Computational Approach to Solvent-Free Synthesis of Ethyl Oleate Using *Candida rugosa* and *Candida antarctica B* Lipases. I. Interfacial Activation and Substrate (Ethanol, Oleic Acid) Adsorption

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This paper presents the results of a MM2 study of the adsorption of oleic acid and ethanol/water in the tunnel and active-site models of lipases from *Candida rugosa* and *Candida antarctica B.* The role of an interface polar/no polar in the opening of *C. rugosa* lipase's lid is also addressed, discussed and analyzed at the level of the conformational changes needed to achieve the lipase open form. The adsorption of oleic acid and alcohols considering *C. antarctica B*, a lipase not interfacially activated, is also presented. In this case, the tunnel is shorter than in case of *C. rugosa* lipase. Two different pockets can be visualized at the active site-tunnel model of *C. antarctica* B lipase: one for the acyl group and another for the alcohol. Wrong location of alcohol and oleic acid severely hinders reaction because it hinders the H-transfer to histidine, a key step in the reaction mechanism. Right location of alcohol decreases the possibility of alcohol inhibition. In the case of *C. rugosa*, no restrictions for ethanol/water location are found. For that lipase, a second adsorption site for oleic acid (outside the tunnel) is presented. This site is the exit tunnel of the ester product when oleic acid is adsorbed in the tunnel. Experimental results of our own that correlate with this study are presented.

1. Introduction

Computer simulations have proven to be a powerful tool in the study of protein structure. Numerous reports have been published on the behavior of proteins in the crystal lattice in a vacuum and in aqueous solution.[1−5] Proteins possess a wealth of conformational states of nearly similar energies.[1] The surrounding medium may influence protein flexibility by altering the energetic barriers separating these states. The results of molecular simulations demonstrated that all the proteins are more flexible in water than in organic solvents and intramolecular forces are stronger in the organic solvents. In aqueous medium, the ratio of surface area to volume of the enzyme decreases and hydrogen bonds and ion-pair interactions increase.[6] When solvent-free medium is analyzed (as it is in the system studied in this contribution), the mixture of substrates becomes the solvent. In particular, in fatty acid esterification reactions, the fatty acid/alcohol mixture, where fatty acid is the major component, becomes the solvent.

Complete understanding of enzyme−substrate−solvent interactions is necessary in order to increase enzyme utility in organic synthesis. Diffusional limitations have been ruled out.[5] The efforts of many researchers have been focused on the study of the binding energy of enzyme−substrate. The reaction intermediates have also been studied carefully, but solvent has not been considered yet.[6,7] The results for solvent-free synthesis, in particular of those with ethanol as substrate, are different from those obtained with organic solvents. Inhibition with ethanol is much stronger and agglomeration effects are determinant. Moreover, the structure of the lipase and the role of hydrophobic/hydrophilic interactions seem to be of huge importance. Several reports from our group demonstrated that, under the same conditions in solvent-free systems, *Candida antarctica B* lipase (CALB) and *Candida rugosa* (or *Pseudomonas fluorescens*) lipase (CRL) are totally different catalysts. At the best condition found, we obtained 76% conversion to ethyl oleate for free or polypropylene-supported CALB whereas only 10−18% was achieved for free and supported CRL. In the case of CRL, activity is strongly dependent on pretreatment (with an oil/water interface, with a bioimprint molecule, etc). In the described reaction medium, CRL lipase has been shown to need the presence of water at such high concentrations as 20% of the oleic acid mass to achieve high conversion.[8−10]

Besides molecular modeling, the methods used to investigate the molecular factors of selectivity combine kinetic and structural data. The best approach is to model the tetrahedral intermediates with the assumption that they are good mimics of the transition states, especially when enantioselectivity is analyzed. Most computer simulations of lipase-catalyzed resolutions have revealed steric interactions between slower-reacting enantiomers and the amino acids of the active site. However, none of them have dealt with the adsorption of oleic acid and ethanol/water, considering the presence or not of water in the active site for such different lipases as those from *C. rugosa* and *C. antarctica*.

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The protocol used in this study can be compared to dynamics simulations under constraints. Since this kind of protocol has been used to discriminate enantiomers, it is clear that it can be used to analyze different adsorption conformations of the same substrate on active sites of different structure or lipase source. Molecular mechanics consists of an analytical function defined in terms of classical physics. It does not treat electrons explicitly; therefore, chemical reactions cannot be studied. Several force fields have been developed such as MM2, MM3, CVFF, CHARMM, AMBER, OPLS, and GROMOS for proteins, sugars, RNA, and DNA.[11] The cross terms present, for example, in MM3 are of minor importance in protein modeling and give rise to unnecessary computations if applied to proteins. Several authors use also molecular dynamics (MD) to search the conformational space for low-energy conformations. Although QM/MM and GRID methods have been reported to be the best approach, there are several points to solve in this approach to consider the effect of substrates, solvent, and residues near the active site. We tried to include an approach to QM/MM. Generally the solvent, or even the complete fatty acid in the case of solvent-free fatty acid reactions, has not been considered. Substrates are modeled by use of simple molecules, like acetic acid; solvent is avoided, and alcohol is methanol. The first application to enzyme systems through the use of explicit solvent and the determination of atomic point charges including the enzyme environment is due to Colombo et al.[12]

In this paper the interaction of the active sites of CRL and CALB with oleic acid and ethanol is presented. Oleic acid being the solvent of the system, the study of the solvent is therefore included. It is known the *C. rugosa* lipase has interfacial activation. Then, the interaction of the flap of *C. rugosa* lipase (obtained from PDB: open 1CRL, closed 1GZ7, other 1TRH) with a string of several CH_2 (as a model of a long hydrocarbon) is analyzed to take into account the requirements to open the flap and the importance of an interface, including water. Different steps for the cis−trans proline isomerization at proline-92 of the lid, a known step for opening the lid in CR lipase, are analyzed and discussed with a small model. Although QM/MM method is considered to be the best approach, the combining of MM and semiempirical methods is useful to give insights on the relative importance of different reactions at the active site, with an appropriate selection of the conformational state.

