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On the interfacial activation of *Candida antarctica* lipase A and B as compared with *Humicola lanuginosa* lipase

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Abstract

The interfacial activation of *Candida antarctica* lipase A (CALA) and B (CALB) has been investigated and compared with that of *Humicola lanuginosa* lipase (HLL). CALB displayed no interfacial activation towards *p*-nitrophenyl butyrate (PNPB) when exceeding the solubility limit of the substrate. No activation was observed towards *p*-nitrophenyl acetate (PNPA) at the addition of sodium dodecyl sulfate (SDS) nor in the presence of a solid polystyrene surface. The catalytic action of CALB was very different from that of *Humicola lanuginosa* lipase, which showed a pronounced interfacial activation with the same substrates. The basis for the anomalous behaviour of CALB is proposed to be due to the absence of a lid that regulates the access to the active site. In contrast to CALB, CALA expressed interfacial activation, but the activation was not as prominent as for *Humicola lanuginosa* lipase (HLL). The structural basis for the activation of CALA is unknown.

Keywords: Emulsion; Water soluble; *p*-Nitrophenyl ester; Adsorption; Sodium dodecyl sulfate

1. Introduction

Triacylglycerol lipases (EC 3.1.1.3) are enzymes that catalyse the hydrolysis of neutral lipids in biological systems. A characteristic feature of triacylglycerol lipases is their activation at water/lipid interfaces [1]. In contrast to esterases, lipases display almost no activity with their substrate in its monomeric state. It was suggested that interfacial activation of lipases is due to a conformational change in the protein leading to increased activity [2]. The three-dimensional crystal structures of several lipases have been determined to date [3–8]. They all have the α/β-hydrolase fold [9]. They contain a catalytic triad similar to the one found in serine proteases and the active site is buried under a lid making the active site inaccessible to the substrate.

The structure of the *Rhizomucor miehei* lipase complexed with an active site inhibitor [10], the co-crystallisation of human pancreatic lipase (HPL) with mixed micelles [11] and the structure of the *Candida rugosa* lipase in an activated conformation without inhibitor [12], demonstrate the opening of the lid making the active site accessible to the ligand.

Enzymes with solvent accessible active sites displaying lipolytic activity have also been found. The structure of a cutinase from *Fusarium solani*, a cutin degrading enzyme, which also has lipolytic activity, has a solvent accessible active site and displays no interfacial activation [13]. From the structure of HPL a model of guinea pig pancreatic lipase (GPL) has been made [14]. GPL and HPL have high sequence identity except a large deletion in GPL, corresponding to the lid region of HPL. GPL displays no interfacial activation and the lack of a lid has been proposed to be responsible for the anomalous characteristic. Cutinase and GPL may be seen as a bridge between esterases and lipases in their ability to display maximal activity towards monomeric substrates as well as at a water/lipid interface.

From the yeast *Candida antarctica* two different lipases, A and B, have been isolated [15]. In the crystal structure of lipase B (CALB), the active site is accessible to solvent and it was discussed whether the lipase is interface activated or not [16]. It was suggested that intermolecular interactions could be responsible for the stabilisation of the open conformation. A short helix, $\alpha 5$, which is ordered/disordered depending on the crystal environ-

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ment, indicating a region in the protein of high mobility, has been pointed out as a possible lid candidate. A similar disorder is observed in the lid of *Humicola lanuginosa* lipase without a transition state inhibitor [17]. A structure of lipase A (CALA) has not been reported.

The influences of an emulsion surface, a solid hydrophobic surface or a detergent, sodium dodecyl sulfate (SDS), have been used to study the activation of porcine pancreatic lipase (PPL) [18–20]. Even below its CMC, SDS have been shown to activate lipases through the formation of a SDS/lipase complex [18].

We have investigated the interfacial activation of lipase A and B from *Candida antarctica* by the partially soluble substrate *p*-nitrophenyl butyrate (PNPB). We have also measured the activity of the lipase against a monomer solution of *p*-nitrophenyl acetate (PNPA) in the presence of a detergent, SDS, or a solid hydrophobic surface, polystyrene. In a study with PPL it was shown by using partially soluble triglycerides, that aggregation of monomers could occur below the solubility limit of the substrate, activating the lipase, and thereby no clear interfacial activation could be seen [21]. For that reason we have included *Humicola lanuginosa* lipase, an enzyme that is known to change conformation upon activation [22], as a reference of interfacial activation in order to be sure of the quality of the assays.

