Volume 332, number 1,2, 143–149 FEBS 13089 October 1993

Topological characterization and modeling of the 3D structure of lipase from *Pseudomonas aeruginosa*

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Received 4 August 1993; revised version received 25 August 1993

Lipase from *Pseudomonas aeruginosa* is a M_r 29 kDa protein with a single functional disulfide bond as shown by a shift in electrophoretic mobility after treatment with dithiothreitol and iodoacetamide. Limited proteolysis of lipase with *Staphylococcus aureus* protease V8 resulted in cleavage after amino acid residues Asp[38] and Glu[46]. Comparison of the lipase amino acid sequence with those of other hydrolases with known 3D structures indicated that the folding pattern might be compatible with the α/β hydrolase fold, thereby allowing us to construct a 3D model which fitted the biochemical properties. The model predicts a catalytic triad consisting of Ser[82], Asp[229] and His[251], and contains a disulfide bond connecting residues Cys[183] and Cys[235]. Residues Asp[38] and Glu[46] are located at the surface of the enzyme, whereas the disulfide bond is rather inaccessible, which is in agreement with the finding that the protein needed to be partly unfolded before a reduction of the disulfide bond could take place. A striking prediction from the model was the lack of a lid-like α-helical loop structure covering the active site which confers to other well-characterized lipases a unique property known as interfacial activation. Experimental determination of lipase activity under conditions where the substrate existed either as monomeric solutions or aggregates confirmed the absence of interfacial activation.

Pseudomonas aeruginosa; Conformational property; Molecular modeling; Interfacial activation

1. INTRODUCTION

Lipases are triacylglycerol ester hydrolases (EC 3.1.1.3) which hydrolyze long chain fatty acid esters of glycerol yielding mono- and diacylglycerol and free fatty acids. A unique property of lipases is their action at oil–water interfaces, the presence of which greatly enhance their enzyme activity (interfacial activation) [1]. Lipases are used for the synthesis of esters and transesterification reactions, and therefore they have become biotechnologically important enzymes used for food refinement, as additives in washing detergents and even as diagnostic enzymes [2]. The lipases used are usually of fungal or bacterial origin, with *Pseudomonas* being the most important bacterial genus [3].

Pseudomonas aeruginosa produces and secretes into the extracellular medium a lipase which has been purified to homogeneity [4] and biochemically characterized [5]. The structural *lipA* gene codes for a pre-protein consisting of the 285 amino acid mature lipase preceeded by a 26 amino acid signal sequence. An additional orf *lipH* codes for a so-called helper protein which is necessary to obtain active extracellular lipase [6]. Several other *Pseudomonas* lipases have been biochemically characterized and the genes have been cloned and sequenced. These lipases form three distinct groups based on structural homology and their M_r, with the 29 kDa *P. aeruginosa* lipase being the prototype enzyme of group II [7]. At least one member of groups I and III has already been crystallized [8–10] but at present, there is no 3D structure reported. The structures of lipases from *Rhizomucor miehei* [11,12], human pancreas [13,14] and *Geotrichum candidum* [15,16] are known. Comparison of these structures with those of other hydrolases yielded a consensus folding pattern known as the α/β hydrolase fold [17], which is characterized by a central core consisting of up to 8 β-sheets connected by α-helices. We used this information, together with data obtained from biochemical studies, to build a 3D structural model of the lipase from *P. aeruginosa*.

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Abbreviations: ECL, enhanced chemiluminescence; DTT, dithiothreitol; ORF, open reading frame; PBS, phoshate-buffered saline.

2. MATERIALS AND METHODS

2.1. *Isolation of lipase*

Lipase was purified from culture supernatants of *P. aeruginosa* PAC1R as described [18]. Preparative isoelectric focusing in a Sephadex-IEF gel matrix (final volume 47 ml) was done in the presence of 3 M urea, 10% (v/v) glycerol and 2.5% (w/v) β-octylglucoside. After IEF, lipase was further purified by concentration and washing with 10 mM HEPES buffer, pH 8.0, containing 0.1% (w/v) β-octylglucoside in a Centricon 30 microcentrator (Amicon, Witten, Germany).

