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Molecular reasons for lipase-sensitivity against acetaldehyde

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Abstract

The molecular reasons for the sensitivity of microbial lipases towards acetaldehyde, emerging as unavoidable by-product from acyl transfer reactions employing vinyl esters as acyl donors, were shown to be associated with specific properties of lysine residues. Since the mechanism of deactivation involves the formation of Schiff bases at the lysine ε-amino groups, the relative reactivity (i.e. nucleophilicity) of each residue was estimated by using an electronic (pK_a value) and a steric parameter (accessible surface area of the side chain). Sensitive lipases, as from *Candida rugosa* and *Geotrichum candidum*, possess several lysine residues that have high pK_a values (> 12) and are highly exposed to the solvent (surface areas of 210–220 Å^2). In contrast, the lysine groups of stable lipases like from *Rhizomucor miehei*, *Candida antarctica* B and *Pseudomonas glumae* have moderate pK_a values (up to 11.6) and are rather buried (surface areas of 130–150 Å^2). A close investigation of *Candida rugosa* lipase revealed that the most exposed lysine residues are located in the lid region (Lys75 and Lys85). The data suggest that Lys75, which is involved in fixing the lid in its open conformation, is presumably the prime target for deactivation by acetaldehyde.

Keywords: Lipase deactivation; pK_a-value; Lysine residue accessibility; Schiff base

1. Introduction

Acyl transfer reactions catalyzed by lipases employing vinyl acetate as acyl donor have become a standard technique for the kinetic resolution of racemic alcohols [2] [1–4] (Fig. 1). However, the lipase has to be chosen carefully because the by-product of this reaction, acetaldehyde, may cause a dramatic loss in activity [5,6]. While the lipases from *Pseudomonas*, *Rhizopus* and *Rhizomucor* strains as well as from *Candida antarctica* and *Chromobacterium viscosum* proved to be remarkably stable, the lipases from *Candida rugosa* and *Geotrichum candidum* lost most of their activity when exposed to acetaldehyde [6]. These data and the fact that both of the latter enzymes are highly homologous [7,8] suggested that the stability of

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[2] *i*-propenyl esters liberate 'innocuous' acetone, but they are more expensive and reaction rates are significantly lower due to steric hinderance when compared to those of vinyl esters [1].

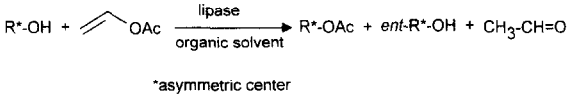

Fig. 1. Kinetic resolution of a racemic alcohol using vinyl acetate as acyl donor.

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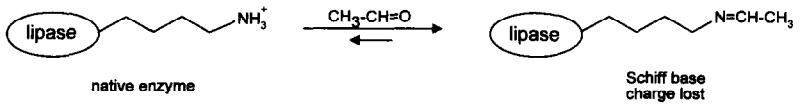

Fig. 2. Schiff base formation between acetaldehyde and the ε-amino group of a lysine residue.

a lipase towards acetaldehyde is related to its molecular structure.

Acetaldehyde is known to act as an alkylating agent on enzymes by forming Schiff bases in a Maillard-type reaction [9]. This reaction can occur on the N-terminus of the peptide, which was excluded due to its generally low accessibility (about 80 $Å^2$ surface area), and on the terminal ε-amino groups of lysine residues [10]. Like the majority of charged amino acids, lysine residues are more likely to be located at the surface of the enzyme rather than in the interior in order to facilitate hydration. By forming a Schiff base a lysine residue looses its positive charge and is, therefore, deprived of the possibility of forming H-bonds as well as of electrostatic interactions, which may cause enzyme deactivation (Fig. 2).

