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Activation in the family of *Candida rugosa* isolipases by polyethylene glycol

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Abstract

We have investigated activation of two isoenzymes (*lip1 and lip3*) from *Candida rugosa* in polyethylene glycol (PEG) media. Aqueous solutions of PEG 8000 and 20,000 activate *lip3* but not *lip1* from *C. rugosa*. Maximum activation (260%) of *lip3* requires 6 h of pre-incubation with PEG 8000 (4%, w/v). PEG seems to shift the equilibrium between the open and the closed forms of *lip3* towards the active conformation. Inhibition experiments demonstrate that ligands have easier access to the *lip3* active site than to the *lip1* active site, both in the presence and the absence of PEG.

The presence of PEG in the crystallization medium is responsible for reported differences in the crystal structures of *lip1* and *lip3*. A comparative analysis of crystallographic models of *lip1* and *lip3* suggests a role for PEG in activation of *lip3* and further stabilization of the activated/open form via dimerization in aqueous media.

Keywords: Lipase; *Candida rugosa*; Cholesterol esterase; Activation; Polyethylene glycol

1. Introduction

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are enzymes that "in vivo" hydrolyze the long chain aliphatic esters of triglycerides to produce fatty acids and glycerol. These enzymes have received extensive attention for their potential use in biotechnology [1].

One can distinguish different modes for activation of lipases at lipid–water interfaces [2]. The simplest case corresponds to displacement of a hydrophobic lid rendering a pre-formed active site accessible to the substrate [3]. In other cases, the oxyanion hole is formed only after the activation process. In the most complex case, a lipase may require the simultaneous presence of a colipase [4]. However, enzymes with lipolytic activity have been identified that do not exhibit the interfacial activation phenomenon. Consequently, the lack of interfacial activation is not a sufficient criterion for distinguishing lipases from esterases. A contemporary definition of lipases – carboxylesterases that are able to catalyze the hydrolysis of long-chain triacylglycerols [2,5] – includes (i) lipases with a lid covering the active site, for which the corresponding oxyanion is formed only after activation [4]; (ii) lipases containing a preformed oxyanion but which do not contain lid, and which do not exhibit activation, for example, *Candida antarctica* B [6] and cutinase [7]; and (iii) lipases having a lid and which exhibit interfacial activation, for example, lipases from *Candida rugosa*.

Interfacial activation of lipolytic enzymes is associated not only with the presence of one or more full-length loops covering the active site, but also the presence of other structural elements that might stabilize closed or open conformations

Abbreviations: E600, Diethyl *p*-nitrophenyl phosphate; PEG, Polyethylene glycol; PNPB, *p*-Nitrophenyl butyrate; INH, Inhibitor

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of the lid [8]. In the case of pancreatic lipase, stabilization and adsorption to emulsified oil droplets are mediated by a preformed lipase–colipase–micelle complex [9,10]. Lipase activation involves different regions of the protein and is thus a complex phenomenon that must be further investigated.

Because isolipases from *C. rugosa* are characterized by lids covering the active site but differ in terms of their interactions with lipidic substrates [11,12], they constitute an appropiate system for further investigation of activation of lipases. A family of at least seven lipase genes has been described for *C. rugosa*, namely *lip1–lip 7*. *lip1* and *lip 3* can be isolated using a simple chromatographic purification process [13]. These isolipases are characterized by a high degree of homology in their sequences, the same molecular mass, and similar amino acid contents (ca. 79%) [13]. They differ with respect to hydrophobicity, specificity and p*I* [12]. *lip1* and *lip 3* also differ in their interactions with substrates at fluid interfaces [14].

lip1 has been crystallyzed in both open [15] and closed conformations [3]. Crystals of an uncomplexed and linoleate-bound cholesterol esterase that is 100% identical in sequence to *lip 3* have also been reported [16,17].

In the absence of any ligand, substrate or product, crystallization media containing polyethylene glycol (PEG) produces the open conformation of *lip3* [18] and the closed conformation of *lip1* [3]. Consequently, we investigated the effects of PEG on the hydrolytic activity of these two different isoenzymes from *C. rugosa* in both the presence and absence of the inhibitor diethyl *p*-nitrophenyl phosphate (E600).