2.2. *Examination of the disulfide bond*

Purified lipase was incubated for 2 min at 100°C with 20 mM

dithiothreitol in the presence of 1% (w/v) SDS. After alkylation with iodoacetamide (final concentration 50 mM) for 15 min at 50°C and addition of sample buffer, the sample was loaded on a 14% SDS-PAGE gel.

2.3. *Treatment with endoproteinases*

Purified lipase (usually 600 ng) in PBS was treated at 30° for 1 h with 1 U of protease arg C or *Staphylococcus aureus* V8 protease, or 10 U trypsin (proteases were obtained from Sigma, Deisenhofen, Germany). Samples were frozen at −80°C, and freeze-dried. Immediately before electrophoresis samples were heated to 100°C for 10 min in SDS-PAGE sample buffer containing 4% (w/v) SDS.

2.4. *Gel electrophoresis and Western blotting*

SDS-PAGE was in 12% gels [19] with sample buffer containing 4% (w/v) SDS, or in 10.5% gels containing tricine [20]. After running the gels for 60 min at 150 V or 100 V, respectively, proteins were transferred to a nitrocellulose membrane (Hybond C, 0.45 μm; Amersham, Braunschweig, Germany) by electroblotting for 40 min at 300 mA [21]. The membranes were incubated with polyclonal anti-lipase antisera raised in rabbits against either denatured whole lipase protein or against a synthetic peptide consisting of amino acids 1–13 of the N-terminal part of the lipase. This peptide was conjugated to bovine serum albumin with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide at a peptide-to-carrier ratio of 90:1, excess coupling reagent removed by extensive dialysis against distilled water, and the conjugate used for immunization. Blots were treated with horseradish peroxidase-conjugated anti-rabbit IgG (Sigma, Deisenhofen, Germany) as the second antibody and developed using the ECL-system (Amersham, Braunschweig, Germany).

2.5. *Lipase activity assays*

Lipase activity was usually detected with *p*-nitrophenyl-palmitate as a substrate with 1 nK at representing about 1.3 ng of pure lipase protein [4]. Interfacial activation of lipase was studied with emulsions of the substrates, triacetin and tripropionin [22], dissolved in 0.3 mM Tris-HCl buffer, pH 8.0, 0.15 M NaCl, and 1.4 mM $CaCl_2$. Lipase activities were measured potentiometrically using a pH-stat (TTT 80 radiometer, Copenhagen).

2.6. *Computer modeling*

The amino acid sequence of *P. aeruginosa* lipase was aligned to the sequences of 35 lipases of different origin obtained from the databases, PIR, SWISS-PROT, GenBank, NRL, and PDB. The 3D structural model was built using the program FRODO [23] by replacing equivalent amino acids in the structure of the acetylcholine esterase from *Torpedo californica* (PDB entry code: 1ACE) [24] which was the only hydrolase for which a complete set of coordinates was available. Energy minimisation was done with the program, XPLOR [25], and final corrections with the program, O [26], and its fragment database. Computer modeling was carried out on an Evans and Sutherland PS 390 or an ESV 10/33 system. Computations were done on a CONVEX C220 computer.

3. RESULTS

3.1. *Lipase contains an intact disulfide bond*

Mature *P. aeruginosa* lipase is a 285 amino acid protein containing two cysteine residues, Cys^{183} and Cys^{235}, which form a disulfide bond. The electrophoretic mobility of purified *P. aeruginosa* lipase decreased when the protein was pretreated with DTT (Fig. 1, lane 1) indicating that the denatured but unreduced protein (Fig.1, lane 2) had a more compact conformation and therefore migrated faster on an acrylamide gel than the denatured and reduced form. A re-formation of the disulfide bond was not observed even without covalent modification of the Cys residues by treatment with iodoacetamide.