In contrast to our first assumptions, the total number of lysine residues of a lipase is not the criterion for lipase stability. Although the unstable lipases of *Candida rugosa* and *Geotrichum candidum* exhibit an enhanced lysine content (about 4% of the total number of amino acids), the stable lipases of several *Pseudomonas* strains, *Candida antarctica*, *Rhizomucor miehei* and *Humicola lanuginosa*, have only 2–3% lysine residues. However, the (stable) lipases from *Rhizopus niveus*, *Rh. delemar* and *Pseudomonas fluorescens* do not fit into this pattern due to their high lysine content of up to 5–6%. Hence it is reasonable to assume that the Schiff base formation does not take place arbitrarily on any of the lysine residues within the whole protein, but rather depends on the structural preferences within the vicinity of particular lysine residues, which makes them more reactive than others. Since the Schiff base formation comprises the reaction of an electrophile (acetaldehyde) and a nucleophile (the lysine ε-amino group), the relative nucleophilicity of an individual lysine residue will determine its relative contribution in the deactivation reaction. As the electrophile (acetaldehyde) always remains the same, the relative reactivity is only dependent on the nucleophilicity of the ε-amino groups. Consequently an unstable lipase is expected to possess one (or more) lysine residues being good nucleophiles and moreover being critical to the function of the lipase. Unfortunately, nucleophilicity per se cannot directly be measured or calculated but it can be estimated for each type of reaction [11]. However, it can be thought of as a combination of an electronic parameter ('basicity') [3] and a steric parameter ('accessibility') [4], which are accurately calculable. By following this approach we calculated the pK_a values and the solvent accessible surface areas of the lysine residues of those lipases whose three-dimensional structure is known.

2. Methods

2.1. Molecular modeling equipment

All calculations were performed on Silicon Graphics workstations. The manipulation of molecules and the graphic evaluations and display were performed employing the molecular modeling package SYBYL [12].

2.2. Structures of lipases

The following three-dimensional structures of lipases were obtained from the Brookhaven Protein Data Bank:

[3] Controlled through thermodynamics.
[4] Controlled through kinetics.

Candida rugosa lipase	open (CRL_o): 1CRL	closed (CRL_c): 1TRH
Geotrichum candidum lipase	open: —	closed (GCL_c): 1THG
Pseudomonas glumae lipase	open: —	closed (PGL_c): 1TAH
Candida antarctica lipase B	open (CAL_o): 1LBS	closed (CAL_c): 1TCA
Rhizomucor miehei lipase	open (RML_o): 4TGL	closed (RML_c): 3TGL

2.3. Supplementary material

A full set of pK_a and surface area values can be obtained via Internet: http://www-orgc.tu-graz.ac.at/fabgroup.

2.4. Calculations

The accessible surface areas were calculated by using the program ACCESS [5] [13] with a designated probe radius of 2.5 Å and evaluated by ACCFMT.

The TITRA program [14] was employed for the calculations of the pK_a values using the following parameters: ion exclusion depth 2.5 Å, ionic strength 0.1 M, solute $\varepsilon = 4$, solvent, $\varepsilon = 78.5$. The pH was set to the pH optimum of the lipase (7.2).

3. Results and Discussion

3.1. pK_a values

The pK_a value describes the equilibrium between the protonated and the deprotonated state of an acid (or base), but it can also be considered as a crude measure for 'Lewis basicity' within a homologous series. The reactivity of an amino group strongly depends on its ionization state. Only the non-protonated (uncharged) amine can act as a nucleophile by possessing a lone electron pair, whereas the protonated ammonium species is unreactive. The distribution of both of them is determined by the pK_a value, which in turn is governed by structural features of the vicinity of this group within the protein, such as polarity, availability of hydrogen bonds, acidic or basic residues etc. As may be deduced from Fig. 3, amino groups exhibiting a high pK_a value are good electron donors and strong nucleophiles, but they are, therefore, highly protonated. They may be characterized as 'few but strong' nucleophiles. The opposite is true for those residues that have reduced pK_a values, i.e. the majority of them is non-protonated; consequently they represent 'many weak' nucleophiles [6].

Thus, lysine residues possessing higher pK_a values are prone to undergo Schiff base formation more easily. In order to locate possible sites for this deactivation reaction, the pK_a values of the ε-amino groups of lysine residues were calculated using the program TITRA [14] for selected lipases whose three-dimensional structure is available. TITRA computes pK_a shifts and titration curves of a protein from its structure. The algorithm starts with the pK_a of each titrating group in a model compound, which can be measured by NMR. If this group is placed in a protein, its pK_a will shift for two reasons: (1) Moving the titratable group from the aqueous environment to the protein will change its free energy for protonation. This term is calculated

[5] The original VAX-version was adapted for UNIX.

[6] For example, from two titratable groups ($pK_a = 10$ and 12, respectively), a molar fraction of 10^{-3} and 10^{-5}, respectively, is in its uncharged form in a medium of pH 7.