2. Materials and methods

2.1. Chemicals

p-Nitrophenyl acetate (PNPA), *p*-nitrophenyl butyrate (PNPB), tributyrin, gum arabic and Mops were purchased from Sigma (St. Louis, USA). Acetonitrile was obtained from Aldrich (Steinheim, Germany), *N*-succinimidyl[2,3-^{3}H]propionate from Amersham (Stockholm, Sweden) and polystyrene cuvettes from Labassco (Stockholm, Sweden). All chemicals used were of analytical grade.

2.2. Enzymes

Highly purified lipases from *Humicola lanuginosa* (HLL) and *Candida antarctica* component A (CALA) and B (CALB) were kind gifts from Novo Nordisk.

2.3. Protein quantification

Protein concentrations were determined spectrophotometrically at 280 nm. (CALB $\epsilon = 3.3 \cdot 10^4$ M^{-1} cm^{-1}; CALA $\epsilon = 5.2 \cdot 10^4$ M^{-1} cm^{-1}; HLL $\epsilon = 4.3 \cdot 10^4$ M^{-1} cm^{-1}).

2.4. Protein labelling

N-Succinimidyl[2,3-^{3}H]propionate was used for labelling the lipases with tritium in the same manner as the Bolton-Hunter reagent is used to iodinate proteins [23]. 1 ml (10 nmol) of the tritium reagent dissolved in toluene was placed in a glass test tube and the toluene was evaporated with a stream of nitrogen. Lipase, 200 μl (30–40 μM, 6–8 nmol) in 0.1 M sodium borate buffer, pH 8.0, was added to the dried reagent and the mixture was gently agitated for 20 min at 0°C. The radiolabelled lipase was separated from low molecular weight reaction products by thorough dialysis against 50 mM Mops pH 7.5.

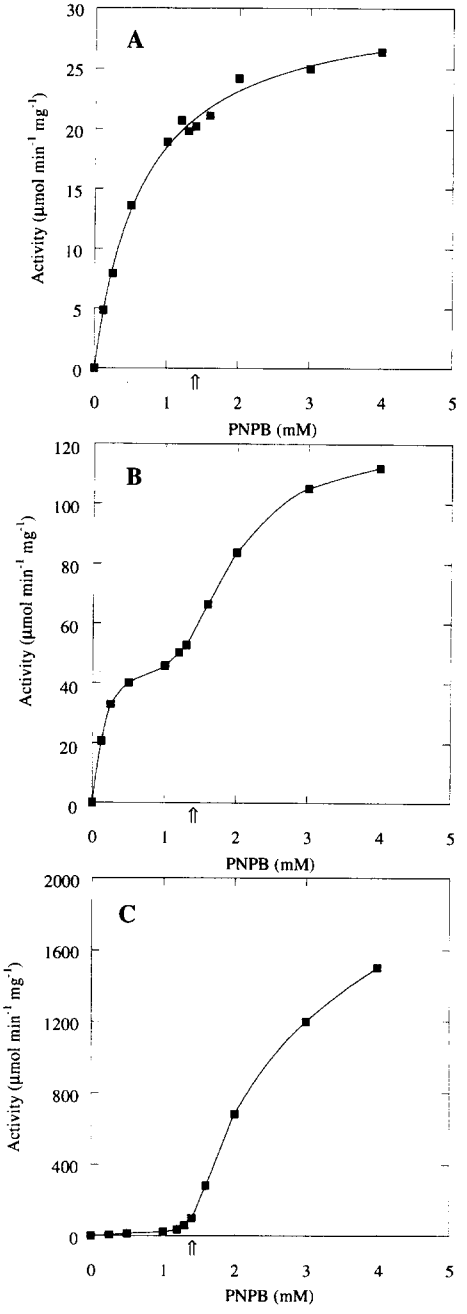

Fig. 1. Influence of PNPB concentration on lipase specific hydrolytic activity. The arrow indicates the solubility limit of PNPB. (A) CALB; (B) CALA; (C) HLL.