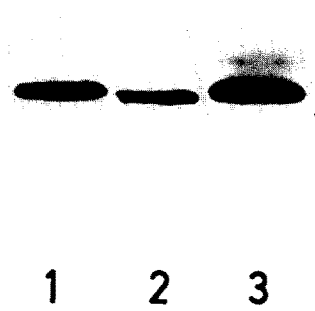


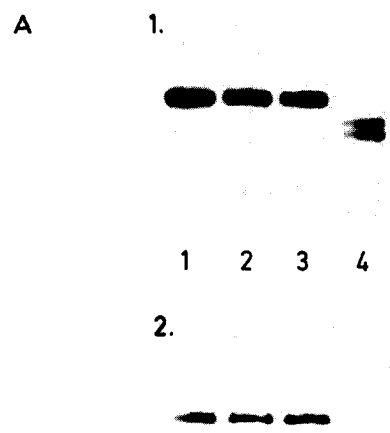
Fig. 1. Examination for an intact disulfide bond in *P. aeruginosa* lipase. Samples were loaded on 14% SDS-PAGE gels, blotted on nitrocellulose membranes, incubated with anti-whole lipase antiserum and blots developed using the ECL system. Lipase samples were denatured with SDS and reduced with DTT (lane 1), not reduced (lane 2), or reduced and treated with iodoacetamide (lane 3).

3.2. *Proteolytic digestion of lipase*

In order to identify potentially surface-exposed domains in the purified lipase protein, we digested lipase with different endoproteases and detected the generated fragments by immunoblotting. Fig. 2A1 shows that, under the conditions used here, neither trypsin nor arg C digestion produced detectable fragments. The 29 kDa band representing mature lipase protein disappeared, however, upon digestion of lipase with *S. aureus* protease V8 yielding two fragments of M_r 26 and 25 kDa, respectively, while the enzymatic activity of the lipase remained unaffected. By Western blotting and detection of the fragments using an antibody directed against a 13 amino acid N-terminal fragment, we could show that the N-terminal part of the lipase was cleaved off by *S. aureus* protease V8 (Fig. 2A2) suggesting that cleavage occured at positions Asp^{38} and Glu^{46} (Fig. 2B).

3.3. *Computer modeling of 3D structure*

Assuming that conserved parts of different lipase sequences preferentially relate to the active site and secondary structural elements, we first aligned 6 different *Pseudomonas* lipase sequences and compared them with lipase and hydrolase sequences from other organisms (Fig. 3). The active site consists of a catalytic triad formed by Ser, His, and Asp (or Glu in *G. candidum* lipase) residues with the Ser residing in a consensus pentapeptide $GlyX_1SerX_2Gly$, where X_1 is His in bacterial lipases. As shown in Fig. 3A, this part of the sequence is highly conserved throughout the lipases, allowing us to assign Ser^{82} as the active site Ser-residue. His^{251} has to belong to the catalytic triad because two other His residues residing in conserved parts of the sequence could be excluded. His^{14} belongs to the oxyanion hole and His^{81} would preceed the nucleophilic Ser^{82} instead of following the order Ser, Asp(Glu), His described for α/β-type hydrolases [17]. Accordingly,



B

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                10          20          30          40          50
N- STYTQTKYPI VLAHGMLGFD NILGVDYWFG IPSALRRDGA QVYVTEVSQL
                60          70          80          90         100
   DTSEVRGEQL LQQVEEIVAL SGQPKVNLIG HSHGGPTIRY VAAVRPDLIA
               110         120         130         140         150
   SATSVGAPHK GSDTADFLRQ IPPGSAGEAV LSGLVNSLGA LISFLSSGST
               160         170         180         190         200
   GTQNSLGSLE SLNSEGAARF NAKYPQGIPT SACGEGAYKV NGVSYYSWSG
               210         220         230         240         250
   SSPLTNFLDP SDAFLGASSL TFKNGTANDG LVGTCSSHLG MVIRDNYRMN
               260         270         280
   HLDEVNQVFG LTSLFETSPV SVYRQHANRL KNASL -C
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Fig. 2. (A) Digestion of *P. aeruginosa* lipase with trypsin (lane 2), arg C (lane 3), and *S. aureus* protease V8 (lane 4) and detection of fragments by Western blotting using antiserum against (panel 1) whole lipase protein and (panel 2) N-terminal peptide of lipase. Lane 1 contains untreated lipase protein. (B) Amino acid sequence of *P. aeruginosa* lipase showing cleavage sites of *S. aureus* protease V8 (arrows) as deduced from A.