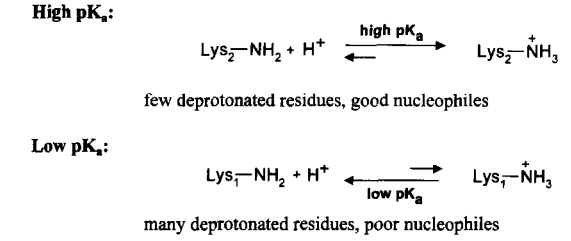

Fig. 3. Influence of pK_a value on thenucleophilicity of amino groups.

from the solvent accessible surface of the amino acid side chain in the protein. (2) Electrostatic interaction among all titrating groups within the protein is pH-dependent. This term is calculated for all of these groups through an iterative self-consistent algorithm.

From the assumption mentioned above, it is expected that unstable lipases (*Geotrichum candidum*, *Candida rugosa*) possess lysine residues with enhanced pK_a values in comparison to the corresponding pK_a values from stable lipases.

Fig. 4 shows that this assumption proved to be correct. The pK_a values calculated for lysine residues within the stable lipases (RML_o, RML_c, PGL_c, CAL_o, CAL_c) are not far from the model pK_a of 10.6, corresponding to an isolated lysine, and they do not exceed a value of 11.5 (Lys109 in RML_c). On the other hand, unstable lipases (GCL_c, CRL_o, CRL_c) possess several lysine residues that exhibit high pK_a values up to almost 13, which makes them strong nucleophiles. In particular, Lys75 in CRL_o is noteworthy with its pK_a of 12.75. The bold frame set to a pK_a of 10.6 corresponds to the model pK_a of an isolated lysine.

3.2. Solvent accessible surface area

Besides the electronic parameters the reactivity of a nucleophile is strongly dependent on its steric requirements because it must be accessible to the electrophile to undergo a reaction. For instance, it is a common technique to increase the steric bulk in order to reduce the nucleophilicity of a base, e.g. alkali *tert*-butoxides. During the Schiff base formation the acetaldehyde is mobile but the lysine residues are comparatively 'immobilized' because they are linked to a macromolecule. As a consequence, the ease of the approach of the electrophile strongly depends on the accessibility of the lysine residue (Fig. 5).

The accessible side chain surface areas of the lysine residues were calculated employing the

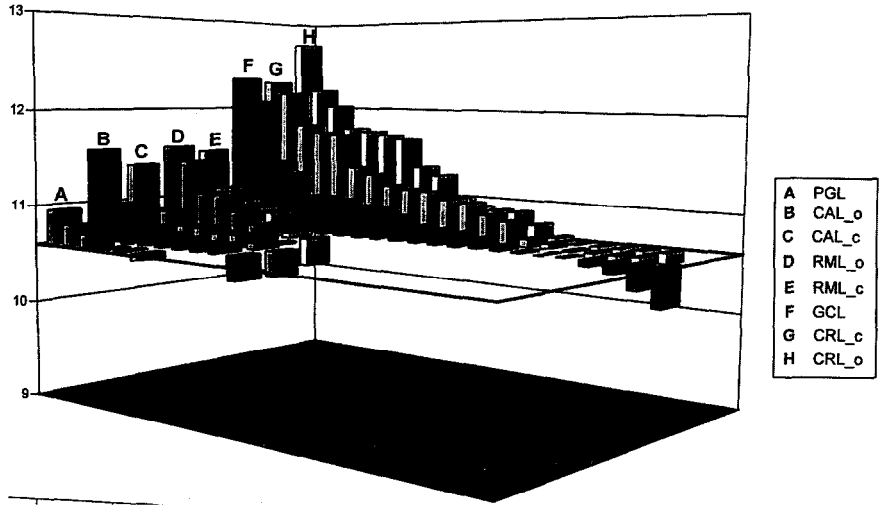

Fig. 4. pK_a values for lysine ε-amino groups of lipases. CAL = *Candida antarctica* lipase, PGL = *Pseudomonas glumae* lipase, RML = *Rhizomucor miehei* lipase, CRL = *Candida rugosa* lipase, GCL = *Geotrichum candidum* lipase. _o = lid open, _c = lid closed.

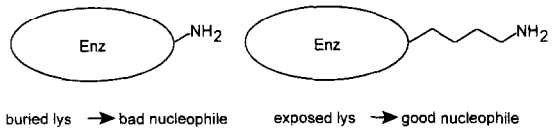

Fig. 5. Influence of the steric requirements on the nucleophilicity of lysine residues.