2. Experimental

2.1. Materials

Lipase type VII from *C. rugosa*, glycerol, diethyl *p*-nitrophenyl phosphate and *p*-nitrophenyl butyrate (PNPB) were obtained from Sigma; 1-pyrenecarboxaldehyde (99%) from Aldrich; and polyethylene glycol 8000 and 20,000 were obtained from Merck.

2.2. Methods

2.2.1. Purification of lipases

Purification of two extracellular lipases from *C. rugosa* was carried out according to the method reported previously [12]. Under the experimental conditions, none of the pure lipase solutions eluted from the chromatographic columns contained significant amounts of dimers. Only monomeric solutions of purified *lip1* and *lip3* were employed in this study.

2.2.2. Enzyme assays

The activities of *lip1* and *lip3* (4.3×10^{-8} M, 0.77×10^{-4} mg/ml) for the hydrolysis of PNPB (1.7×10^{-3} M) were measured in sodium phosphate buffer (0.1 M, pH 7) by following the accumulation at 30 °C of PNPB at the isosbestic point of the nitrophenol/nitrophenolate couple (346 nm, $\varepsilon = 4800\ M^{-1}\ cm^{-1}$).

2.2.3. Activation of enzymes with PEG

lip1 and *lip3* (1.3×10^{-5} M) were incubated with 4% (w/v) PEG at 25 °C. Two types of PEG (8000 and 20,000) were used in 0.1 M sodium phosphate buffer (pH = 7). The hydrolytic activities of both isoenzymes were measured at different incubation times over an 8 h period. For reference, the activity of the enzyme in the PEG-free medium was taken as 100%.

2.2.4. Enzymatic inhibition

Isoenzymes *lip1* and *lip3* (1.3×10^{-5} M) were first incubated for 6 h in phosphate buffer (0.1 M, pH 7, 25 °C) in both the presence and absence of 0–5% (w/v) PEG 8000. Subsequently, sufficient E600 was added to the enzyme solution to produce a 0.26 mM solution. The activities of these two enzyme solutions were measured at different times after addition of inhibitor. The residual activities are referenced to the corresponding enzyme activity before any treatment with activator/inhibitor.

2.2.5. Fluorescence study

Fluorescence of 1-pyrenecarboxaldehyde (3.3×10^{-4} M) was used to detect changes in the dielectric constant of the medium. Fluorescence spectra of 1-pyrenecarboxaldehyde (3.3×10^{-4} M) in the different incubation media used to treat the enzymes were recorded at 30 °C. A Perkin-Elmer fluorescence spectrometer (LS 50B) with excitation at 288 nm was used to obtain emission spectra at 250–600 nm.

3. Results

3.1. Effect of PEG on the activity of lip3

Incubation of monomeric *lip3* with 1–5% (w/v) PEG 8000 for two hours increased the hydrolytic activity of this isolipase in aqueous solution (Table 1). The maximum increase in activity was observed following incubation with 4% (w/v) PEG.

Table 1
Activity of *Candida rugosa lip3* (0.06 mg/ml) after incubation for two hours at 25 °C with different percentages of PEG 8000 in 0.1 M sodium phosphate buffer (pH 7)

PEG 8000 (%, w/v)	Hydrolytic Activity ($Umg^{-1} \times 10^{-3}$)	Activity (%)
0	2.95	100
1	3.95	134
2	4.80	163
3	5.15	174
4	5.93	201
5	5.0	169

