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MODELING OF SOLVENT EFFECTS IN THE ACTIVATION OF THE LIPASE FROM *RHIZOMUCOR MIEHEI*

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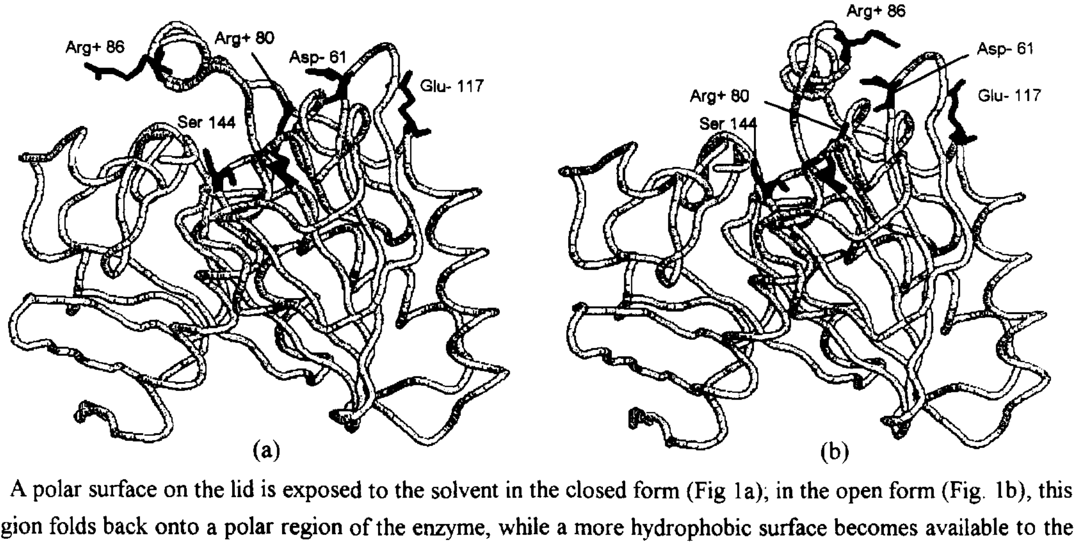
Abstract: the effects of water and hydrophobic solvents on the stability of the open and closed forms of *R. miehei* lipase were evaluated with different solvent models. Desolvation of arginine 86 at the water-lipid interface plays a key role in the activation process, while the contribution from hydrophobic stabilization of the open form is less important. Copyright © 1996 Elsevier Science Ltd

Rhizomucor miehei lipase is an excellent model for understanding the mechanism of lipase interfacial activation.[1] The crystal structures of both the native ("closed")[2] and inhibited ("open")[3] forms of the enzyme are available. In the closed form the catalytic site is buried under an α-helical loop (the "lid"); the lipase is thus inactive in its native form. In the open structure, the lid is displaced from the catalytic site by a bending at two hinge regions: the α-helix of the lid moves approximately 8 Å away, and this allows the substrate to reach the serine 144 catalytic group (Fig. 1).

Figure 1. Closed (a) and open (b) forms of the lipase from *R. miehei*. The inhibitor is removed from the open form.



A polar surface on the lid is exposed to the solvent in the closed form (Fig 1a); in the open form (Fig. 1b), this region folds back onto a polar region of the enzyme, while a more hydrophobic surface becomes available to the solvent. The opening of the lid is thus accompanied by an increase, by approximately 750 Å^2, of the hydrophobic surface accessible to the solvent and stabilization of this surface by the nonpolar lipid phase was proposed as the driving force in the activation.[4] More recently, *in vacuo* molecular dynamics and mechanics calculations have suggested that the open form of the lipase is strongly stabilized by intramolecular electrostatic interactions, mainly between the Arg+ 86 residue of the lid and Asp- 61 on the surface of the enzyme (Fig 1b).[5]

In this communication we describe a theoretical study on the effects of polar and hydrophobic environments on the stability of the open and closed forms of the enzyme. Calculations were carried out by molecular mechanics[6] and the effect of the solvent was evaluated, at different levels of sophistication, by three models: *i.* a simple scaling of electrostatic interactions modulated by the dielectric constant ε in the electrostatic term of the force field potential; *ii.* a discrete solvent model including, for simplicity, a limited number of solvent molecules localized in proximity of the lid and *iii.* a more sophisticated polarizable continuum model (PCM).[7]

The crystal structures of the native[2] and inhibited[3] lipase, obtained from the Brookhaven Protein Data Bank (PDB), were used as starting geometries for the closed and open forms, respectively; water and the inhibitor were removed and the structures were optimized[6] after addition of hydrogen atoms, at pH=7.[8] Calculations were repeated with an effective dielectric constant $\varepsilon = 1$ - to simulate a hydrophobic environment - and with $\varepsilon = 10$, representing, in this simplest model, the effect of a polar environment.[9]

