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Molecular modeling of the mechanism of ethyl fatty ester synthesis catalyzed by lipases. Effects of structural water and ethanol initial co-adsorption with the fatty acid

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ABSTRACT

This manuscript presents an MM2 modeling of the esterification of model fatty acids (oleic acid and 9-decenoic acid) with ethanol using lipases from *Candida antarctica* B (CALB) and *Candida rugosa* (CRL). The role of water and ethanol as part of an H-bonding network acting at the adsorption of the fatty acid, the stabilization of intermediaries and the acyl enzyme and on the regeneration of the Serine OH are taken into account.

The model includes the consideration of the tunnel and the catalytic triad/oxyanion hole – the large active site – for CRL and CALB-using structures of the open lipases from the PDB. Besides, an additional molecular modeling of the H-bonding distances found in the intermediaries and the acyl enzyme using CALB was carried out. The fatty acid esterification demonstrated to be activated for CRL and almost non-activated for CALB—in the context of the large active sites. When the results of the additional MM2 modeling (for a shorter model) were analyzed. H-bonding at the level of the strong and weak H-bonding between Histidine, water, ethanol and O from Serine were found. Molecular modeling results also revealed that the presence of a molecule of water at the catalytic triad of both lipases promotes a significant change in the calculated energy profiles to lower energies and a potential too strong adsorption of substrates and products. There is qualitative agreement between the theoretical modeling and the experimental work published elsewhere.

1. Introduction

Lipases are attractive biocatalysts to synthesize fatty esters in mild conditions and with high selectivity. The open literature shows a great number of experimental reports devoted to parametric analysis aimed to maximize ester yield. Besides, there are several kinetic modeling and reactor simulation contributions dealing with enzymatic fatty acid esterifications [1–4]. Molecular modeling appears as an attractive tool to complement experimental and reactor/kinetics simulation data of lipase-catalyzed fatty ester synthesis. Solvent-free systems are particularly attractive for enzymatic synthesis because of the avoidance of the solvent and downstream purification/separation steps and high productivities per mass unit of enzyme.

By using lipase's structure information provided by Uppenberg et al., Grochulski et al. and others on several lipases, molecular modeling has been successfully applied to study interactions of lipases with different compounds [5–8]. In this contribution molecular modeling is applied to study the steric interactions in the mechanism of reaction of an enzymatic solvent-free fatty ester synthesis. The model reaction chosen for the MM2 calculation is the lipase-catalyzed synthesis of ethyl oleate from oleic acid and ethanol [9]. The described reaction has been thoroughly studied by our group at laboratory scale in solvent-free system. Then, the reaction media included only oleic acid and ethanol with different initial amounts of water, without solvent. The experiments revealed a wide range of esterification activities depending on the biocatalyst used and the amount of water present initially [10–13].

In general, the need of some amount of water in non-aqueous medium to assure lipase activity in esterification reactions has been explained in terms of the contribution of water to the structural integrity, active site polarity, stability of the protein [14] and the increase in enzyme flexibility, even to distinguish between enantiomers [15]. The presence of a molecule of water at the active site of lipases was hypothesized by Haeffner et al. and presented in context in the review of this author on the topic of molecular modeling of lipases [16 and references therein]. The authors pointed out the importance of the presence of water in the tetrahedral transition

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state to the acyl enzyme formation in the lipase-catalyzed esterifications. In the case of the lipase-catalyzed aminolysis of esters a molecule of water could be necessary in some cases to facilitate the transfer of the proton to the catalytic Histidine, part of the active site [17]. Other authors have proposed a role in the substrate-assisted catalysis through an H-bonding between the substrate and the imidazolium of Histidine in the final activity and enantioselectivity in the hydrolysis of racemic esters in organic solvents saturated with water [18].

Even the sophisticated calculation methods are carried out in gas phase or in a continuum model of a solvent. Generally, the solvent interaction at the level of the catalytic triad is not explicitly considered. We are aware of the limitations of our MM2 approach. However, we think that the results presented here may be an interesting input for more sophisticated modeling approaches. Even more, this modeling gives some insight on the potential impact of water in the local structure around the catalytic triad and the water's role in the substrates coordination and the intermediates/acyl enzyme stabilization in the model reaction.

However, in the real life of lipase catalysis, there are plenty of potential for the substrates to do H-bonding with exposed lateral residues of the amino acids of the lipase (lid, tunnel, etc.). Besides H-bonding, there are repulsive and attractive forces, hydrophobic forces, etc., acting during the path of the substrates to the catalytic triad of the lipase. These interactions are also present in the exit of the products from the catalytic triad and through the tunnel of the lipase. The interactions of intermediates and acyl enzymes are mainly with the catalytic triad and its environment and not so much with the tunnel and lid of lipases. The extent of the interaction of the substrates with the catalytic triad and its environment depends on the structures of the substrates.