2. Methodology

Structures of these enzymes were obtained from the Protein Data Bank, by use of the Swiss−PDB viewer program, and analyzed with the MM2 and PM3 methods from Chem3D software of Cambridge Soft at the active site and the access to the active site.[13a] The MM2 in Chem3D force field (from here MM2 applied in this work) has been demonstrated to produce similar potential energies than CVFF force field when methyl-hexylphosphonate binding (1*R* and 1*S*) to an active-site model for lipases (Ser, His, Glu) was analyzed. This methodology is similar to the common study in inorganic computational chemistry of planes of perfect metal/oxides clusters as models of otherwise infinite, defective inorganic solids. The study of partial models including only the active site as mobile portion, maintaining fixed the neighborhood and the rest of the protein for semiempirical calculation, can provide insights at the local level of the effects of supports, solvents, and inhibitors. After a MM2 minimization of the tunnel and catalytic triad models of the lipases, a PM3 study was carried out to address the electronic configuration at the active-site level. The PM3 method is a semiempirical molecular orbital calculation that takes into account changes in electronics to compare different conformers. The output of PM3 is $\Delta H_f°$. The MM2 minimization provides the steric energy. This approach was selected to avoid undesirable changes in the conformational space of the enzyme that would occur with a PM3 overall minimization. The number of atoms (more than 100) makes it difficult to apply density functional theory (DFT), with the known problems of structural minimization in these cases. When DFT is used, the minimum is dependent on the initial structure since all the atoms in the protein cannot be used. PM3 minimization can include only a few atoms and the results are highly dependent on the initial structures because only electronic effects are addressed, and sometimes with not well-selected parameters or basis set. The protocol is similar to constrained molecular dynamics. The effect is even worse for DFT.

From the X-ray structure of the lipase from the PDB and by isolating the portions of the enzyme in which we are interested (lid, tunnel, active site, side-chain residues), we can study *at the local level* the effects of support—in this case, polypropylene—water, solvents, and inhibitors (if present).[14−18] As previously stated, this can be compared to a constrained dynamics simulation. In other words, the MM2 is applied to the bigger portion and the PM3 to the smaller one. This approach tries to be a quantum mechanical/molecular mechanical (QM/MM) approach, obviously with limitations because PM3 is not an ab initio method. The force field MM2 in Chem 3D includes a new implementation of the force field of MM2 by Norman L. Allinger, based mainly on the work of Jay Ponder of Washington University. Parameters were provided by N. Allinger and additionally by J. Ponder.[13c]

3. Structure of Active Sites and Tunnel of CR, CALB, and Other Lipases

C. rugosa lipase includes 534 amino acids with an active Ser 209−His 449−Glu 341 catalytic triad embedded in an environment of primarily hydrophobic amino acids and covered by a lid formed by an amphipathic α-helix. The PDB entries are 1CRL (open) and 1TRH (closed). This lipase has been carefully studied. The oxyanion hole is formed by Gly 124 and Ala 210.[19] The formation of the oxyanion hole does not depend on the repositioning of amino acid residues following the opening of the lid. Unique to this lipase is the involvement of the carbohydrate moiety (Asn 351) in the stabilization of the open conformation, through its interactions with the open lid. This lipase belongs to a family of enzymes whose composition can vary according to the

growth conditions. The *C. rugosa* family has a tunnellike binding site.[20] The part of the flap of CR involved in the rearrangement encompasses the region from Glu 66 to Pro 92. During the opening, significant changes in the secondary structure take place: the helical turn 69−72 unwinds to a more extended conformation, while residues 86−90 refold into a short α-helix. The center of one of the helixes travels a distance of 19 Å to become the part of the flap more exposed to the solvent. The flap maintains its amphiphatic character, in agreement with Cygler and Schrag.[19] Region 66−92 makes more hydrogen bonds in the open than in the closed form. The movement of the flap is associated with a rearrangement of its secondary structure and the cis−trans isomerization of the peptide bond of Pro 92. It appears that the more affected residues in the change from closed to open conformation are Glu 66, Thr 68, Lys 85, and Pro 92, which changes from cis to trans. It is evident from the structure analysis that lid from *C. rugosa* has no arginine residue but an asparagine 79.[19]

Comparing *C. rugosa* lipase with that from *Ps. fluorescens*, the second one is smaller and nonglycosylated. Moreover, it has two salt bridges involving calcium ions, which seem to be important to stabilize its structure. The process of refolding of *C. rugosa* lipase can be a rather complex one, whereas for lipase from *Pseudomonas* it is claimed to be easier. Lipases from *Ps. fluorescens* are highly selective, especially for hydrolysis of esters of secondary alcohols. Those lipases accept less-bulky ligands than lipases from *Candida* do, but they have been described to be very selective for narrow open-chain substrates with chiral center located near and remote from the reaction center. *Ps. fluorescens* monomeric lipase has amine residues distributed homogeneously on the surface of the lipase and not concentrated in a specific area of the protein.[20−21] *C. rugosa* lipase tends to aggregate through its very hydrophobic lid, making its handling difficult when dispersed enzymes are desired. No lysine enrichment regions are found in this enzyme (only six lysine residues), a fact that, compared with other enzymes, has promoted slower immobilization rates on inorganic solids. In this lipase the binding site is located in the tunnel inside the protein, which is at least 22 Å long with an internal diameter of about 4 Å. This tunnel can hardly be predicted because it is blocked by side chains. It is formed by Gly 124, Phe 125, Ser 209, Ala 210, Met 213, Val 245, Pro 246, Phe 296, Ser 301, Leu 302, Arg 303, Leu 305, Leu 307, Phe 345, Tyr 361, Phe 362, Ser 365, Phe 366, Val 409, Leu 410, Leu 413, Gly 414, Phe 415, Phe 532, and Val 534. The length of the total tunnel fits a fatty acid of C17. The alcohol binding site is not limited by a wall. The substrate accesses the binding pocket from the right-hand side [see ref 22 for an excellent review of this (and other) active sites of lipases].

In the case of lipase from *Mucor meihei*, it has a catalytic triad with a chymotrypsinlike triad Ser−His−Asp. Interfacial activation implies the displacement of the lid to expose the active site. For this enzyme, activation is enhanced in a hydrophobic environment.[22] The fatty-acyl chain of the substrate ester docks into the groove, aligning its ester bond with the catalytic triad sitting at the bottom of one end of the cleft. In this sense, the active site is crevicelike near the surface. Activation is controlled by Arg 86 and Asp 91 in the inactive conformation, Asp 91 experiences repulsive forces and pushes the lid toward the open conformation. Upon activation Arg 86 and Asp 61 approaches each other and in the active conformation these residues form a salt bridge that stabilizes the open conformation.