Both the closed and open forms correspond to energy minima at $\varepsilon = 1$ and at $\varepsilon = 10$. The two open forms minimized at $\varepsilon = 1$ and $\varepsilon = 10$ are almost identical (r.m.s. = 0.22 Å) and closely similar to the original X-ray structure (r.m.s. = 0.72 Å), and the same is also true for the closed forms (r.m.s. = 0.12 Å and 0.83 Å, respectively). Total energies, together with their bond, non-bond (dispersion and repulsion) and electrostatic contributions are reported in Table 1. At $\varepsilon = 1$, corresponding to a non polar environment, the open form is more stable by 13.2 kcal/mol, while at $\varepsilon = 10$ the stability order is reversed and the closed form becomes more stable by 31.4 kcal/mol (entry 3). It can be seen (entry 3) that the stabilization of the open form in the nonpolar environment ($\varepsilon = 1$) is entirely due to the intramolecular electrostatic component ($\Delta E_{el} = -56.7$ kcal/mol) while both bond (27.8 kcal/mol) and non-bond contributions (16.3 kcal/mol) favour the closed form. As to the relative stability of the two forms, the present results are in qualitative agreement with those previously obtained with other force fields.[5]

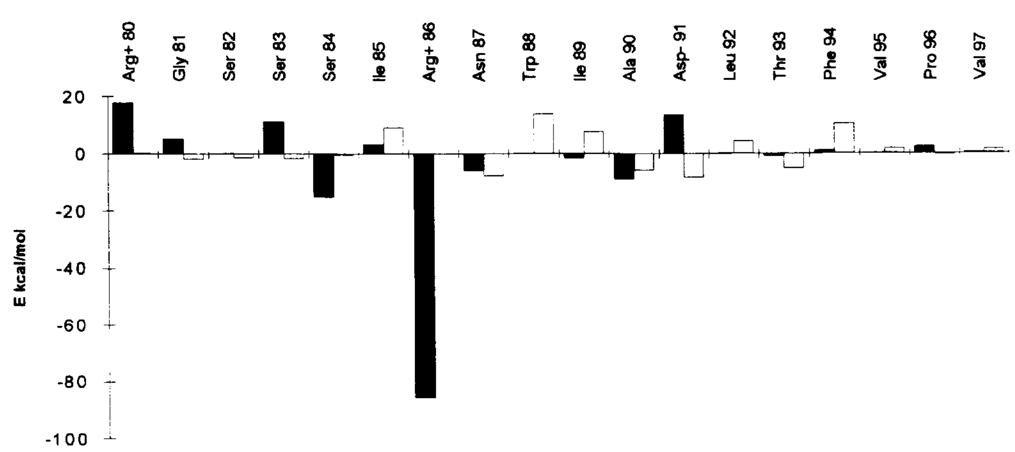
Table 1

	E (kcal/mol); $\varepsilon = 1$				**E (kcal/mol); $\varepsilon = 10$**			
	Total	**Bond**	**Non bond.**	**El.**	**Total**	**Bond**	**Non bond.**	**El.**
1. Closed	-172.4	208.1	-9.6	-370.9	118.6	167.3	-22.2	-26.5
2. Open	-185.6	235.9	6.7	-427.4	150.0	197.8	-18.0	-29.8
3. **ΔE (open-closed)**	**-13.2**	27.8	16.3	-56.7	**31.4**	30.5	4.2	-3.3

We have also evaluated the non bonding and electrostatic interactions between each residue of the lid and the rest of the protein with the program INTEN,[10] that allows partitioning of the total energy, and its components, into the contributions due to interacting pairs of aminoacids; calculations were carried out at $\varepsilon=1$ for both the open and closed form and the results are shown in Fig. 2. The electrostatic stabilization of the open form is largely due to interactions involving Arg+ 86; the salt bridge with Asp- 61 (Fig 1b) accounts for over 35% of the total contribution of this residue, 18% being due to an interaction with Glu- 117 (Fig. 1b). In the closed form

Arg+ 86 moves away (Fig. 1a) while another arginine residue on the hinge region of the lid (Arg+ 80) rotates toward Asp- 61. This interaction is seen to stabilize to some extent the lid in its closed conformation (Fig. 2).

Figure 2. Interaction energy differences ($\Delta E = E_{open} - E_{closed}$) between residues of the lid and the rest of the protein calculated with the INTEN program (full bars: $\Delta E_{electrostatic}$; empty bars: $\Delta E_{nonbond}$.)