Strictly, all these interactions should have to be considered to discuss properly the energetic of the enzymatic esterification. It would be the only way to compare theoretical results with experimental ones. In this sense, only QM/MM methods are the right approach. However, the molecular mechanics calculations may give insight on the Van der Waals interactions of the substrates, known intermediates and products with the catalytic triad-tunnel and lid of the lipases. For example, it is known from the XRD studies that the *Candida antarctica* lipase B (CALB) has a water molecule tightly associated with the catalytic residue Asp 187 through a hydrogen bond in solid state [6]. Calculations of water-binding sites in the modeled acyl enzymes of *Rhizomucor meihei* lipase, *Candida rugosa* lipase (CRL) and *Thermomyces lanuginosa* lipase showed that the hydrophilic site may accommodate a water molecule in a position that is favorable for an in-plane nucleophilic attack on the carbonyl carbon atom of the acyl group. This water molecule may donate 1 hydrogen bond to the imidazole of the catalytic triad, which is proposed to act as a base in the catalytic reaction [7].

The questions that the manuscript tries to answer using simple MM2 calculations tools are:

(1) Has 1 water molecule some kind of impact in the steric energy approximate profile of an esterification reaction with ethanol and oleic acid using a catalytic triad/tunnel model of CALB and CRL?
(2) May gas-phase theoretical modeling data (obtained with the consideration of the oleic acid, ethanol and water since the beginning of the reaction) be related *qualitatively* to data obtained in liquid solvent-free system with water present since the beginning of the reaction with no experimental external or internal diffusional restrictions? The last assumption has been tested experimentally [13a and 13b].
(3) May gas-phase data obtained with limited computational tools give useful input on the differences found experimentally using different lipases of different structures, such as CALB and CRL in solvent-free systems? In the framework of the used computational tools the steric restrains and the geometric details of the catalytic triad have been taken into account. Besides, neighborhoods of the catalytic triad and its interaction with substrates/products/intermediates/water have been properly considered.

We have also performed extensive experimental work and reaction kinetics simulation studies, many of them in collaboration with other authors. The studies mentioned explored the impact of water on the kinetics and the equilibrium composition of solvent-free ethyl oleate synthesis [8,10–12]. In our case, experimentally, we used 3 g oleic acid and 0.5 g ethanol (being the molar ratio oleic acid:ethanol near 1:1). Therefore, no solvent was included in the theoretical modeling and one ethanol was included with one oleic acid in the neighborhood of the catalytic triad.

This work tries to present additional theoretical evidence for the proposed roles of water and ethanol considering the experimental evidence presented above and taking into account the well-known general mechanism of esterification with lipases [16–20].

2. Methodology

2.1. The software

The MM2 approach was selected to avoid undesirable changes in the conformational space of the enzyme with a semiempirical/ab initio minimization, using a simplified model. Structures of lid-opened CRL (1crl) and CALB (1tcb) were obtained from the Protein Data Bank (PDB) using the Swiss-PDB viewer program. Hydrogens were added and the overall structures of tunnel (if present) and the active sites of CRL and CALB were minimized by the use of MM2. The analysis was performed with the Chem3D 5.0 Ultra and 11.0 (from Cambridge Soft). Models built this way are quite good for the qualitative understanding of the interactions, at the local level only, although they are not useful in quantitative terms since the described approach employs force field methods and only a portion of the enzyme is included in the calculation. We carried out:

(a) steric energy MM2 calculations of the interactions of the oleic acid/ethanol/ethyl oleate with the tunnel and the active site of CALB and CRL modeled complete (the large active site or LAS) and
(b) steric energy MM2 calculations of the interactions generated using 9-decenoic as the fatty acid and the catalytic triad and the oxyanion hole of CALB (the short active site or SAS).

2.2. Models of the actives sites of CRL and CALB

The active sites of CRL and CALB were modeled considering large active site (LAS) models for the determination of the minimal steric energy conformation with MM2. A short active site model (SAS) was used in the case of CALB for the analysis of H-bonding lengths found in the intermediates and the acyl enzyme. LAS of CRL includes the complete catalytic triad and the tunnel: Gly124, Phe125, Ser209, Ala210, Met213, Val245, Pro246, Phe296, Ser301, Leu302, Arg303, Leu305, Leu307, Phe345, Tyr361, Phe362, Ser365, Phe366, Val409, Leu410, Leu413, Gly414, Phe415, Phe532 and Val534. In the case of CALB, SAS includes the Ser105–His224–Asp187 catalytic triad and the oxyanion hole (Thr40 and Gln106). The LAS of CALB additionally includes Asp134, Ser105, Thr138, Ile189 and Val190 on its left hand side, Gln157 and the oxyanion hole residue Thr40 on its right hand side, Gln157, Val154, Ile285, Leu144 and Val49 [5–7].