When *C. antarctica* B lipase is analyzed, the sequence includes 317 amino acids, which folds in an $\alpha\angle\beta$ hydrolase fold. The size is approximately 30 × 40 × 50 Å. Ser 105−His 224−Asp 187 triad is responsible for enzymatic activity. NAG 401, NAG 402, and BOG (octyl β-glucoside) stabilize the structure. CALB has a molecular weight of 33 000 and a p*I* of 6.0. The PDB entry used was 1TCB.The substrate fatty acid binding pocket of CALB is an elliptical, steep funnel of 9.5 Å × 4.5 Å. The substrate is oriented parallel to its long axis. The lid constitutes the right-hand wall (10.5 Å). The funnel has a hydrophilic bottom formed by Asp 134 and Ser 105. It is lined with Thr 138, Ile 189, and Val 190 on its left-hand side and by Gln 157 and the oxyanion hole residue Thr 40 on its right-hand side. Near Gln 157 the fatty acid kinks and follows the left-hand wall. From C7 to C13 it becomes hydrophobic. This hydrophobic binding site is formed by Val 154, Ile 285, Leu 144, and Val 49. Its width decreases with its minimum at C13 of the fatty acid chain. At 13.5 Å above the bottom, it reaches the end of the funnel and turns into the hydrophobic surface of the enzyme, which is in contact with the hydrophobic interface. The height of the wall at the alcohol binding site is 10.5 Å.

There is a common pattern Gly-X1-Ser-X2-Gly for the nucleophile serine and the oxyanion hole residue X2; an exception is CALB, with Thr-Trp-Ser-Gln-Gly. The oxyanion hole consists of two residues, which donate their backbone amide protons to stabilize the substrate in the transition state. One residue is located in the structurally conserved nucleophilic elbow. The other oxyanion hole residue is not located in a region with conserved sequence and structure. In all lipases, a glycine is conserved that contacts the nucleophilic elbow. Pleiss et al.[22] found that all lipases can be assigned to two different kinds of oxyanion hole: GX type (with G = glycine and X being the oxyanion hole residue), and the GGGX. In the case of CALB, which belongs to the GX type, X is Thr (hydrophilic).[20,22] In the case of *C. rugosa* lipase, the oxyanion is of GGGX type, and X is Phe. In the open form, the conformation of the dipeptide GG changes upon activation but GX does not. It has been proposed that a water molecule plays a role in the change. In the case of CALB, the X residue interacts with an anchor residue by hydrogen bonding (Gln 157). In fact, in CALB there are three anchor residues (Gln 157, Gln 106, and Leu 73). The oxyanion hole residue and the anchor residue line the bottom of the deep hydrophobic cleft to which the scissile fatty acid binds.

Figure 1 shows the relative size of CALB and CR lipases with respect to oleic acid and ethanol. A scheme of the active-site structures is also presented in Figure 2.

4. Results and Discussion

4.1. Opening of the Lid. CRL is active upon interfacial activation. Interfacial activation implies the opening of the

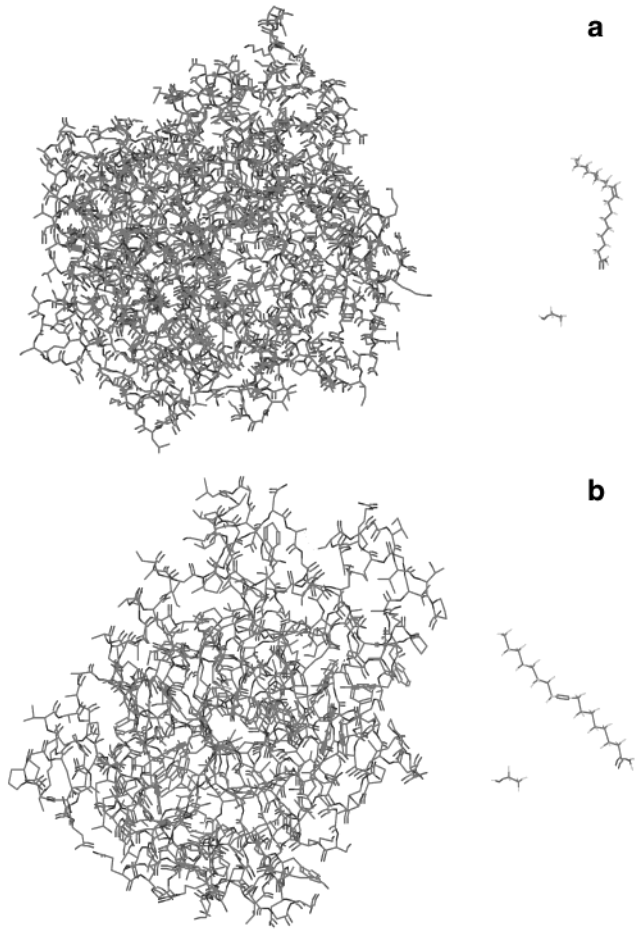

Figure 1. (a) Size comparison of (a) CRL and (b) CALB with oleic acid and ethanol. To be clearer, the hydrogen atoms of lipase were not depicted.

lid, with the cis−trans isomerization of Pro 92 and the shift of the lid almost 19 Å, before the oleic acid can be adsorbed on the active site. CALB works without interfacial activation.[23−26] This enzyme can be regarded as an intermediate between a lipase and an esterase. CALB has a very limited available space around the active-site pocket. The active site includes two channels, the acyl channel being more spacious than the alcohol channel. When secondary alcohols are analyzed, CALB is especially suited to produce the *R* enantiomer because of steric hindrance in the case of the *S* enantiomer transition state. In the following discussion, only the interfacial activation of CRL will be analyzed.

4.1.1. Steps for Opening of the CRL Lid with and without Water/PP: Effect of a Polar/Nonpolar Interface. The lid of CRL does not contain arginine, but it includes 3 Pro, 4 Glu, 1 Gly, 1 Thr, 1 Tyr, 1 Asn, 3 Leu, 2 Lys, 3 Ala, 1 Asp, 3 Val, 1 Met, 1 Gln, 3 Ser, and 1 Phe. A recent work of our group analyzed the interaction of amino acids with a model of polypropylene (PP).[27] The order in the interaction of the lateral chain of the amino acid with PP in terms of the attraction PP-lateral chain was

Glu > Leu > Ile > His > Val > Trp > Ser > Thr > Ala > Pro > Gly > Met > Asp > Lys > Tyr > Phe > Gln ≫ Arg.

The interaction of the lateral chain of the amino acid with water, considering the lateral chain nonionized, was

Arg > Glu > Asp > Asn > Trp > Phe > Gln > Lys > His > Ser > Met > Thr > Leu > Ile > Pro > Val > Ala > Gly.