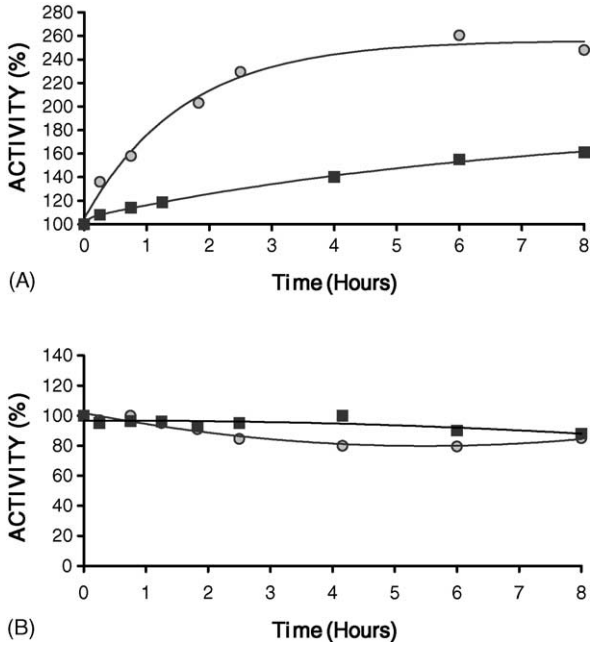

Fig. 1. Variation of the activity of *C. rugosa* isoenzymes (1.3×10^{-5} M) in presence of PEG. Conditions: 4% PEG (w/v) in sodium phosphate buffer (pH 7), 25 °C. (A): ○, *lip 3* + PEG 8000; ■, *lip 3* + PEG 20,000 and (B): ○, *lip 1* + PEG 8000; ■, *lip 1* + PEG 20,000.

3.2. Effect of the molecular weight of PEG on the activities of C. rugosa isolipases

Incubation in solutions containing 4% (w/v) PEG 8000 and PEG 20,000 increased the hydrolytic activity of *lip3* (Fig. 1A). The increase in activity was larger for PEG 8000 than for PEG 20,000. The extent of activation of the lipase following incubation with PEG also depends on the incubation time. The optimum incubation time was 6 h for *lip3* (260% activation). Maximum activation of *lip3* (161%) with PEG 20,000 requires 8 h of pre-incubation.

Unlike *lip3*, *lip1* was not activated by incubation in solutions containing PEG (Fig. 1B).

3.3. Inhibition of pretreated lipases

Inhibition of both isoenzymes by 0.26 mM E600 was studied in the presence and the absence of 4% (w/v) PEG 8000 (Fig. 2).

In the absence of PEG, incubation of *lip3* with the inhibitor for 5 min decreased the activity of *lip3* to 81% of the original value. By contrast, no change in activity was observed for *lip1*. These results indicate that in aqueous solution access of either substrates or inhibitors to the active site is easier for *lip3* than for *lip1*. The presence of PEG in the incubation medium increases inhibition of *lip3* by E600 and leads to a residual activity of 37% after 5 min (Fig. 2). This effect was not observed for *lip1*.

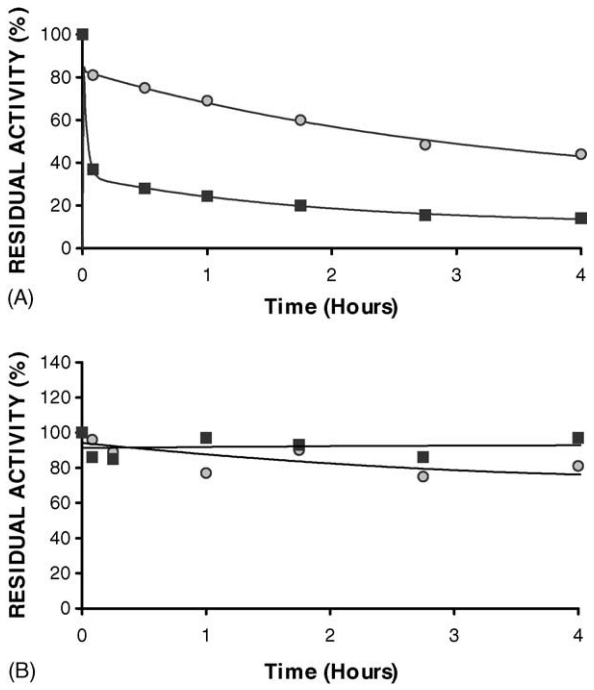

Fig. 2. Enzymatic activity after incubation of the isolipase with an inhibitor in the presence and absence of PEG. Conditions: 25 °C, 1.3×10^{-5} M lipases in 0.1 M sodium phosphate buffer (pH 7), 0.26 mM E600; (A): ○, *lip3* + INH; ■, *lip3* + INH + PEG 8000 and (B): ○, *lip1* + INH; ■, *lip1* + INH + PEG 8000.

