.

To support further these experimental results, *in silico* calculations were performed: Figure 5 shows the optimized geometry of S-MLABe and R-MLABe and of the active site of CpLip2 obtained after molecular docking, molecular dynamics equilibration, energy minimization by DFTB and DFT quantum chemical calculations as described in the *Computational methods*

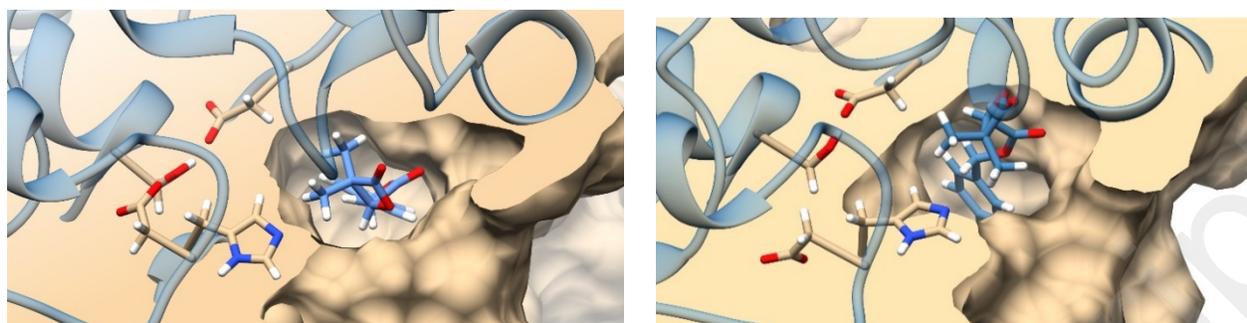
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3 section. ETS-NOCV analysis (extended transition state energy decomposition analysis combined  
4 with the theory of natural orbitals for chemical valence)<sup>33</sup> by DFT calculations (BP86-  
5 D4(EEQ)/DZ level of theory) indicated that the main orbital interaction energy contribution  
6 between MLABe and the active site residues was associated to a strong hydrogen bond (-29 kJ/mol  
7 and -23 kJ/mol with S- and R-MLABe, respectively) between the  $\epsilon$  N atom of histidine 365 and  
8 one of the “acidic” protons of MLABe (Figure 5).  
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24 **Figure 5.** Optimized geometry of the active site of CpLip2 in complex with S-MLABe (left) and  
25 R-MLABe (right). The two amino acid residues evidenced are the catalytic serine (S180) and  
26 histidine (H365). Interaction energies between the ligand and the active site residues were  
27 calculated by DFT (BP86-D4(EEQ)/DZ with water as implicit solvent) according to the ETS-  
28 NOCV theory. The colored meshes show the deformation of the electron density (depletion in red  
29 and accumulation in blue; cut-off 0.007 a.u.) associated to the main NOCV, corresponding to a  
30 hydrogen bond between H365 and a proton of MLABe (-29 kJ/mol with S-MLABe and -23 kJ/mol  
31 with R-MLABe).  
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36 This result corroborates the hypothesis of a preferred interaction between MLABe and the catalytic  
37 histidine instead of the catalytic serine 180, and supports the proposed mechanism of  
38 polymerization of MLABe involving histidine, thanks to the presence of “acidic” protons (Figure  
39 2A).  
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42 Finally, we tried to polymerize the diMeMLABe in the presence of CpLip2, CpLip2\_S180A and  
43 imidazole in a catalytic amount in Tris HCl buffer pH7. In all these cases, we did not observe the  
44 formation of the corresponding PdiMeMLABe, and unpolymerized and undegraded diMeMLABe  
45 was recovered (Figures SI.2C and SI.2D). It is to be noted that molecular docking simulations with  
46 (R) and (S)-diMeMLABe and the active site of CpLip2 showed that the steric hindrance brought  
47 by the two methyl groups of the lactone ring should prevent the substrate from getting close enough  
48 to the catalytic residues (Figure 6). Nevertheless, the impossibility of polymerizing diMeMLABe  
49 in the presence of imidazole as initiator, i.e. without the steric hindrance problem encountered  
50 with lipases, demonstrates that the absence of “acidic” protons prevents the enzymatic  
51 polymerization of these lactones.  
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**Figure 6.** Geometry of the catalytic pocket of CpLip2 in complex with S-diMeMLABe (left) and R-diMeMLABe (right) obtained by molecular docking and energy minimization by molecular dynamics of the whole CpLip2 structure in explicit water. The amino acid residues displayed correspond to the catalytic triad (S180, H365 and D332) and to the D90 residue interacting with the catalytic serine in CpLip2.

All these observations are thus in favor of the proposed mechanism, namely the abstraction of one of the two “acidic” protons of the lactone’s ring by the catalytic histidine leading to the formation of a monobenzyl fumarate which then initiates the polymerization of the remaining monomers.

**CONCLUSIONS.** The obtained results lead us to conclude that the lipase-catalyzed polymerization of MLABe does not follow the canonical mechanism, but results from the abstraction of one of the two “acidic” protons of the lactone’s ring by the histidine of the catalytic triad leading to the formation of an activated monomer, *i.e.* monobenzyl fumarate (Figure 2B), responsible for the polymerization of MLABe. In other words, the lipase acts only as a catalyst to form an activated monomer. However, it would also be responsible for particular conformation adopted by PMLABe chains, thus inducing the presence of crystalline zones even when racemic MLABe are polymerized, conclusion confirmed by the observed melting temperatures measured for PMLABe obtained by enzymatic polymerization of racemic MLABe [unpublished results]. However, additional studies (X-rays, for example) still need to be carried out to elucidate the organization of macromolecular chains induced by the presence of the lipase.

#### AUTHOR INFORMATION

##### Corresponding Author

\* sandrine.marion.1@ensc-rennes.fr

##### Present Addresses

† Université Clermont Auvergne, CNRS, SIGMA Clermont, ICCF (Institut de Chimie de Clermont-Ferrand) - UMR 6296, 63000 Clermont-Ferrand, France

##### Author Contributions

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### ABBREVIATIONS

diMeMLABe: benzyl dimethylmalolactonate

MLABe: benzyl malolactonate

PdiMeMLABe: poly(benzyl dimethylmalate)

PMLABe: poly(benzyl malate)

ROP: Ring Opening Polymerization

### SUPPORTING INFORMATION

SEC chromatograms of PMLABe obtained under different experimental conditions (Table 2 and Table 3), <sup>1</sup>H NMR spectra of pure diMeMLABe and after reaction under various conditions.

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# For Table of Content Only - Lipase catalyzed ring-opening polymerization of benzyl malolactonate: an unusual mechanism?

Hubert Casajus, Eric Dubreucq, Sylvain Tranchimand, Véronique Perrier, Caroline Nugier-Chauvin, Sandrine Cammas-Marion