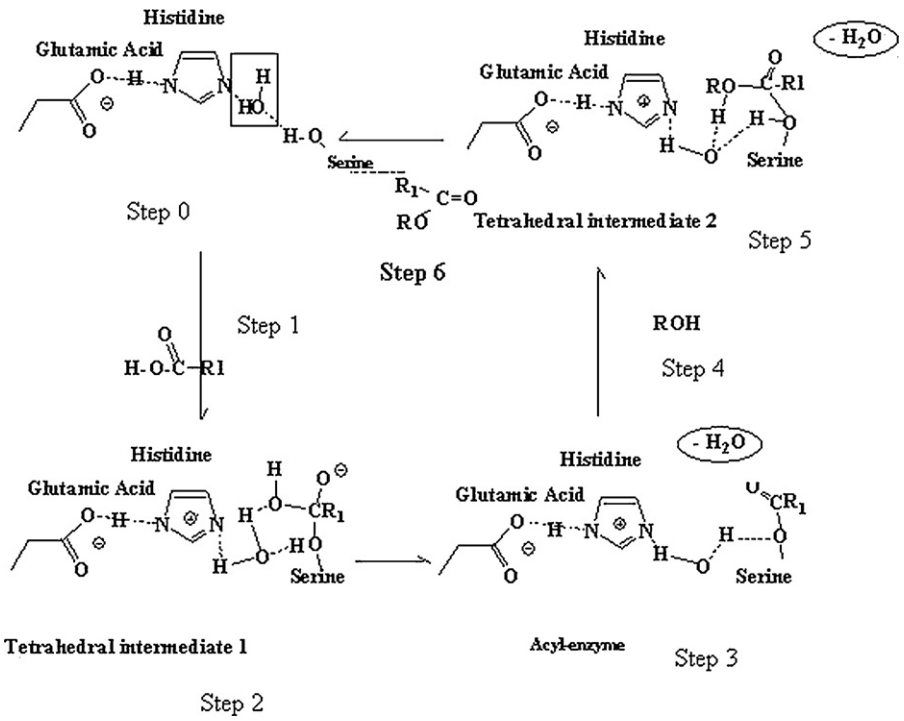

Fig. 1. Ping Pong Bi-Bi mechanism of ethyl oleate synthesis catalyzed by CRL, considering the presence of water between Serine 209 and Histidine 449 since the beginning. The water molecule released due to the acyl enzyme formation – circled – may also be H-bonding in the neighbors of the active site. Step 2 and step 5 are indicated. At these steps the substrates approaches the catalytic triad.

2.3. Modeling of mechanism of ethyl oleate synthesis catalyzed by CRL and CALB

The mechanism chosen was the Ping Pong Bi-Bi, which has been widely used in the kinetic modeling of lipase-catalyzed fatty acid esterifications [3–4]. Fig. 1 shows a scheme of the mechanism of ethyl oleate synthesis catalyzed by CRL. In the case of CALB glutamic acid in the active triad of the lipase must be replaced by aspartic acid. The tetrahedral intermediates were considered with the oxygen from opened carbonyl modeled as an alkoxide (charge −1) using Chem3D 5.0 Ultra (from Cambridge Soft, 1999). The oxygen in Serine at the tetrahedral intermediate was assigned as oxonium (+1 charge, tricoordinated). The presence of a molecule of water at the active site of lipase between Histidine and Serine has been considered following the proposals analyzed and reported in the review of Haeffner et al. [16 and references therein]. Even though one water molecule is also produced by each esterification reaction its location is different from that of the water trapped in the lipase structure and specifically placed near its catalytic triad.

2.4. Initial positions of the substrates

Energy minimization of each substrate was performed to generate a low-energy conformation with suitable bond distances and angles. The oleic acid or 9-decenoic acid was then manually docked into the substrate-binding sites in the CRL and CALB models. After that, we performed a MM2 minimization procedure. This approach has support in the recent work of Kwon et al. [21].

Once the position of the fatty acid was obtained in a conformer associated to minimum steric energy, the ethanol was located. The alcohol was placed initially in such a way that the orientation was related to the position of the polar groups in the catalytic triad and the oleic acid substrate and again minimized with MM2. One water molecule was placed between the Serine and the Histidine and again minimized by MM2 to compare with the situation without this water molecule. Several different initial positions were selected considering the known facts about approaches of the ethanol and the minimizations produced one of them as the most probable initial situation for the adsorption of the fatty acid near the catalytic triad.

The initial location of the acyl groups of the substrates was guided by the conformation of the known inhibitors of lipase. Botta et al. have presented results where the computational step corresponding to the molecular docking of ligands to enzymes was simplified since the functional groups of the substrates interacting with the macromolecule and the amino acid residues of the catalytic machinery were well known in advance. Thus, the docking problem was essentially reduced to locate the possible conformations of the ligand within the active site of its target enzyme. When dealing with lipases from *C. rugosa* and *Pseudomonas cepacia*, they used the XRD structures of the phosphonyl group of an inhibitor to guide the building of the tetrahedral oxyanion intermediates providing a good template to adjust torsional angles of the substrate [22]. Even more, studies such as from Torrance et al. show that cooperativity is involved strongly in enzymatic catalysis. The authors conclude that enzyme function does not require a single residue with finely tuned geometry but rather a whole cascade of interactions. Their results for 42 enzyme structures demonstrated that the geometry of productive interactions closely resembles the one seen in non-catalytic hydrogen bonds with distances and angles in the normal range. For example, the authors reported that a long hydrogen bonding geometry is seen between the Ser–His pair in Ser–His–Asp catalytic triad. Ser–His in their analysis did not display an unusual hydrogen bonding geometry although they may have a weak hydrogen bonding strength [23]. The main studies from structural facts in CALB are related to its enantioselectivity and stereoselectivity toward many secondary alcohols [24].