2.5. Lipase adsorption

Solutions of labelled lipases, 1 ml (Mops 50 mM; pH 7.5; 0–1250 nM HLL or 0–890 nM CALA or 0–855 nM CALB), were incubated for 30 min in new polystyrene cuvettes. Subsequently the solutions were removed and the cuvettes were washed twice with 50 mM Mops buffer (pH 7.5). The amount of lipase adsorbed on the cuvette was analysed in 14 ml Ready safe™ (Beckman Instruments, Fullertone, CA) with lipases still bound to the cuvette or removed from the surface by treatment with SDS (10%, w/w). A Packard Tri-Carb® 1500 liquid scintillation analyser was used. The two methods gave the same results.

2.6. Lipase activity measurements

The specific lipase hydrolytic activity towards tributyrin and *p*-nitrophenyl butyrate (PNPB) was measured at 25°C and pH 7.5 with a pH-stat (Radiometer VIT90 videotitrator, ABU 91 auto byrette). The substrate solution was emulsified by sonication for one minute. The reaction was started by the addition of enzyme solution to the stirred substrate solution (1.5 ml, 0.2 M tributyrin or PNPB, 5% gum arabic and 0.2 M $CaCl_2$).

In the assay of interfacial activation, hydrolysis of PNPB was monitored at 400 nm with a spectrophotometer (Varian Cary 5E UV-VIS-NIR) at 25°C. PNPB was dissolved and diluted in acetonitrile and was added to the buffer solution (Mops 50 mM, pH 7.5) to the desired concentration of PNPB, keeping the acetonitrile concentration constant at 1% (v/v). After sonication for 2 min the reaction was started by the addition of enzyme solution (10 μl) to the substrate solution (990 μl) in a quarts cuvette. The solubility limit of PNPB was determined spectrophotometrically at 800 nm.

The PNPA assay was performed in a similar way. It was empirically found that no sonication was needed in this case, a thorough shaking of the cuvette was enough. In the study of the influence of detergent, SDS (10%, w/w) was added to the substrate solution with subsequent stirring for 2 min. For the activity measurements in polystyrene cuvettes, 3 ml lipase solution (Mops 50 mM; pH 7.5; 0–1200 nM HLL or 0–1150 nM CALA or 0–1100 nM CALB) was left for 30 min in the cuvettes before the total activity was measured. The activity from adsorbed lipase was analysed by pouring out the lipase solution from the cuvette, washing with buffer (50 mM Mops, pH 7.5) twice, and then adding PNPA in a buffer solution. To minimise diffusion limitations a magnetic stirring device was used during the activity determinations.

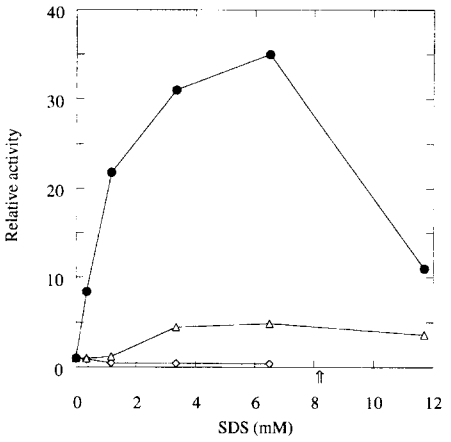

Fig. 2. Influence of SDS concentration on lipase hydrolytic activity towards PNPA. The arrow indicates the CMC of SDS [16]. ◇, CALB; △, CALA; ●, HLL.

3. Results

Lipase B from *Candida antarctica* (CALB) displayed no interfacial activation while lipase A from *Candida antarctica* (CALA) displayed a marginal interfacial activation using the partially soluble substrate *p*-nitrophenyl butyrate (PNPB) (Fig. 1). The *Humicola lanuginosa* lipase (HLL) displayed a very pronounced interfacial activation with PNPB (Fig. 1), indicating that no substantial aggregation of substrate molecules occurred below the solubility limit of the substrate. Hydrolysis of monomeric *p*-nitrophenyl acetate in the presence of SDS (Fig. 2), or with lipase adsorbed to a polystyrene solid surface (Table 1), gave a similar picture as the PNPB assay, no activation of CALB, a low activation of CALA and a high activation of HLL.