Asp^{229} was identified as the most likely candidate participating in the catalytic triad based on conservation of sequences, as shown in Fig. 3B. The model therefore contains a catalytic triad consisting of residues Ser^{82}, Asp^{229} and His^{251}. Another strictly conserved structural element is the region forming the oxyanion hole which contains a central Gly residue (Fig. 3C), which is Gly^{15} in *P. aeruginosa* lipase. The distance predicted by the model between residues Cys^{183} and Cys^{235} allowed us to connect them by a disulfide bridge. A Ramachandran plot [27] (Fig. 4A) indicated that ϕ/ψ; angles were in allowed regions. Calculation of a 1D/3D profile [28] revealed positive peaks (Fig. 4B) and gave a score of 86 relative to a score of 125 expected for an X-ray structure and a minimum score of 56 expected for a model structure. Fig. 5 shows the secondary structure and a ribbon plot of the 3D structural model for *P. aeruginosa* lipase.

3.4. *Determination of interfacial activation*

Examination of the 3D structural model did not give any indication for a lid-like surface loop covering the active site which has been found in most of the lipases studied so far [29]. Interaction of this lid with the hydrophobic substrate is responsible for interfacial activation, which is represented by a sharp increase in enzyme activity in the presence of an oil–water interface. We therefore examined this property with the lipase substrates triacetin and tripropionin, which can be used in concentrations exceeding substrate saturation. Fig. 6 clearly indicates that *P. aeruginosa* lipase did not show interfacial activation, thereby resembling an esterase rather than a lipase from its kinetic behaviour.

4. DISCUSSION

The lipase from *P. aeruginosa* contains a unique disulfide bond connecting residues Cys^{183} and Cys^{235}. Lipases from *P. cepacia* [30] and *P. glumae* [31] belonging to homology group I also contain two Cys residues. The position of both Cys residues appears to be conserved throughout homology groups I and II, whereas no Cys residues have been found in *P. fluorescens* lipase belonging to homology group III [32]. Initially, no effect was observed upon treatment of *P. aeruginosa* lipase with