The presence of CH_2 groups in the lateral chain of the amino acids is important to understand the interactions with PP and with water. Reported results of our group about the conformational change of the lid during the opening upon interaction with PP includes an analysis of the change in location of the lateral groups of the amino acids.[27] In the native form, CRL is exposed to water hydrophilic lateral chains and because of the hydrophobic effect the lateral hydrophobic chains are located to the inside of the lipase. Upon comparing the open and closed forms of CRL, it is clear that several hydrophobic lateral groups are exposed to the outside (to the solvent). It is obvious that the leading force for that behavior *must* be hydrophobic in nature to stabilize the location of these lateral groups. The role of the interface in the shift of the lid appears to be clear when the shift is modeled in several steps (up to 20 steps), as we performed and published in ref 27. These steps include the cis to trans isomerization of proline 92 (a real hinge point) and the conformational change of the major part of the residues of the lid. The isomerization implies an unfold−refold of the lid and the presence of an intermediate (tetrahedral) at proline 92, probably because of protonation due to the donation of a proton group from inside the protein. Clearly polar surroundings must be present, in addition to hydrophobic medium. As Cygler and Schrag[19] reported, the ϕ and ψ angles almost do not change for Met 82, Val 81, Leu 80, Asp 79, Leu 78, Ala 77, Ala 76, and Lys 75. The other hinge point is Pro 65. The conformational change of Asn 72, Leu 73, *cis*-Pro 74, and Lys 75 generate the precursor of the open lid and condition the opening of the lid. The presence of an interface, including up to eight water molecules, trapped in the interface PP−lid are enough to strongly stabilize the open lid. In the open conformation, several polar residues are located to the inside of the protein, just the opposite of the hydrophobic effect. We modeled in 10 steps the total conformational change. Considering the vacuum, the opening of the lid involves a change in steric energy of nearly −15 kcal/mol. If a model of PP is considered, the change achieves −25 kcal/mol.

4.1.2. CRL Mechanism for Cis−Trans Isomerization of Proline 92: Why the Interface? The only way that proline in the CRL's lid can suffer isomerization is upon protonation and change of the amide N to a transition state with an ammonium character. The best candidate for proton donation is a hydroxyl group of one of the serines in the protein structure. Water or even buffer of pH 7 is not acidic enough to protonate N from proline. We analyzed two serine residues as H donors: Ser 59 (located in the neighborhood of Pro 92) and Ser 91. When Ser 59 is considered as the H donor, the cis−trans isomerization of proline 92 implies a change in steric energy of +5.2 kcal/mol and a change in PM3 electronic energy of +2.8 kcal/mol.[28,29] When Ser 91 is considered as the H donor, we found that the change in steric energy is −9 kcal/mol and the change in PM3 electronic energy is −6.22 kcal/mol. Then, the donation of the proton from Ser 91 is energetically favored.

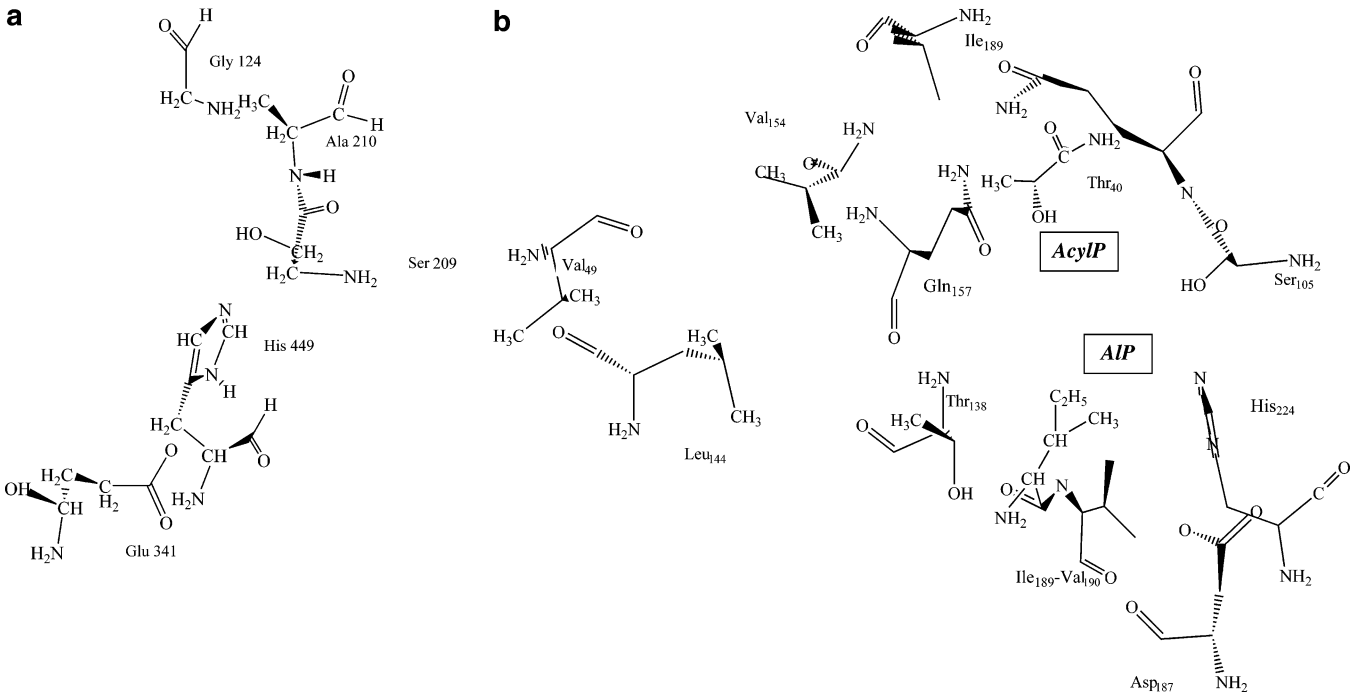

Figure 2. (a) CRL active site. (b) CALB active site and tunnel. AcylP, acyl pocket; AlP, alcohol pocket.

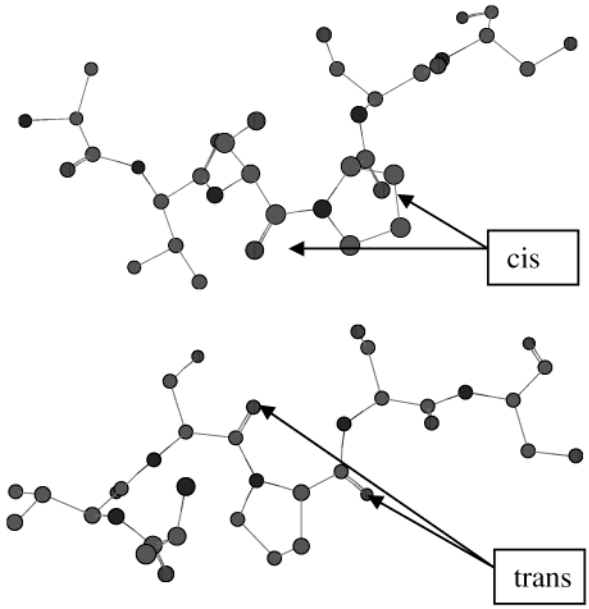

Figure 3. Cis−trans isomerization of proline 92 in the CR lid.