In the simplest electrostatic model the solvent merely shields electrostatic interactions between aminoacid residues by a factor corresponding to the effective dielectric constant. In an attempt to describe the contribution of specific interactions, such as hydrogen bonding, that might be important in aqueous medium, a mixed model was considered in which explicit solvent molecules were introduced to mimic the first layer of the solvent directly in contact with the protein. To simplify the treatment, only a limited number of solvent molecules were introduced in the area, in proximity of the lid, where structural changes between the closed and open forms are larger; in this area, presumably, the contribution of specific interactions with the solvent to the relative stability of the two forms is more important.[11] For the same reason methane was chosen as a simple model of a hydrophobic solvent, mimicking a general aliphatic hydrocarbon. The two forms of the lipase were thus covered with a 2.5 Å layer of solvent (119 molecules of water and methane) in an area that includes the aminoacids of the lid and those having at least one atom within 3Å from the lid[12] and then optimized. The enzyme structure was initially kept fixed and solvent molecules were allowed to relax on the enzyme surface; finally the optimization was repeated allowing the relaxation of both enzyme and solvent together; all calculations were carried out at ε = 1. Results are in Table 2.

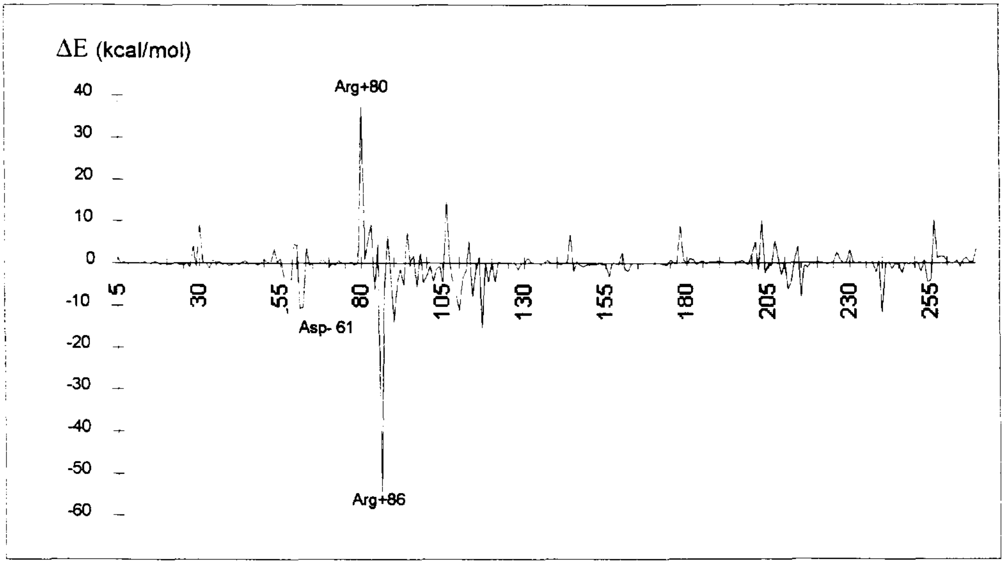
Table 2

	ΔE (open-closed) (kcal/mol)							
	Water				Methane			
Entry	Total	Bond	Non bond.	El.	Total	Bond	Non bond.	El.
1. Enzyme	-15.2	24.9	12.8	-52.9	-11.9	25.6	16.1	-53.6
2. Enzyme-solvent	62.8	-	5.9	57.0	12.0	-	9.1	2.9
3. Solvent	-46.8	2.5	19.2	-68.5	-7.0	-0.8	-6.5	0.2
4. Total	**0.8**	27.3	37.9	-64.4	**-6.9**	24.7	18.7	-50.3

Comparing the results for the water-solvated lipase (entry 4) and for the unsolvated enzyme (Table 1, entry 3, $\varepsilon = 1$), it can be seen that the introduction of a relatively small number of water molecules localized around the lid is sufficient to increase the relative stability of the closed form by approximately 14 kcal/mol with respect to the unsolvated enzyme. The closed form is actually stabilized by interactions with the solvent in both media (Table 2, entry 2) but in methane this effect is not large. Solvation by water, on the contrary, stabilizes the closed form by 62.8 kcal/mol (entry 2), which reduces to 16 kcal/mol taking into account unfavourable interactions between solvent molecules (entry 3); this is sufficient to overcome the intrinsic stability of the open form.

Specific interactions between water and each aminoacid residue of the enzyme were then analyzed with the INTEN program[10] (Fig. 3): particularly large energy differences, indicating a change in solvation between the open and closed structures, are found for two residues on the lid. Arg+ 86 is highly stabilized by water in the closed form, while Arg+ 80 is strongly solvated in the open form. Arg+ 86 and Arg+ 80 form of a salt bridge with Asp- 61 in the open and closed structure, respectively (Fig. 1 and 2):[2-4] the interaction energies shown in Fig. 3 clearly indicate that formation of the salt bridge is accompanied by a substantial desolvation of the arginine residues involved.