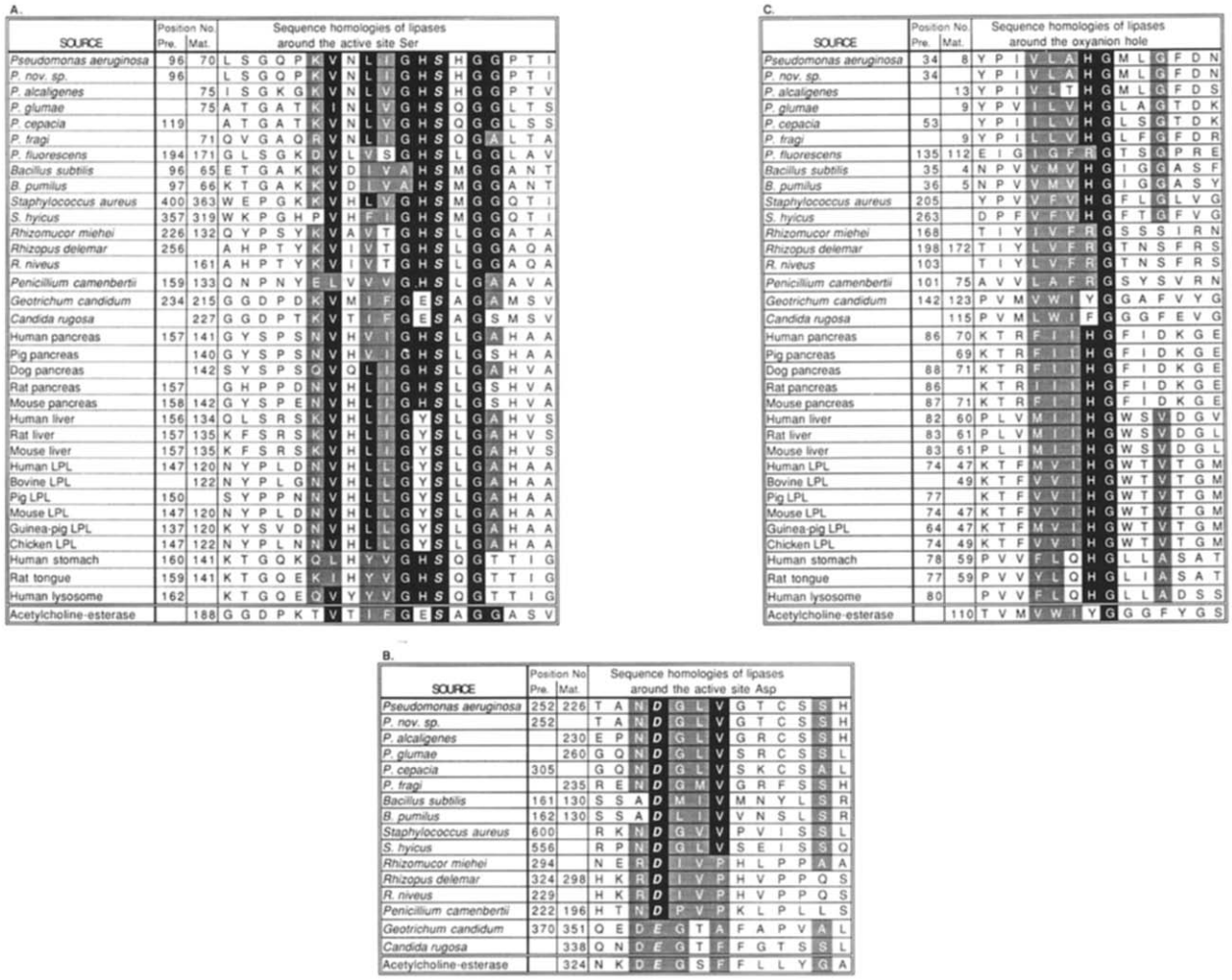

Fig. 3. Comparison of amino acid sequences of *P. aeruginosa* lipase with those of hydrolases from different origin. Homology is shown for regions around (A) the catalytic Ser residue, (B) the catalytic Asp residue, and (C) the oxyanion hole. Pre., preprotein including signal sequence; Mat., mature protein.

DTT suggesting the absence of a functional disulfide bond [4]. We repeated these experiments and found that the conformation of enzymatically active lipase had to be partially unfolded by treatment with non-denaturing concentrations of SDS (e.g. 0.025% w/v). This pretreatment gave a rapid and complete decrease of lipase activity in the presence of disulfide reducing agents (K. Liebeton and K.-E. Jaeger, unpublished results), suggesting that the disulfide bond is not readily accessible for the reducing agent. This observation is in agreement with the 3D structural model where the disulfide bond is not completely surface exposed. From the presence of this disulfide bond which obviously stabilizes the enzymatically active conformation of lipase it might even be speculated that a protein disulfide isomerase analogous to the product of the *E. coli* gene *dsbA* [33], should be involved in the periplasmic maturation of lipase which is known to be transported via a periplasmic intermediate (K.-E. Jaeger and S. Lory, unpublished results) thereby following the 2-step pathway described for *P. aeruginosa* secretory proteins [34].