The donation of the proton of serine to amide N produces an N ammonium with the H endo to the structure (the H comes from an amino acid residue of the protein). This is a very favored reaction, with a change in steric energy of −113.5 kcal/mol. However, if the H is not donated by an amino acid residue but comes from outside the protein (in this case the H is placed exo), the structure is not so favored as when the H comes from the protein. This intramolecular reaction must take place in a polar local environment, where even the partial donation of H of serine could be possible. In absence of polar surroundings, H donation or bonding is not possible.

In Figure 3, the cis and trans forms of proline are presented. The amino acid string depicted was obtained from the PDB and analyzed with MM2. Discussion of the importance of cis−trans isomerization is found in ref 30. The total string involves residues from Ala 89 to Ser 94, including Val 90-Ser 91-Pro 92-Ser 93-Ser 94[31] (see Figure 3).

When the change in the lid is analyzed, it becomes clear that the keys to the opening of the lid are two: first, the cis−trans proline 92 isomerization, through a possible intramolecular H bonding (or donation) from serine 91; and second, the change from endo to exo of lateral hydrophobic groups and the change from exo to endo of lateral hydrophilic groups, in opposition to the known hydrophobic effect, as we proposed in ref 8. The interface role is now clear. A polar/nonpolar interface at optimum relation is needed in order for the hydrophobic *and* hydrophilic forces to act together and open the lid, at different points in a concerted mechanism.

4.2. Adsorption of Substrates. 4.2.1. CRL Adsorption of Oleic Acid and Ethanol in the Tunnel: Role of Water. The adsorption of oleic acid in the lipase tunnel was modeled by considering the whole tunnel of the lipase. The length of the tunnel of CRL and the length of the fatty acid molecule are very similar. In Figure 4 it is evident that the structure of the oleic acid fits exactly in the structure of the tunnel of the lipase. Oleic acid has a Connolly molecular area of 359.3 Å^2. Although the length of the fatty acid fits almost exactly in CRL's tunnel, when placed near the serine group, the oleic acid occupies only about 60% of the tunnel area.

Adsorption of oleic acid into the CRL tunnel, with no water in the neighborhood of the active site, is endothermic by 29 kcal/mol. If a water molecule is included near the serine residue, the adsorption is exothermic by 1 kcal/mol. The presence of ethanol in the neighborhood of the His group of the catalytic triad is exothermic by about 3 kcal/mol. *These facts reveal that polar molecules such as water and ethanol facilitate the adsorption of oleic acid's polar head in the tunnel.* It is clear that in a solvent-free medium the polar

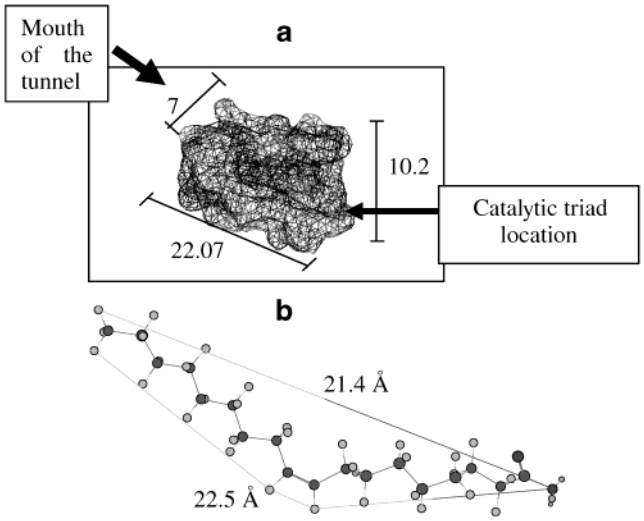

Figure 4. (a, top panel) Molecular surface of CRL tunnel (without substrates). The horizontal arrow indicates the position of the active site. Of the length of 7 Å shown, just 4 Å are really available. (b bottom panel) Dimensions of oleic acid, considering the shape of the molecule (22.5 Å) or not (21.4 Å).

head of oleic acid is surely solvated by water (if present)/ethanol. We proved experimentally that the presence of water improves the activity of free CRL; in fact, free CRL is hardly active in the absence of water.[8−10]

The beneficial effect of water seems to be far more important than just the formation of an interface and an emulsion/biphasic medium. It seems that, when adsorbed with oleic acid/ethanol substrates, water changes the conformation of the neighborhood of the catalytic triad of CR. Published experimental details of the structure of the open CRL agree with this finding, especially from the work of Grochulski and co-workers.[29,30]

Figure 5 shows the front and lateral views of *C. rugosa* lipase. The squared area shown in Figure 5a is analyzed in Figure 6, with only the short "tunnel" walls of this secondary site. The lid and active-site triad have been labeled in Figure 5b.

4.2.2. Adsorption of Oleic Acid outside the Tunnel: The "Ester Exit" Tunnel. Figure 6a shows the position of the open lid, the catalytic triad, and a proposed exit for the ester, once formed, in *C. rugosa* lipase. Pleiss et al.[22] recently published that mutations inside the tunnel of *C. rugosa* demonstrated that chain length specificity toward fatty acids can be explained by a simple mechanical model of blocking side chains from binding. Mutations at the entrance of the tunnel introduce challenging properties. Mutant L304F did not accept C4 and C6 chains but hydrolyzed longer chain lengths > C8. The authors explained these observations by assuming the existence of an alternative binding site outside the tunnel. Short-chain scissile fatty acids preferably bind to the tunnel, while medium- and long-chain scissile fatty acids can bind either to the tunnel or to an alternative scissile fatty acid binding site outside the tunnel. This site outside the tunnel has been suggested to explain the switch of enantioselectivity for chiral acids, which are too bulky to bind into the tunnel of the homologous *G. candidum*. The site outside the tunnel also explains the effect of mutations at the entrance of *G. candidum* lipase.[32]

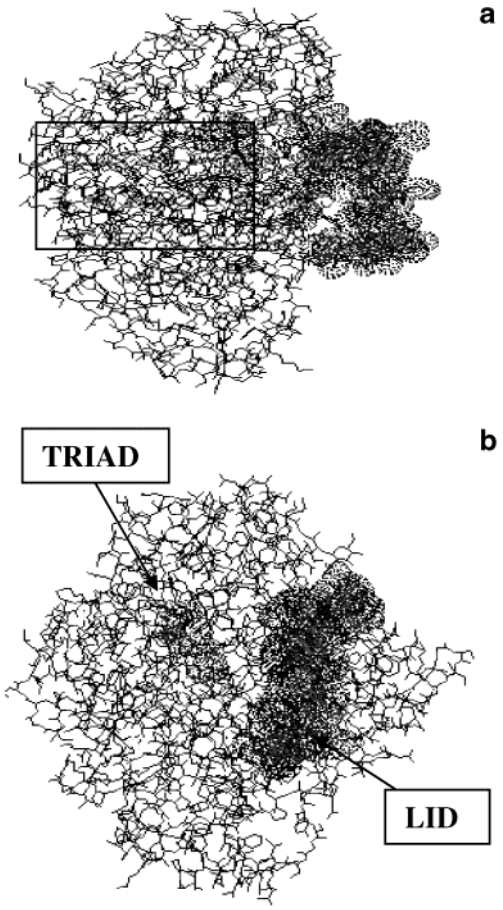

Figure 5. (a) Lateral and (b) front view of CRL.