Figure 3. Interaction energies between aminoacid residues and water. Energy differences ($\Delta E = E_{closed}-E_{open}$) are plotted vs residue number.



The simplest electrostatic model presented earlier does not take into account specific interactions and can not distinguish between regions that are available to the solvent and those that the solvent can not access. The discrete model, on the other hand, is useful to analyze the key role of specific interactions with water in the area around the lid but, due to the limited number of solvent molecules, can not give a quantitative description of the overall effect of solvation. In order to overcome these limitations and obtain a more realistic description of solvent effects, the free energies of solvation of the closed and open structures of the lipase in water and in a nonpolar solvent were calculated using the polarizable continuum model.[7] In this model the solute molecule is placed in a cavity formed by the non overlapping parts of atomic VdW spheres surrounded by a polarizable continuum characterized by the solvent's macroscopic dielectric constant ε.[7] The model was initially applied to both the open and closed structures of the lipase, minimized at $\varepsilon = 1$. Solvation energies in water were calculated with $\varepsilon = 78.5$[13] and a mean radius of the water molecule of 1.68 Å;[7] for the non polar medium, an average value of $\varepsilon = 1.6$[13] and a radius equal to 3.0 Å were chosen as representative of a generic aliphatic hydrocarbon. Results are in Table 3.

Table 3

Entry	$\Delta\Delta G_{sol}$ (open-closed) (kcal/mol)	
	Water	Hydrocarbon
1. Enzyme	44.1	-0.2
2. Enzyme+Discrete water	46.5	

The differences between the Gibbs free energies of solvation of the open and closed forms calculated by the PCM (entry 1) indicate very clearly the ability of water to solvate the more polar closed form of the lipase. The nonpolar medium, on the contrary, does not display a particular ability in stabilizing the open form, in spite of the increased hydrophobic surface exposed to the solvent. The calculation was then repeated for the system consisting of the enzyme and 119 discrete water molecules (entry 2). The value found for this contribution is closely similar to that found for the enzyme alone and suggests that a large stabilization of the closed form is provided by unspecific interactions, in addition to specific interactions accounted for by the discrete model (Table 2). The PCM model thus confirms that solvation by water of the closed form is responsible for the higher stability of this form in the aqueous medium. The hydrocarbon solvent, on the contrary, does not discriminate between the two forms; thus the open conformation, which is intrinsically more stable (Table 1) is adopted in the lipid phase.

In conclusion the results obtained with different methods of treatment of solvent effects are in good qualitative agreement and indicate that the stability of the closed form of *R. miehei* lipase in water is due to the solvation of the charged residues on the enzyme's surface. In addition water also acts as a shield in cutting off the strong electrostatic self-stabilization of the open form, which, in the absence of solvent, is intrinsically more stable. Strong interactions are found, in the inactive enzyme, between water and Arg+ 86 on the lid and between Arg+ 86 and Asp- 61 in the active form. Desolvation of Arg+ 86 at the lipid-water interface is thus seen to play a leading role in the activation process while stabilization of the hydrophobic surface of the open form by the nonpolar environment does not appear to be important.

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9. The simple scaling electrostatic model can not distinguish between solvent accessible and non-accessible regions. This inadequacy is, to some extent, corrected by the arbitrary choice of an intermediate value of the effective dielectric constant ε for the polar medium (ε = 10), corresponding approximately to a 90% reduction of all intramolecular electrostatic interactions, which mimicks the concurrence of solute-solvent interactions. The use of the macroscopic value of the dielectric constant for water (ε = 78.5) leads to a further reduction of electrostatic interactions, but leaves the overall picture unchanged.
10. This program allows to calculate the interaction energy (electrostatic + Van der Waals) between two regions of a molecule using a DISCOVER input format file. Interaction energy is defined as follows:

$$E_{int} = \sum_{i}^{n}\left(\sum_{j}^{N}\frac{q_i Q_j}{\varepsilon \cdot r_{ij}} + \sum_{j}^{N}\left(\frac{A_{ij}}{r_{ij}^{12}} - \frac{B_{ij}}{r_{ij}^{6}}\right)\right)$$

    where n and N are the number of atoms in each region. The parameters q_i, Q_j (atomic charges), A_{ij} and B_{ij} were obtained from ref. 6.
11. The assumption is based on the observation that the structural differences between the two forms, in areas remote from the lid, are negligible both in the crystal structures and in the minimized structures of this work (r.m.s.= 0.42 Å, excluding the lid (residues 80 to 97)).
12. Sixty-four residues were considered for direct solvation: 27 to 33, 57 to 63, 79 to 98 (lid), 106 to 114, 143 to 146, 117, 204 to 215, 254 to 258, 266.
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