3.4. Study of the dielectric constants of different media

Several authors [19–21] have indicated that the dielectric constant of the medium is responsible for conformational changes accompanying interfacial activation of lipolytic enzymes. Changes in the dielectric constant of the enzymatic solutions arising from the presence of PEG 8000 and 20,000 have been investigated by fluorescence (Table 2). Addition of glycerol (50%, v/v) produced shifts in the emission wavelength of 1-pyrenecarboxaldehyde similar to those observed for PEG 8000. However, the activity of *lip3* is not affected by addition of glycerol to the buffer. Neither activation nor

Table 2
Fluorescence of 1-pyrenecarboxaldehyde (3.3×10^{-4} M) at 30 °C in different media (excitation at 288 nm)

Medium	Dielectric constant (ε)	Emission peak (nm)
Methanol	32.7	455.5
PEG 6000 4% (w/v)[a]	–	465
PEG 8000 4% (w/v)[a]	–	468
Glycerol 50% (v/v)[a]	60.25[b]	470
PEG 20,000 4% (w/v)[a]	–	473
Sodium phosphate buffer 0.1 M	–	575
Twice distilled water	78.5	579

[a] 0.1 M sodium phosphate buffer containing the indicated amount of PEG or glycerol.

[b] $\varepsilon_{water} = 78.5$; $\varepsilon_{Glycerol} = 42$; $\varepsilon_{mixture} = (50 \times 78.5) + (50 \times 42)/100 = 60.25$ (if ε of the 0.1 M sodium phosphate buffer is equal to the value of ε for the twice distilled water).

deactivation of this isoenzyme was observed in this system when *lip3* (1.3×10^{-5} M) was incubated for 27 h at 25 °C in 0.1 M sodium phosphate buffer (pH 7) containing 50% (v/v) glycerol.

3.5. Structural analyses of *C. rugosa* lipases

Analysis of the structures of these two isoenzymes reveals a large number of intramolecular interactions in *lip1* that favor stabilization of either the open or the closed forms of these isoenzymes (the Lys 85-Glu 88 salt bridge in the open form and the Ser 450–Ser 84 hydrogen bond and two aromatic interactions of Phe 344 in the closed form of *lip1* are not present in *lip3*). Consequently, transitions between open and closed forms occur more readily in *lip3* than in *lip1*. In aqueous solutions, the open form of *lip3* is stabilized via dimerization of two protein molecules (primarily via hydrophobic interactions of a region involving the internal face of the lid) [18].

Protein–PEG complexes have been observed in the case of the FixJ receiver domain [22] and in a peptide deformylase [23] by using X-ray crystallography. In both proteins, non-specific hydrophobic and hydrogen bond interactions are established between the protein and the PEG.

4. Discussion

Comparison of the crystallization conditions and the conformations observed for a variety of lipases suggests that the conformation of the protein is determined by the dissolution medium [19–21]. However, closed structures of some lipases have been obtained in media that stabilize open forms of other lipolytic enzymes. *C. rugosa lip1* requires the presence of an organic co-solvent (e.g., 2-methyl-2,4-pentanediol) to crystallize in open form [15]. By contrast, *lip3* crystallizes in its open conformation in aqueous media without the need for an organic co-solvent [18]. It seems that in *C. rugosa* isoenzymes the protein structure plays an important role in the activation process in media of relatively high dielectric constant.