The exit of ethyl oleate molecule must be easy in order to avoid product inhibition. In this sense, it is difficult to think of the ester *walking down back* in the tunnel. The exit of ethyl oleate was modeled from the catalytic triad to the outside of the lipase through the road shown in Figure 6b. The change in steric energy from ethyl oleate placed at the mouth of this second tunnel (inside the lipase) to ethyl oleate located outside the lipase was near 4.65 kcal. This minimum was found by MM calculation. This exit tunnel for ethyl oleate also can be a binding site for oleic acid, ethanol, and solvent. The analysis of the structure shows that the exit tunnel's mouth and exit are polar in nature and the inside is hydrophobic. Therefore, oleic acid adsorption is favored at the mouth, but the path followed to reach the catalytic triad involves a total change in steric energy of +11.9 kcal. In this sense, for C18:1, binding in this site would be favored only if the original tunnel is blocked. Therefore, C18 in the absence of water/ethanol may adsorb by the open lid through the tunnel or by this secondary site. This can be a reason for substrate inhibition, because the exit of the ester is severely hindered when oleic acid is adsorbed in this secondary site. Our experimental results showed that a pretreatment of PP-supported CR lipase with *oleic acid* deactivates *C. rugosa* lipase completely. Subsequent addition of ethanol/water mixture did not recover lipase activity.

Figure 6 panels c and d show the position of oleic acid at the entrance of the ester exit and adsorbed near the catalytic triad. The structure of *C. rugosa* lipase seems to be designed to include the two channels mentioned above. The molecular

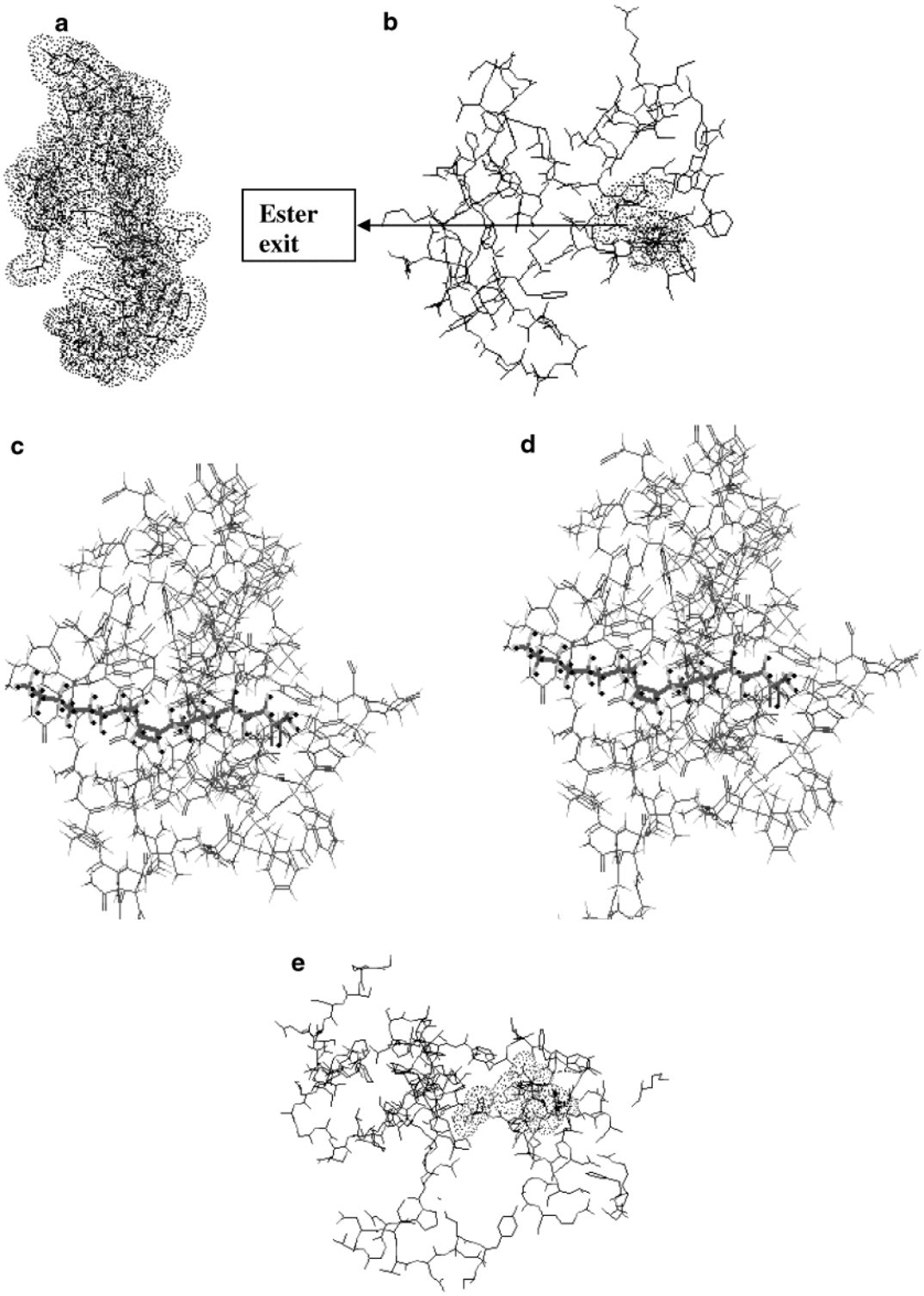

Figure 6. (a) CRL's lid. (b) Ester/oleic exit/second adsorption site. (c) Oleic acid adsorbed at the mouth of the secondary adsorption site, and (d) oleic acid adsorbed at the neighborhood of Ser 209 of CRL; the oleic acid chain is shown in bold. (e) View of alternative adsorption/exit.

mass of this lipase is in line with this proposal: 60 kDa vs 35 kDa for the other kinds of lipases. The ester exit when esterification is analyzed becomes the fatty acid exit if hydrolysis is considered.