ACCESS program. For each atom listed in the input file the program calculates the surface area in Å^2 that is accessible to a probe sphere of a radius of 2.5 Å in this case, which corresponds to the van der Waals radius of acetaldehyde. The results from several lipases — given as the sidechain surface area in Å^2 accessible to the probe — are shown in Fig. 6.

The sidechain surface areas of lysine residues show a strikingcoincidence with lipase stability. Whereas the lysine residues of stable lipases (*Rhizomucor miehei*, *Candida antarctica* B, *Pseudomonas glumae*) are rather buried by showing maximal surface area values of 130–150 Å^2, the unstable lipases (*Candida rugosa*, *Geotrichum candidum*) possess several residues, which are extremely exposed (210–220 Å^2) and, therefore, are highly nucleophilic.

Our interest was focused on *Candida rugosa* lipase, because this enzyme has been more

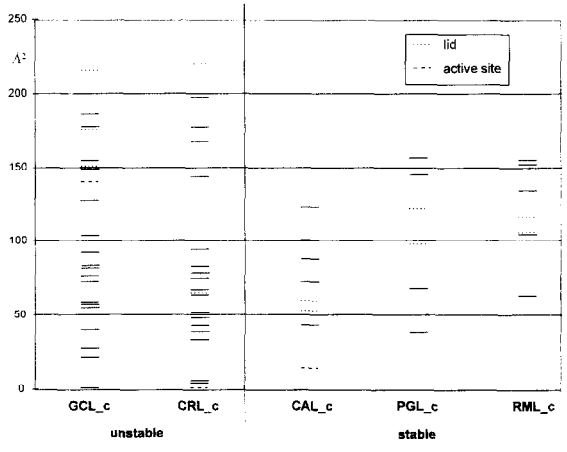

Fig. 6. Sidechain surface areas of lysine residues accessible to a spherical probe (radius 2.5 Å). CAL = *Candida antarctica* lipase, PGL = *Pseudomonas glumae* lipase, RML = *Rhizomucor miehei* lipase, CRL = *Candida rugosa* lipase, GCL = *Geotrichum candidum* lipase. _o = lid open, _c = lid closed. Dashed lines = Lys at active site region, dotted lines = Lys in the lid.

widely used as a biocatalyst for the transformation of non-natural compounds as compared to the *Geotrichum candidum* lipase. Aiming at the identification of those lysine residues, which are prone to be attacked by acetaldehyde, we investigated two special regions in more detail: (i) the active site and (ii) the lid region.

3.2.1. (i) Active site region

Considering the accessible surface areas of the lysine residues located near the active site there is no correlation with the stability of a lipase (Fig. 6, dashed lines). For example, Lys404 and Lys431 which are located within an 8 Å range of the active site region of the unstable *Candida rugosa* lipase cannot be considered as highly reactive due to their restricted accessibility in the open as well as in the closed conformation (surface area values < 1 and 5 Å^2, respectively). Comparable low surface area values were found in stable lipases. Lys136 of the lipase from *Candida antarctica* B exhibits surface areas of less than 18 Å^2 for both conformations and the lipases from *Pseudomonas glumae* and *Rhizomucor miehei* do not even possess any lysine residue within the range of 8 Å of the active site serine residue.

3.2.2. (ii) Lid region

A different picture emerged from lysine residues from the lid region (Fig. 6, dotted lines). Whereas the lysine residues in the lid region of the stable lipases show reduced surface area values (CAL_o/CAL_c Lys271 and Lys290, ca. 60 Å^2; PGL_c Lys22, 97 Å^2; Lys43, 121 Å^2; RML_o/RML_c Lys106 and Lys109, 95–120 Å^2), the residues of both unstable are highly exposed (CRL_c Lys75, 218 Å^2; CRL_o Lys85, 204 Å^2; GCL_c Lys75, 175 Å^2; Lys81, 216 Å^2; Lys96, 150 Å^2).

In order to identify the most nucleophilic lysine residues, where Schiff base formation is likely to occur, both the electronic and steric parameters are combined following our proposal discussed above. Fig. 7 shows a typical graph

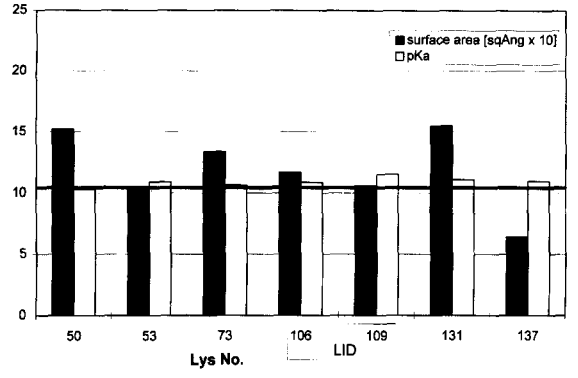

Fig. 7. pK_a and surface area values for *Rhizomucor miehei* lipase (RML_c).