PEG is a common component of solutions employed for crystallization of proteins. Crystals of *C. rugosa* lipases have been grown in PEG media [3,16,18]. The present study demonstrates the role of PEG 8000 and 20,000 in inducing conformational changes involving displacement of the lid covering the active site of *lip3*. The activation effect of displacement of the lid in *lip3* has been quantified. Displacement increases the hydrolytic activity of *lip3* by a factor of 2.6. The effects of inhibition of *lip3* by E600 in the presence and absence of PEG have also been compared. The results suggest that PEG increases the displacement of the lid and facilitates inhibition of *lip3* by E600. This effect of PEG was observed only for *lip3*. By contrast, the closed conformation of *lip1* is not activated by PEG, nor is this enzyme significantly inhibited by E600 in media containing PEG.

Lip3 and *lip1* from *C. rugosa* have homologous structures as a consequence of their very high degree of sequence homology. However, for *lip3* the open form seems to be more favored than is the case for *lip1*. In the absence of PEG, *lip3* is significantly inhibited by E600, while the activity of *lip1* is not affected by this solute (Fig. 2). The dimers of *lip3* are very stable in aqueous solutions relative to the corresponding situation for *lip1*. This fact agrees with reported observations that in both hydrophobic chromatography and reverse micelles, *lip3* from *C. rugosa* establishes stronger interactions than *lip1* with the matrix and the micellar interface, respectively [11,12]. This aspect of the behavior of *lip3* is also responsible for inhibition of this isoenzyme by E600 in the absence of PEG (Fig. 2). In addition, the experimental results can be explained in terms of the net effect of PEG molecules, which shifts the equilibrium between the closed and the open forms of *lip3* towards to the open form. Hence, access of the inhibitor to the active site in *lip3* is easier in the presence of PEG than in its absence. For *lip1*, where activation was not observed, the lid remains closed and the inhibitor does not enter the active site.

Failure to observe activation of *lip3* in a glycerol–water medium with a dielectric constant similar to that of the PEG solution (Table 2) suggests a direct role for PEG in the activation process, rather than an effect of the polarity of the medium. Computational studies of the conformations of three different lipases (*H. lanuginosa*, *Rhizomucor miehei* and *R. delemar*) suggest that the electrostatic interactions between residues located in the vicinity of the loop (the substrate contact zone) are relevant for lipase activation in hydrophobic environments [24]. In these three lipases, significant shifts in p*K* values for some residues in the loop have been observed when the lipases are activated in media of different dielectric constants [24]. Unlike the corresponding results obtained in non-polar media, our studies in aqueous media containing PEG demonstrate that the dielectric constant is not the factor responsible for activation of *lip3*.

Just as is the case for the FixJ receiver domain and the aforementioned peptide deformylase (see Section 3), a non specific interaction of PEG with *lip3* from *C. rugosa* facilitates displacement of the lid, and the resulting open form of *lip3* is subsequently stabilized via formation of relatively strong hydrophobic interactions with the partner molecule of the dimer. Solvation studies of *R. miehei* lipase have suggested an important role of the desolvation of Arg 86 in activation phenomena [25]. In *C. rugosa* lipases, the presence of PEG could be responsible for drastic changes in the solvation of one or more of the protein residues directly involved in activation of the lipase.

In *lip1*, a stabilization effect of the open form has been attributed to the carbohydrate attached at Asn 351 [26]. *lip1* and *lip3* do not differ with respect to the indicated glycosylation site [3,18].

The cholesterol esterase activated with bovine bile salt formed dimers with the active sites facing one another [27]. Similarly, crystallization of dimers of both *lip1* and *lip3* from

C. rugosa have been reported [17]. However, only in the case of *lip3*, has dimerization of the protein in aqueous solution been described [18]. As is the case for *lip3*, the hydrophobic side of the lid of *lip1* interacts with the symmetry related molecule in the dimer crystal. However, in *lip1*, the corresponding intermolecular forces do not determine the stability of the open conformation, since the intramolecular interactions between residues on the hydrophilic face of the lid and the adjacent protein surface are more extensive [15].

In summary, we have demonstrated that in aqueous solution, PEG activates *lip3* but not *lip1*. The net effect of the PEG in solution is a shift of the equilibrium between the open and the closed forms of *lip3*. The presence of PEG in the crystallization medium could have determined the previously reported closed [3] and open [18] conformations of *lip1* and *lip3* from *C. rugosa*, respectively.

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