2.5. Additional considerations

There are no diffusional effects or phase transfer effects considered in this modeling. The lack of diffusional effects has been

Table 1
Description of the steps of ethyl oleate reaction mechanism (based on Ping Pong Bi-Bi mechanism) for the MM2 calculation. Final conformations (with main structural facts) associated to these steps when water is included in the catalytic triad shown in Fig. 1.

Step	Description
0	Initial state: native enzyme model and substrates far away of the enzyme model.
1	Adsorption of oleic acid at the active site of the enzyme model—ethanol is modeled far away from the coordinated fatty acid.
2	Formation of tetrahedral intermediary 1 a) with ethanol modeled far away from the intermediary, or b) with ethanol modeled close to the intermediary (distance lower than 3A).
3	Water release and acyl enzyme formation (Ethanol near the active site – or not – from here and thereafter). Water located near the catalytic triad.
4	Adsorption/coordination of ethanol in the active site enzyme model.
5	Formation of tetrahedral intermediary 2.
6	Regeneration of the active site and ethyl oleate desorption.
7	Release of ethyl oleate from the structure.

tested experimentally. From the experimental and simulation work from our group we are aware that there is phase transfer of substrates/products between the organic and the aqueous phase. This transfer takes place especially in the biphasic reaction media in the oleic acid esterification with ethanol in the presence of water [13]. Therefore it is important to consider this study as an additional tool to complete the explanation of the experimental results presented in Refs. [8,10–13].

3. Results and discussion

3.1. Mechanism of ethyl oleate synthesis. Steric energy profile using LAS models of CRL and CALB

The steps of the Ping Pong Bi-Bi mechanism considered in the modeling of the steric energy profile of the mechanism of ethyl oleate synthesis are those included in Table 1, and the conformers were analyzed with and without one water molecule between Histidine and Serine. Moreover, the influence of the distance (far/close) of the alcohol molecule to the catalytic triad of the lipase at the initial steps 1, 2 and 3 (see Table 1) has also been evaluated. Results for the ethyl oleate synthesis with the LAS model of CRL have been included in Fig. 2.

As it is illustrated in Fig. 2, the steric energy values calculated for every step of the mechanism with a molecule of water placed at the catalytic site of CRL are lower than those calculated without one structural water at the active site. As shown in Fig. 2 the reduction of steric energy was observed irrespectively of the location of the

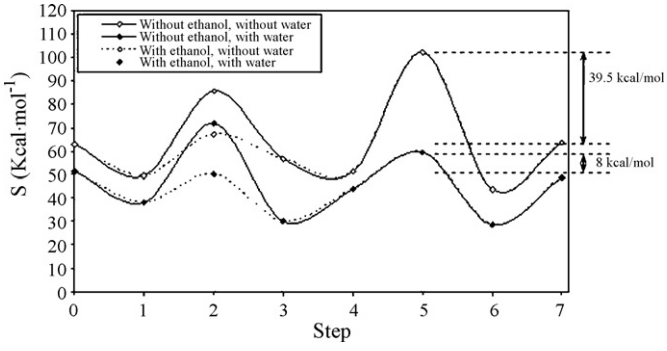

Fig. 2. Evolution of the steric energy involved in the mechanistic steps of ethyl oleate synthesis with/without a water molecule placed in the catalytic triad of CRL. Effect of ethanol in the neighborhood of the active site at the formation of tetrahedral intermediate 1.

alcohol. The reported steric energy values were as obtained from the MM2 minimization. This first observation may be considered as an alternative or additional possible explanation for the well-known beneficial effect of water on lipase activity. In this case the effect would not be due to macroscopic variables, but to structural reasons related to the conformation of the active site of lipases.

Under the implicit assumption that almost no enthalpic changes take place, and considering only steric energy relationships, subtraction of the steric energy involved in steps 5 and 0 gives a first approximation of the energetic barrier of the reaction. For the mechanism with no water present at the catalytic triad the described calculation provided a steric energy barrier of 39.5 kcal/mol. On the other hand, if water is considered as a part of the active site of CRL, then the calculated energetic barrier was 8 kcal/mol. Thus, for ethyl oleate synthesis with the LAS model of CRL, the inclusion of a water molecule implies a more favorable pathway in MM2 steric energy terms. However, it must be pointed out that this energetic barrier is not an accurate activation energy because calculations were not performed with an ab initio software (i.e. Density Functional Theory, DFT). The profile of steric energy vs. mechanistic steps found in this contribution resembles the data of Hu et al. [25] who used Hartree–Fock (HF) and DFT calculations for the hydrolysis of methyl formate considering that we are looking to esterification and not hydrolysis. Hult et al. did not include water at the catalytic triad. Moreover, Norin and coworkers reported that they found that the four-membered ring transition state is energetically less favored than the water-assisted six-membered ring transition state in calculations on water-assisted hydrolysis of methyl acetate under neutral conditions [26]. Our results are in qualitative agreement with these authors results.