For the *Candida* lipases the hydrolytic activity towards PNPA was linear with the concentration of lipase (mea-

Table 1
Specific activity of CALB, CALA and HLL against tributyrin, PNPB and PNPA

Lipase	Tributyrin [a] (μmol min^{-1} mg^{-1})	PNPB [a] (μmol min^{-1} mg^{-1})	PNPA [b] (μmol min^{-1} mg^{-1})	
			dissolved [c]	adsorbed [c]
CALB	445 ± 41	31 ± 3	27 ± 2	16 ± 2
CALA	420 ± 35	105 ± 15	9.6 ± 2	15 ± 2
HLL	5600 ± 350	3290 ± 300	< 10 [d]	540 ± 60

[a] Titrator, 200 mM emulsion pH 7.5. [b] Spectrophotometer, 3 mM PNPA pH 7.5. [c] Activity of the lipases when dissolved in aqueous buffer or adsorbed to a polystyrene surface. [d] The activity of *Humicola* lipase in solution is probably overestimated due the large contribution from lipase adsorbed to the cuvette.

sured up to 600 nM for CALB and 700 nM for CALA) but for HLL the activity followed a saturation behaviour (Fig. 3). The adsorbed activity followed a saturation behaviour for all lipases and correlated with bound [^{3}H]lipase (Fig. 3, Table 2). The experimental adsorption data were fitted to a Langmuir adsorption isotherm, which has been used for PPL adsorbed to siliconized glass beads [19] (Table 2). All lipases showed a higher activity towards emulsified tributyrin than towards PNPB (Table 1). CALB displayed the largest difference in activity, 15-fold, between PNPB and tributyrin. The *Candida* lipases showed saturation kinetics below the solubility limit of PNPB (Fig. 1). With PNPA a linear relationship between lipase activity and substrate concentration was observed up to 3 mM (at 3.5 mM PNPA a solid phase appears) for all three lipases. The activity towards PNPA (3 mM) compared to PNPB (200 mM emulsion) was lower for CALA and HLL but the same for CALB (Table 1).

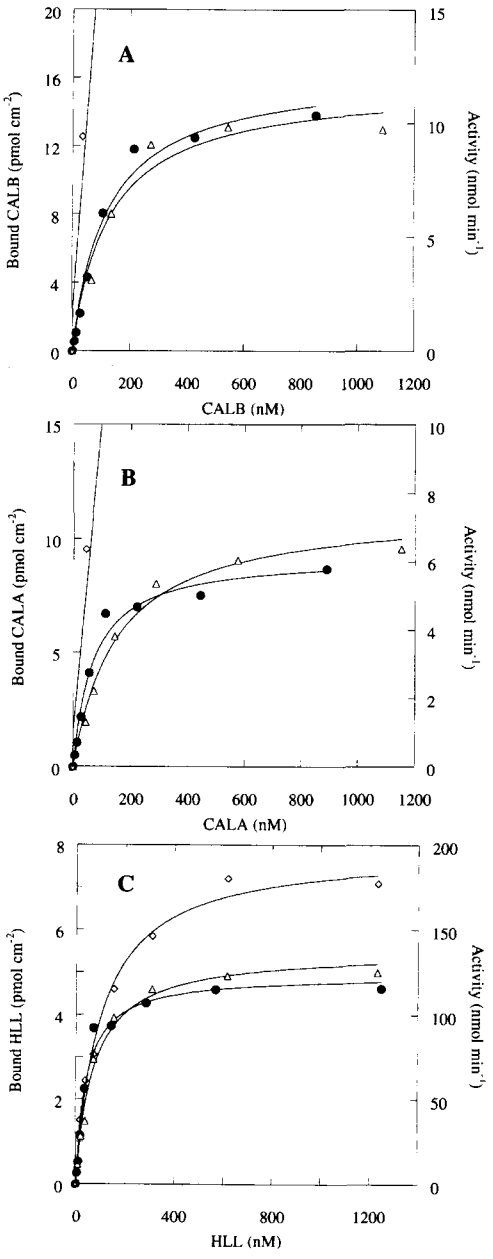

Fig. 3. Influence of lipase adsorption to a polystyrene surface on the hydrolytic activity towards PNPA. ◇, Total activity; △, adsorbed activity; ●, ^{3}H-labelled lipase. The activities refer to a 3 ml solution with 1 mM PNPA. The total activity was linear with the lipase concentration for the *Candida* lipases (measured up to 600 nM for CALB and 700 nM for CALA). (A) CALB; (B) CALA; (C) HLL.