Proteolytic digestion of the lipase with different proteases did not generate multiple peptide fragments, as theoretically expected from an analysis of the amino acid sequence which contains 18 cleavage sites for trypsin, 11 for endoproteinase Arg C, and 23 for *S. aureus* protease V8. The reason for that might be (i) the occurrence of purified lipase in a protein–detergent micelle or (ii) the presence of residual LPS which is known to form micellar aggregates with *P. aeruginosa* lipase [5]. In both cases, the lipase appears to be embedded in micelles, with only small parts of the protein being surface exposed and accessible to protease cleavage. Fragmentation was observed only upon digestion with *S. aureus* protease V8, indicating that amino acid residues Asp^{38} and Glu^{46} were accessible. Further experiments using buffer systems favouring cleavage behind Glu residues [35] indicated that Asp^{38} should be better accessible than Glu^{46} (data not shown) which is in agreement with the predictions from the 3D structural model. Asp^{38} is located at the end of β-strand 2 and appears to be easily accessible, while Glu^{46} is located in the middle of β-

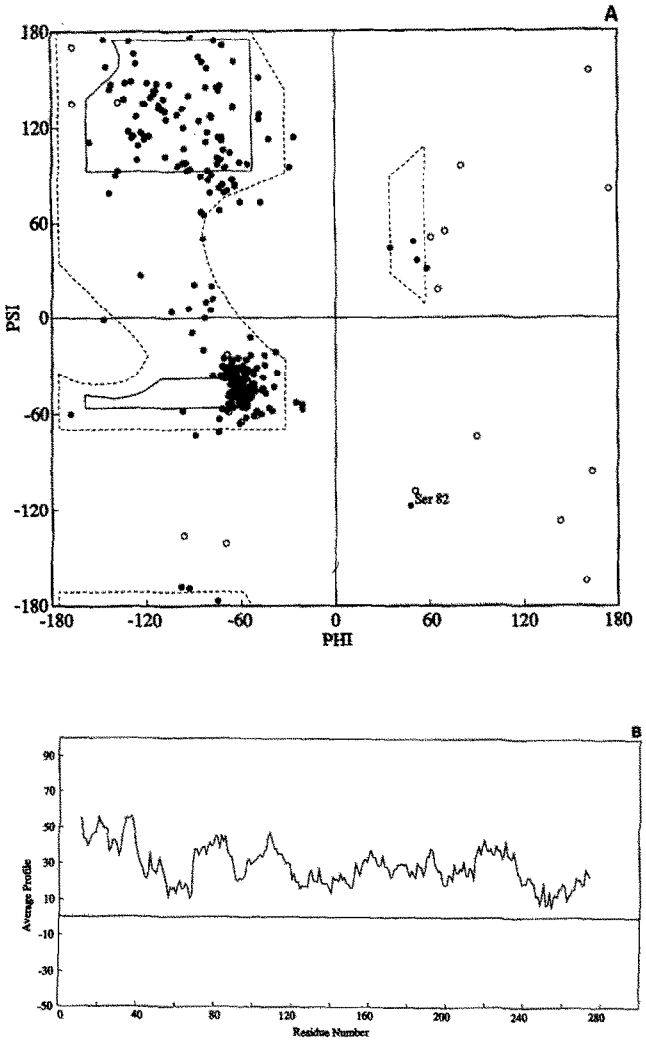

Fig. 4. (A) Ramachandran plot [27] and (B) Eisenberg plot [28] of amino acid residues as predicted from the 3D model for *P. aeruginosa* lipase.