displaying pK_a values and the surface areas for a stable lipase (RML_c). Although the data of the open and the closed conformations differ slightly, the same general tendencies can be found. The majority of the pK_a values are in a range of 10.6 to 11, one is even lower and only two exceed 11. The surface areas are moderate, too. Therefore, these residues are not likely to act as strong nucleophiles. Very similar graphs are obtained for all stable lipases being calculated.

The pK_a values and the surface areas of all lysine residues of *Candida rugosa* lipase are depicted in Figs. 8 and 9, corresponding to the open and closed conformations. Comparing both figures two residues deserve special attention: Lys75 (closed: pK_a 11.3, 218 Å^2; open: pK_a 12.8, 0 Å^2) and Lys85 (closed: pK_a 11.1, 64 Å^2; open: pK_a 11.7, 204 Å^2). Lys75 and Lys85 possess both elevated pK_a values as well as outstanding surface areas, which make them good nucleophiles. Moreover, both residues are part of the lid of *Candida rugosa* lipase playing a role in the catalytic process. This is reflected by the fact that Lys75 and Lys85 undergo a significant conformational change during the lid movement. Lys85 is highly accessible in the open (204 Å^2) but rather buried in the closed form (64 Å^2). It does not form any H-bonds in either of the conformations.

The most intriguing aspect is related to Lys75. It represents the most accessible lysine residue of the whole protein in the 'lid-closed' conformation (218 Å^2), but it is completely hidden when the lid is open (< 1 Å^2). In this conformation it forms H-bonds with the backbone carbonyl groups of Asn292 and Glu70 as well as a bifurcated H-bond with the ω-carboxylate of Glu71 as outlined in Fig. 10. This tight network of H-bonds is considered to lock the lid in its

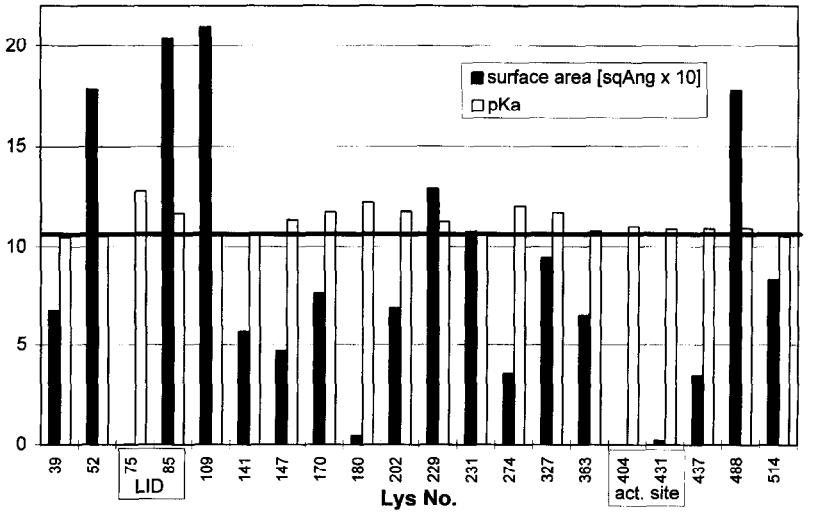

Fig. 8. pK_a and surface area values for *Candida rugosa* lipase (CRL_o).