Fig. 2 also shows that the most important differences found upon consideration of the presence of water at the active site of CRL arise from the formation of the acyl enzyme (step 3), and from its reaction with ethanol (step 5). Presence of water at the initial step of approach of oleic acid/ethanol provides a potential network of H-bonding that might decrease the intermediates and transition states energies.

Results of Fig. 2 in dotted lines show the effect on the steric energy profile of the explicit consideration of ethanol in the neighborhood of the active site at the step of the formation of the tetrahedral intermediate 1 (step 2). For both conditions modeled the co-adsorption of ethanol at the step of oleic acid adsorption contributes to the reduction of the steric energy involved in the formation of the tetrahedral intermediate 1 by around 20 kcal/mol (see Fig. 2). Then, ethanol also contributes to the fatty acid coordination by adsorption due to the stabilization of the bonds through multiple H-bonding, an aspect that is being more and more cited in the literature [18].

Steric energies calculated for every step of the mechanism of ethyl oleate synthesis for the LAS model of CALB have been included in Fig. 3. Ethanol was co-adsorbed since the step 1 because otherwise no minimum could be found. Comparison of molecular modeling results evidences that esterification catalyzed by the CALB model is much more favorable in steric energy terms than the ethyl oleate synthesis mediated by the CRL model. In the case of the results obtained with the LAS model of CALB, the reaction is carried out forward almost without energetic barrier. Therefore, the ethyl oleate exit is the only endothermic step.

Comparison of steric energies with and without structural water shows that water promotes reaction by lowering the steric energy of all mechanistic steps of the reaction. The steric energy difference between steps 7 and 6 when the LAS model of CALB is analyzed implies +21.1 kcal/mol. This value is very similar to the one obtained for the ester *desorption* considering the LAS of CRL (+20.1 kcal/mol). The steric energy of *adsorption* of the product to LAS of CRL or CALB would be then −20.1 to −21.1 kcal/mol.

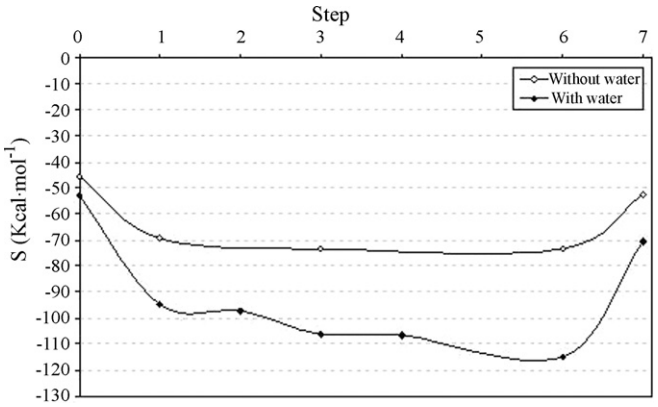

Fig. 3. Evolution of the steric energy involved in the mechanistic steps of ethyl oleate synthesis with/without a water molecule placed in the catalytic triad of CALB. Effect of ethanol in the neighborhood of the active site at the formation of tetrahedral intermediate 1.

3.2. The MM2 calculation on a short active site or SAS of CALB. Analysis of H-bonds lengths (bonding and non-bonding)

We performed an additional MM2 calculation on the SAS of CALB. This calculation included the main facts of the oxyanion hole and the catalytic triad, 9-decenoic acid and ethanol, with water present (or not) between Histidine and Serine. Fig. 4 presents the schematic picture of the tetrahedral intermediate 1 at the catalytic triad of CALB. This figure includes a water molecule between Histidine and Serine since the beginning of the reaction. Important distances are marked with dashed lines and numbered from 1 to 13. Fig. 5 shows the schematic picture of the acyl enzyme when water is present between Histidine and Serine and Fig. 6 shows the schematic picture of the intermediate 2 with two water molecules. One water molecule is located between Histidine and Serine and the other is the one generated because of the acyl enzyme formation. These water molecules are placed around the catalytic triad of CALB. The different distances of H-bonding for the different conformers found after the MM2 minimization are presented in Table 2.