Structure of this alternative tunnel implies the following residues: Ser 212, Asp 337, Gln 338, Asn 339, Asp 340, Glu 342, Thr 343, Phe 344, Phe 345, Gly 346, Lys 404, Ser 407, Ala 408, Leu 410, Gly 411, Asp 412, Gly 414, Phe 415, Thr 416, Leu 417, Arg 419, Arg 420, Asn 516, Phe 517, and Arg 518. This tunnel includes mainly polar residues but also strings of nonpolar residues (from Phe 344 to Gly 346) (see Figure 6e).

4.2.3. CRL Adsorption of Oleic Acid and Ethanol at the Active Site: The Stereoespecific Pocket and Its Function in Adsorption. The adsorption of oleic acid in the lipase tunnel was carried out considering the whole tunnel. It was evident that the structure of the oleic acid fits almost exactly in the structure of the tunnel.

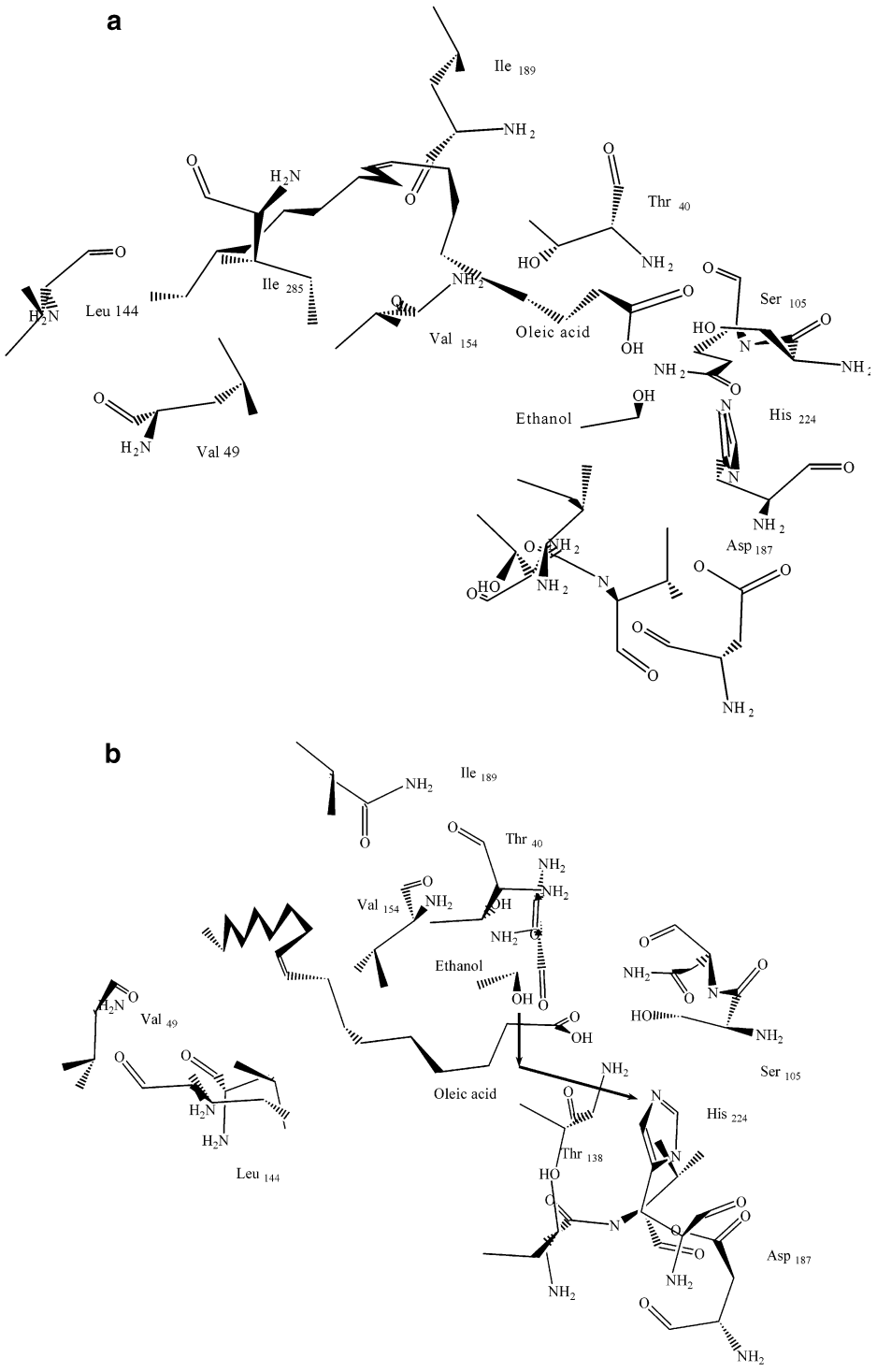

Figure 7. Ethanol and oleic acid adsorption on the CALB active site. (a) The oleic acid is adsorbed in the acyl pocket and the ethanol is adsorbed in the alcohol pocket. (b) The oleic acid is adsorbed in the alcohol pocket and the ethanol is adsorbed in the acyl pocket. The arrow shows the direction of ethanol movement in the approach to histidine when ethanol is located in the acyl pocket and not in the alcohol pocket.

Once the fatty acid has reached the active site, in the modeling of the adsorption of oleic acid at the active site, the tunnel was ignored and only the amino acids surrounding the active site were considered. The adsorption was evaluated by locating the chain in the tunnel direction and outside the tunnel. In this last case the adsorption of the fatty acid on the active site would be through the lateral solvent adsorption site of lipase, with the solvent pocket located near the active triad.

Results showed that adsorption of the COOH at the active site by the tunnel is strongly favored with respect to adsorption outside the tunnel by the solvent lateral adsorption site. Presence of water does not introduce any constraint on the adsorption since the change in steric energy is always

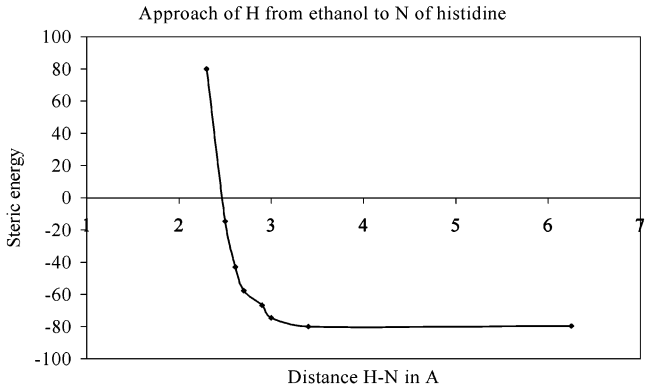

Figure 8. Dependence of the steric energy of the interaction H (ethanol)···histidine with H (ethanol)···histidine distance, when the wrong substrate adsorption occurs.