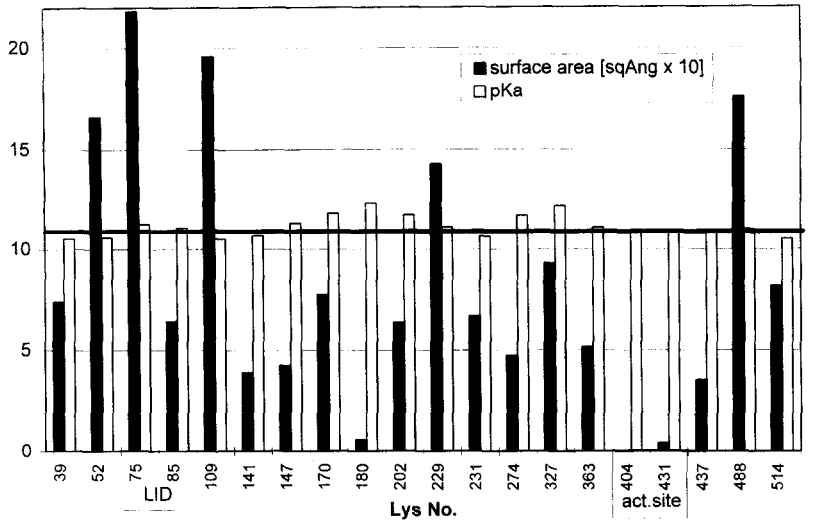

Fig. 9. pK_a and surface area values for *Candida rugosa* lipase (CRL_c).

open conformation. A related lid-locking mechanism involving a tryptophan residue was reported for *Rhizomucor miehei* lipase [15]. In the closed form Lys75 is too distant from the other three residues to form any H-bonds (spread over a 35 Å range).

Considering the facts discussed above, it is anticipated that the deactivation caused by acetaldehyde will most likely involve Lys75 for the following reasons: In the 'lid-closed' form, Lys75 can undergo Schiff base formation most easily because it has not only the greatest surface area but also the highest pK_a value of all lysine residues. During the formation of the Schiff base, the positive charge is removed, which would impede the formation of the hydrogen-bonding network required for fixing the lid in the open conformation [7].

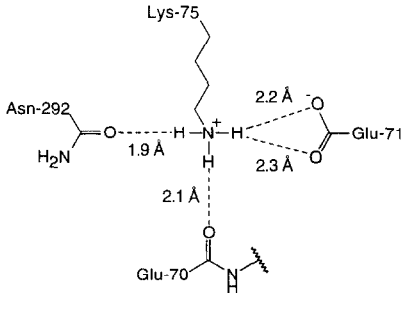

Fig. 10. Schematic representation of H-bonds involving Lys75 in lid-locking position (CRL_o).

4. Summary and outlook

The molecular reasons for the sensitivity of microbial lipases towards acetaldehyde emerging as unavoidable by-product from acyl transfer reactions employing vinyl esters as acyl donors were shown to be associated with specific properties of lysine residues. The reactivity of the ε-amino groups of lysine residues being prone to undergo Schiff base formation during the deactivation reaction was estimated describing the non-calculable nucleophilicity by a calculable electronic (pK_a value) and a steric term (side chain surface area). Both terms are in good agreement with lipase-stability data. The calculations show the following: (i) The lysine residues of stable lipases (e.g. from *Rhizomucor miehei*, *Candida antarctica* B, *Pseudomonas*

[7] Unfortunately the crystal structure of the open conformation of *Geotrichum candidum* lipase is not yet available. Therefore, it will stay a matter of speculation whether lysine residue 81, the structural counterpart of Lys75 from CRL, will act in a similar manner.

glumae) are moderately exposed (130–150 Å^2) and exhibit only a few residues possessing moderately elevated pK_a values ($\leq$ 11.5). (ii) On the contrary, unstable lipases (e.g. from *Candida rugosa* and *Geotrichum candidum*) possess highly accessible lysine residues (210–220 Å^2), and exhibit extremely high pK_a values ($>$ 12) for some residues. Attention is focused on Lys75, which is involved in fixing the lid in its open conformation, while exhibiting not only a great accessible surface area of 218 Å^2 but also an elevated pK_a value of 11.3 in the closed conformation. This extreme accessibility combined with the high 'basicity' makes it a good nucleophile prone to deactivation by acetaldehyde.

In order to stabilize *Candida rugosa* lipase by genetic engineering substitution of the lysine residue at position 75 by arginine seems to be most reasonable for the following reason (compare Fig. 10): Lys75 assists in fixing the lid in its open position through H-bonds with Glu70, Glu71 and Asn292. Schiff base formation on this residue removes all hydrogens and the lone electron pair from its ε-nitrogen atom. Consequently the formation of H-Bonds will be impossible. Furthermore, the Schiff base exerts steric hindrance which impedes the lid to switch from the closed to the open form. Arg instead of Lys at position 75 is largely inert towards Schiff base formation, but it would be able to form a hydrogen bonding network that is required for locking the lid in its open position. Thus, it should be possible to stabilize *Candida rugosa* lipase by genetic engineering to be used in acyl transfer reactions employing vinyl acetate as acyl-donor. A study directed towards this goal is in progress.

Acknowledgements

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