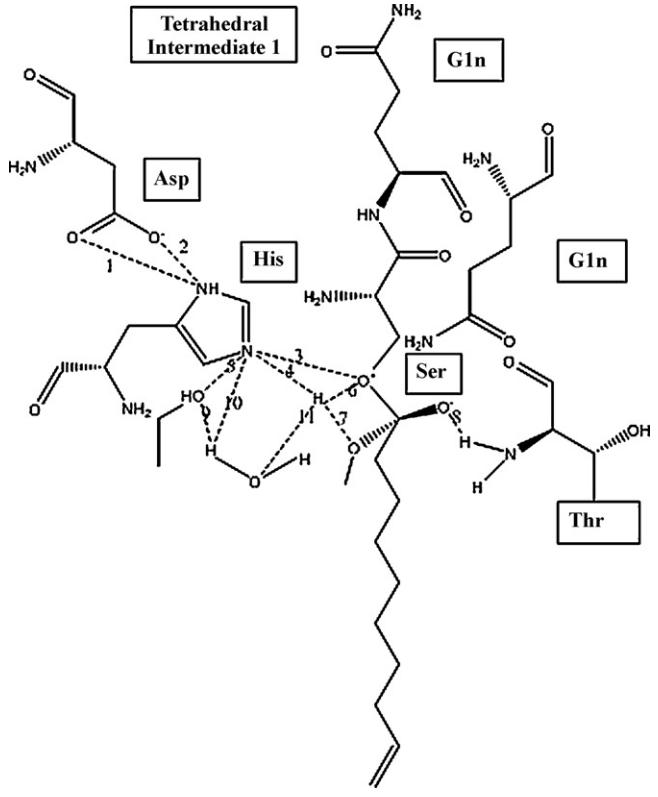

Fig. 4. Schematic picture of the tetrahedral intermediate 1 at the catalytic triad of CALB—that includes a water molecule between Histidine and Serine since the beginning of the reaction. Important distances are marked with dashed lines and numbered from 1 to 13 (see Table 2).

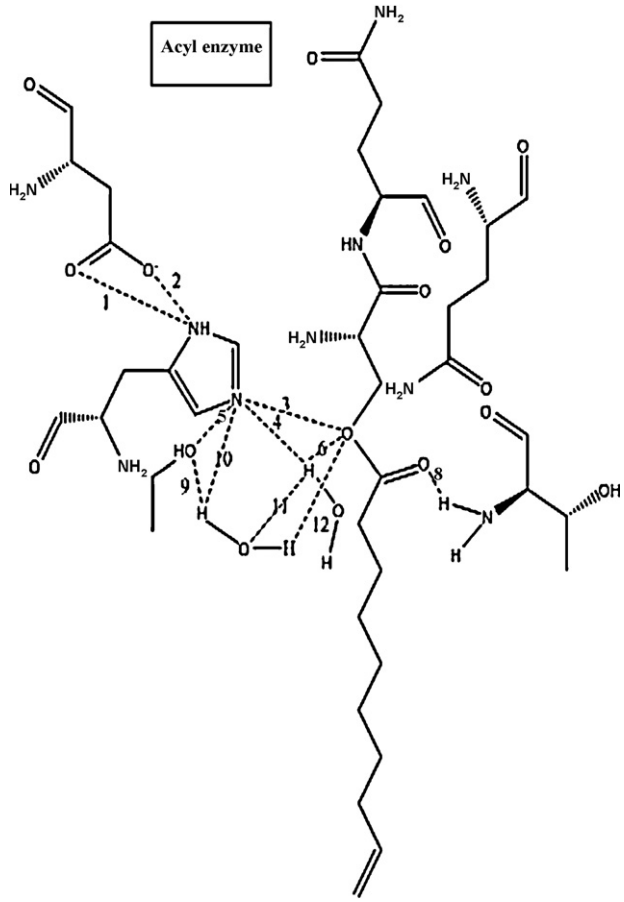

Fig. 5. Schematic picture of the acyl enzyme at the catalytic triad of CALB—that includes a water molecule between Histidine and Serine since the beginning of the reaction. Important distances are marked with dashed lines and numbered from 1 to 13 (see Table 2).

As expected, the presence of water affected the distances Asp–His–Ser and the H-bonding distances at the intermediates and at the acyl enzyme structures. The distances 1–8 in Table 2 are in general longer when water is present. However, new bonds are created because of the presence of water. These new bonds are numbered 9–13 in Table 2. These bonds are in the range from 1, 9 to 3 Angstroms (Å), characteristics of H-bonding. Considering the data from Noris et al. some of the distances here found for the intermediate 1 without water are in the range reported for the RML transition state complexes for acid substrates with lipases. These published distances are 2.4–2.5 Å for 2; 3 Å for 3; 1.9 Å for 4 and from 1.8 to 3.6 Å for 8, being 2, 3, 4 and 8 entries of Table 2. In fact, the results for the oxyanion hole stabilization imply a short distance between the O alkoxide and the Hs from NH_2 of Thr40 of CALB [24].

The tetrahedral intermediate 1 and the acyl enzyme are surrounded by an H-bonding network, more important when water is present in the active site. The corresponding data are included in Table 2.