−13.3 kcal/mol. With respect to ethanol, there is no hindering for the approach of ethanol to any of the residues of the catalytic triad. This fact is closely related to the known (and confirmed in our experiments) strong deactivation suffered experimentally by *C. rugosa* in the presence of ethanol.

4.2.4. CALB Adsorption of Oleic Acid and Ethanol at the Active Site. For *C. antarctica* B lipase, the modeling includes the tunnel plus the catalytic triad and ethanol (water)/oleic acid. In the case of the intermediary acyl enzyme, there is a water molecule placed at the location where calculations showed a minimum of energy. For this enzyme, adsorption of oleic acid and ethanol can take place in different pockets: the acyl pocket and the alcohol pocket (see Figure 2b, AcylP and AlP, respectively).

Adsorption of the acid in the acyl pocket and the alcohol in the alcohol pocket was the first situation studied. Adsorption of the alcohol in the acyl pocket and the acid in the alcohol pocket was also addressed.

(a) If oleic acid is adsorbed in the acyl pocket and ethanol in the alcohol pocket, the steric energy of the model is −69.5 kcal/mol. In this conformation, H from ethanol is located at 2.2 Å from N of histidine. The intermediary acyl enzyme has a steric energy of −73.4 kcal/mol and the change in the steric energy is −3.9 kcal/mol. Ethyl oleate plus water has a total steric energy of −73.6 kcal/mol. The reaction of ethanol with histidine (beyond the H transfer) is sterically hindered because the ethanol is placed parallel to histidine, pushed away by the oleic acid chain. No place to locate the ethanol is available (if it reacts with histidine by opening of the N═C bond) (see Figure 7a).

(b) If oleic acid is adsorbed in the alcohol pocket and the alcohol is adsorbed in the acyl pocket, the steric energy of the residues is −76.3 kcal/mol. The acyl enzyme intermediary has an steric energy of −79.6 kcal/mol and the change in the steric energy is −3.3 kcal/mol. Distance as high as 3.401 Å are found at minimum steric energy from alcohol to N from histidine. With this approach, the H transfer to histidine is severely hindered (see Figure 7b). Therefore, this approach is unproductive to generate ethyl oleate, but it can deactivate the active site because the acyl enzyme can be formed. It has been found that CALB can be deactivated by the fatty acid under selected reaction conditions. Figure 8 shows the repulsive interaction of H (ethanol) with histidine when the ethanol is approaching histidine to initiate the H transfer.

4.3. Role of Different Amino Acids in the Stabilization of the Open Form of the Lid: Revision of Published Literature. Examination of the amino acid sequence of the lid regions of lipases from *Rhizomucor meihei*, porcine pancreas *H. lanuginosa*, and *C. rugosa* showed the presence of an arginine residue only in those lipases that were found to be inhibited by guanidine. In this sense, *C. rugosa* has no arginine in the lid[31,33,34] and no inhibition was found. On the other hand, the additional stabilization of the open form found when there is arginine in the lid structure is also lost. The role of the protein as a macromolecular ligand has been studied in the case of oxidoreductases, especially peroxidases and cytochrome 450 that includes a hemo group as prosthetic group, with powerful software (intermediate neglect of differential overlap/restricted open-shell Hartree−Fock/configuration interaction, INDO/ROHF/CI) and it was demonstrated that the electronics is severely affected by the protein conformation and primary structure.[35] It has been published that cis−trans isomerization of proline is intramolecularly assisted in polypeptides containing His or Arg near the Pro. Moreover, carbohydrate linked to Asn 351 is proposed to play a role in stabilization of the open lid in the case of CRL. The main kinetic phase in the folding of acylphosphatase, AcP, is associated with the formation of a functional and catalytically active enzyme, confirming previous findings that a large fraction of AcP molecules fold in a cooperative two-state manner. Given that the isomerization of the Gly 53−Pro 54 peptide bond is the origin of the slow phase of the acylphosphatase folding, the question naturally arises as to the effect of cis−trans isomerization of the Ser 70−Pro 71 peptide bond in the folding of this small protein. The cis to trans isomerization appears to have to take place in the unfolded state prior to folding and to be the rate-determining step for the folding of these molecules. Folding processes associated with proline isomerization have been studied in detail and are best understood in ribonucleases A and T1. Both proteins contain two cis X−Pro peptide bonds in the native protein. Under strongly refolding conditions, all unfolded molecules of ribonuclease T1 can convert very rapidly into native or nativelike intermediates regardless of the configuration of their proline residues.[36] The reversible protein phosphorylation on serine or threonine residues that precede proline (pSer/Thr-Pro) is a key signaling mechanism for the control of various cellular processes, including cell division. It is interesting to note that Pro 92 in CRL belongs to a sequence Ser 91-Pro 92-Ser 93 in the lid, a short sequence found also in AcP and ribonucleases. Recent published papers on protein structure and solvent effects pointed out the importance of sequence−function correlations.[37−38]

5. Conclusion

The interfacial activation showed by *C. rugosa* lipase is achieved through the opening of the amino acid lid that covers its active site. Molecular modeling has showed that the key for the opening of CRL's lid is the isomerization of

Pro 92. The only way for proline's isomerization to take place is through the protonation of its N. The best H-donor for the protonation of proline seems to be the Ser 91 residue. Water or buffer of pH 7 is not acidic enough to be able to protonate Pro 92. The CRL's lid opening involves the relocation of some hydrophobic groups from endo to exo and of some hydrophilic groups from exo to endo. A polar/nonpolar interface is needed in order for hydrophobic and hydrophilic forces to act together and ensure the opening of CRL's lid.

Adsorption of oleic acid in the tunnel of CRL in the absence of water is an endothermic process, whereas in the presence of ethanol/water the adsorption is exothermic. Polar molecules have been shown to facilitate the adsorption of the polar head of oleic acid on the lipase tunnel. The adsorption of oleic acid molecule at the active site of lipase through the lipase tunnel is energetically favored with respect to its adsorption through the solvent lateral site. The existence of a tunnel through which the ester produced leaves the lipase, and through which the fatty acid adsorption may also occur, has been proposed.

With respect to CALB's active site, it has two different pockets in which the oleic acid and the ethanol may be adsorbed. The formation of ethyl oleate is favored by the adsorption of the fatty acid in the acyl pocket and the ethanol in the alcohol pocket. Adsorption of the acid in the alcohol pocket and ethanol in the acyl pocket may cause lipase deactivation due to oleic acid.

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