Table 2
Calculated binding parameters for the adsorption of CALB, CALA and HLL to an aqueous-polystyrene interface

Lipase	K_d [a] (nM)		Maximal adsorption (pmol cm^{-2})
	^{3}H-labelled	native	
CALB	122±21	129±39	16±1
CALA	70±13	152±24	9.3±0.5
HLL	43±7	73±9	4.9±0.2

[a] Binding studies were performed in a Mops buffer (50 mM, pH 7.5). K_d determinations, using the Langmuir isotherm, were based on radioactivity measurements of ^{3}H-labelled lipase and activity measurements of native lipase. Maximal adsorption was based on ^{3}H-labelled lipase.

4. Discussion

The crystal structure of CALB has been suggested to represent an 'open conformation', stabilised by intermolecular contacts [16], in analogy with what has been found for the open activated conformation of *Candida rugosa* lipase (without active site inhibitor) [12]. A small helix (α5) in CALB has been suggested to act as a lid because of its observed disorder in some crystal structures, indicating a region of high mobility [16]. The proposed lid is a 5 residue long hydrophobic helix in which Glu-145 makes hydrogen bonds to Ser-150 and Thr-158, respectively, in the ordered structures. This is in analogy with the stabilisation of the open conformation of HLL by polar interactions between the lid and the core of the protein [22]. However, in HLL the central part of the lid is an α-helix more than 10 residues long, which is much more amphiphilic than the α5 helix in CALB.

Our results showed that *Candida antarctica* lipase B (CALB) did not display any interfacial activation towards PNPA and PNPB in the presence of an interface. The catalytic behavior of CALB was more typical of an esterase than of a lipase such as *Humicola lanuginosa* lipase that showed unambiguous interfacial activation with the same substrates (Fig. 1, Fig. 2 and Table 1). Interfacial activation of *Humicola lanuginosa* lipase is associated with a large conformational change of the lid [22]. We propose that the α5 helix in CALB is not involved in any conformational change regulating the access to the active site. Instead this mobile helix may be seen as part of the

lipid binding surface, anchoring the lipase to a water/lipid interface. It has been shown that CALB is active at the water/lipid interface using the monomolecular film technique [24]. CALB should thus be classified as a lipase, which displays maximal activity at water/lipid interfaces as well as towards monomeric substrates.

Candida antarctica lipase A behaved as an intermediate of CALB and HLL with a low interfacial activation. It is not known if the activation of CALA is due to a conformational change similar to that of HLL as no structural data are available.

The data above are in good agreement with the inhibition of the lipases using serine hydrolase inhibitors [25]. The hydrophobic (lipase) inhibitor, *n*-hexyl chlorophosphonate ethyl ester, which may form micelles, was much more efficient to inhibit HLL than the classical protease (esterase) inhibitor, diethyl-*p*-nitrophenyl phosphate (DNP). For CALB the inhibitors were equally efficient.

The activity towards emulsified PNPB compared to tributyrin were 7%, 25% and 60% for CALB, CALA and HLL, respectively. The low hydrolytic activity of CALB towards PNPB is suggested to be due to difficulties to accommodate the rigid *p*-nitrophenol leaving group in the narrow and deep active site. Even though PNPB is a bad substrate for CALB, this is not believed to influence the relative activity below and above CMC compared to a good substrate. It has been shown that PPL clearly displays interfacial activation towards methyl butyrate, which is hydrolysed at a rate of only 3% of that of tributyrin [20].

The binding characteristics of the lipases towards a solid polystyrene surface were similar (Table 2). The maximal adsorption was higher for the *Candida* lipases compared to HLL, which probably is due to the differences in p*I*. The p*I* is 7.5, 6.0 and 4.4 for CALA, CALB and HLL, respectively. It is well known that proteins show maximal adsorption when pH is near p*I* [26], and in this case the adsorption isotherms were performed at a pH far from the pI of HLL. The average area covered by one lipase molecule based on the adsorption experiments was calculated to be 1800 Å^2, 1040 Å^2 and 3400 Å^2 for CALA, CALB and HLL, respectively. With the dimension of CALB ($30 \times 40 \times 50$ Å) the adsorbed amount of protein corresponds to a close-packed monolayer, which probably is true also for CALA with its higher molecular weight. The slightly larger HLL ($35 \times 45 \times 50$ Å) may form a more loose-packed layer.

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