There are several interesting points to analyze in the tetrahedral intermediate 2 structures with or without additional water:

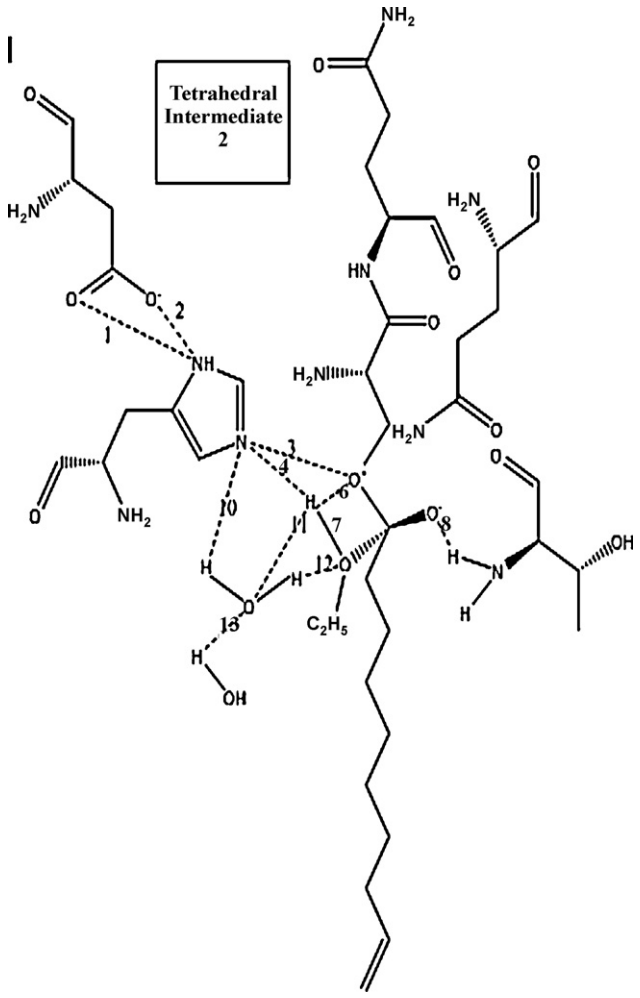

Fig. 6. Schematic picture of the tetrahedral intermediate 2 at the catalytic triad of CALB—that includes a water molecule between Histidine and Serine since the beginning of the reaction. Important distances are marked with dashed lines and numbered from 1 to 13 (see Table 2).

(a) The distance H (from coordinated EtOH) to N from Histidine is 4.5 Å when no water is present between Serine and Histidine. This distance is long; but the distance H (from coordinated ethanol) to O (from Serine) is 2.31 Å (see Table 2, entries 4 and 6). Looking at this situation the transfer to Nε (of Histidine) of an hydrogen would not be favored in terms of distance. The transfer to the Serine seems favored instead.

(b) If water is present at the tetrahedral intermediate 2 the main H to be transferred to Histidine would be from water. Water would receive the proton from ethanol, would transfer a proton to Histidine that would transfer the H to regenerate the Serine OH. The final step would be the release of product ethyl oleate. Distances 6 and 11 are of paramount importance in the context of this mechanism analysis. With water released from the acyl enzyme formation and located near the catalytic triad, the distance 11 (1.83 Å) is shorter than the 6 (2.31 Å). The distance H–N or distance 10 is near 3.2 Å.

(c) If water is present between Serine and Histidine in the tetrahedral intermediate 2, two water molecules may be at once near the active site when the tetrahedral intermediate 2 is generated. N(His) distance to H from the closest water would be 2.19 Å, whereas distance for direct transfer to Serine residue would be 2.38 Å (see Fig. 6).

The water between Histidine and Serine and/or the water released by the acyl enzyme formation may be both active part of an H-bonding network. This network affects the stabilization of the intermediates and/or the mechanism of proton transfer to Histidine and after that to Serine. Specifically, the transfer of an H to Histidine seems to be through water in an H-bonding network and not a direct transfer to O of Serine from the coordinated alcohol in the tetrahedral intermediate 2. A conformer with the Histidine protonated (N–H = 1.05 Å) presented a long distance N(His)–O(Ser) of 4.49 Å (results not shown in Table 2). Distances lower than 4.5 Å in Table 2 may be assigned to H-bonding. H-bonding up to 2.30–2.5 Å have been correlated with energies of near 33–40 kcal/mol, whereas from near 2.5–3.3 Å the energies found have been in the 25–2 kcal/mol. The energy will be higher when the atoms involved in H-bonding are N and O. Between 3.5 and 4.5 Å for the H-bonding, the energies found are in the range of the interactions found between hydrocarbons and polar compounds with SH or OH bonds [27].

Experimentally, using 50 mg of CRL/PP with no water present in the beginning in the solvent-free (or SF) reaction media the molar conversion of 3 g of oleic acid (at molar ratio oleic acid:ethanol = 1:1) to ethyl oleate is near 4%. This conversion is similar to the non-catalyzed reaction. However, it is near 9% when 20% (w/w) oleic acid of water is present. The conversion was tested for all cases after 7 h at 45 °C (with excellent reproducibility). For free CRL (using equivalent amounts of lipase to CRL/PP) the total conversion is almost the same with or without water present than the obtained with the CRL/PP. Using 50 mg of CALB/PP an important impact of the initial water content in the reaction kinetics is found. With CALB/PP the conversion is near 11% with no water added initially or 70% with 20% (w water/weight) oleic acid present initially. For equivalent amounts of free CALB the total conversion of oleic acid to ethyl oleate is near 14% without water or 78% with 20% (w water/weight) oleic acid present initially [13]. The conversion found without water added for equivalent amounts of free CRL or CALB is then 4% and 11%, respectively. But, we have near 8 times more conversion with CALB than with CRL (at the same conditions, with high initial water contents). Also, we have almost 6.5 times more conversion for free or immobilized

Table 2
Distances as shown in Figs. 4–6: labelled 1–13, in Angstrom-NW = no water included between Serine and Histidine-W = Water included between Serine and Histidine. The Intermediate 2 (Int 2-NW) has a water molecule coordinated—from the release of water due to the acyl enzyme formation (see Fig. 6).

Model CALB	1	2	3	4	5	6	7	8	9	10	11	12	13
Int 1[a]-NW	3.19	2.61	3.44	2.38	3.04	1.15	2.65	2.47	No	No	No	–	–
Int 1[a]-W	3.46	2.49	3.82	2.75	4	1.15	2.58	2.48	2.01	2.83	2.46	–	–
Acyl enz[b]-NW	3.54	2.53	3.87	2.18	2.60	4	0.94	2.05	No	No	No	–	–
Acyl enz[b]-W	4.16	3.48	5.32	2.23	3.41	4.39	0.94	2.66	1.993	3	2.02	1.98	–
Int 2[c]-NW	4.52	2.71	5.54	4.5	No	2.31	0.98	2.37	No	3.20	1.83	–	–
Int 2[c]-W	3.83	2.32	4.98	5.84	No	2.38	0.99	2.37	No	2.19	4.24	3.53	1.93

[a] Int 1, tetrahedral intermediate 1.
[b] Int 2, tetrahedral intermediate 2.
[c] Acyl enz, acyl enzyme.

CALB with water present initially compared to no water present initially. CRL only increased near 2.5 times the conversion comparing the results without water or with the optimum amount of water used. It is interesting that esterification with CRL is activated whereas CALB is not from our molecular modeling, when ethanol and water are present. However, the differences between the structures of CRL and CALB are important in terms of lid, tunnel and active site surroundings [8]. The presence of water affects CRL and CALB in the experimental solvent-free esterification of oleic acid with ethanol and this simple molecular modeling is in agreement with these experimental results. CALB seems very sensitive to the amount of water present initially in this solvent-free esterification of oleic acid. The results reported here may be *part* of an explanation of the results presented above. These results include phase transfer effects in the biphasic system oleic acid–ethanol/water. The MM2 results give also support to an additional role of the water. This additional role of water is beyond simple needed hydration and overall conformational flexibility for CRL and CALB. The MM2 results then are in qualitative agreement with the experimental reports and simulation studies from our group [8,10–13].

4. Conclusion

A simple MM2 approach applied to the molecular modeling of enzymatic ethyl ester synthesis of model fatty acids allowed the determination of steric energy for conformers associated to each step of the mechanism of reaction using different models for CRL and CALB. In the case of the CALB model, the esterification reaction is carried forward with no energetic barrier, and considering the MM2 results, the ethyl oleate exit is the only endothermic step. On the other hand, in the case of the MM2 simulation using the LAS model of CRL, results showed a steric energy barrier of 39.5 kcal/mol without water present between Histidine and Serine and only 8 kcal/mol if water is considered as a part of the active site of CRL.

Water and ethanol are thought to provide a network of H-bonding that lowers the energy of each step of the reaction considered, implying a more favorable pathway. But potentially they also stabilize too much the adsorption of substrates and intermediates, being then their effects complex. Answering the questions presented in the introduction:

(1) One water molecule has impact in the steric energy profile of the esterification reaction of oleic acid with ethanol using models of CRL and CALB.
(2) Gas-phase theoretical data obtained with enough care considering the structural main facts of the catalytic triad/oxyanion hole/tunnel of the lipases may be used to qualitatively explain solvent-free esterification data of fatty acids with ethanol and
(3) Even limited computational tools in the context of an MM2 calculation may provide useful information if the limitations and strengths of the procedure are taken into account. Complete complementary experimental work must be available, including the study of the role of substrates/products transfer between phases and diffusional effects, especially in biphasic systems.

The impact of ethanol and water in the mechanism of enzymatic esterification of fatty acids with ethanol using lipases is complex in nature. Both molecules play a role on local interactions at the level of the catalytic triad and the formation of an H-bonding network. Our modeling results are in qualitative agreement with our experimental results in solvent-free esterification using CRL and CALB-free and immobilized on PP.

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