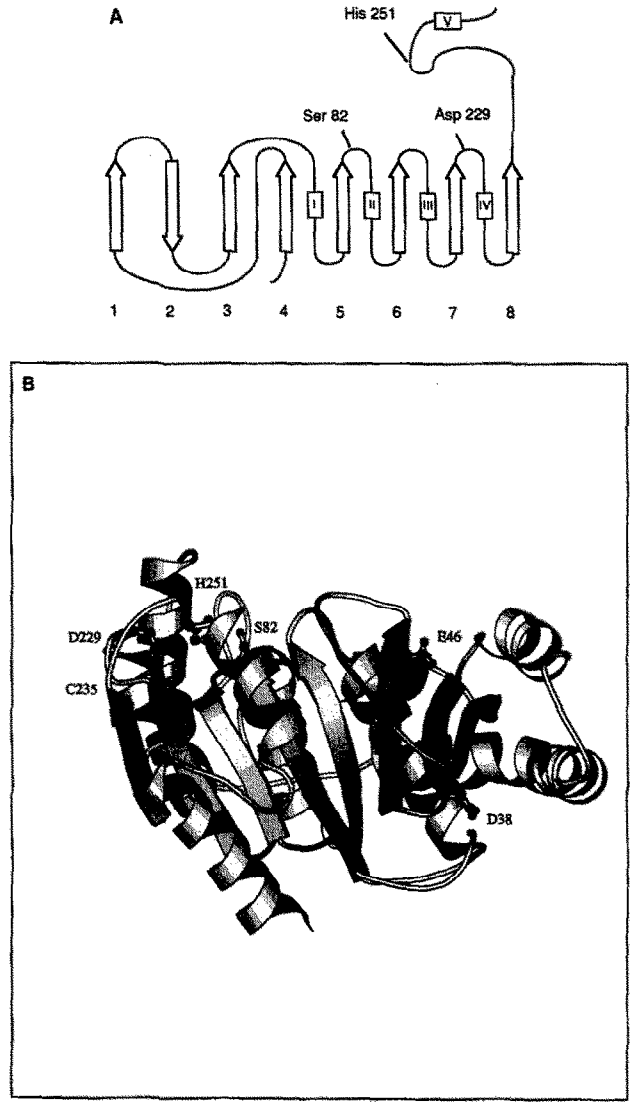

Fig. 5. Structural model of *P. aeruginosa* lipase. (A) Secondary structure (amino acid residue numbers) consisting of β-strands 1 (26–31); 2 (33–38); 3 (40–48); 4 (8–15); 5 (75–82); 6 (101–107); 7 (220–226); 8 (241–246); and α-helices I (55–70); II (83–95); III (174–191); IV (231–236); V (270–285). (B) Tertiary structure shown as a ribbon plot. Side chains of residues Asp^{38}, Glu^{46}, Ser^{82}, Asp^{229}, His^{251}, Cys^{183} and Cys^{235} are displayed in ball–stick representation.

strand 3 which is not completely surface-exposed. After digestion with *S. aureus* protease V8 the enzymatic activity of lipase remained unchanged, suggesting the presence of enzymatically active fragments. Our attempts to isolate these active fragments by isoelectric focusing were not successful, indicating that, after cleavage with *S. aureus* protease V8, the N- and C-terminal parts of the lipase remained non-covalently bound, preserving enzymatic activity. Dissociation of these two fragments, e.g. upon heating in the presence of SDS, may then lead to two inactive fragments. Furthermore, our 3D structural model suggests that the N-terminal part of the lipase, which is no longer present in both 26 and 25 kDa fragments, comprises the oxyanion hole as a structurally and functionally important part of the lipase which should be absolutely necessary for enzymatic activity.

Interfacial activation, which was thought to be a unique property of true lipases, mediated by a lid-like α-helical structure covering the active site [29], appears to be missing in *P. aeruginosa* lipase, as does the lid in our 3D structural model. It is interesting to mention that another hydrolytic enzyme, the cutinase from *Fusarium solani*, does not show interfacial activation. The X-ray structure of cutinase refined at 1.6 Å resolution revealed the absence of a lid-like structure [36]. Recently, a lipase from guinea pig pancreas was described which also did not show interfacial activation. This enzyme carried a deletion in a so-called lid-domain, suggesting that the absence of a lid is responsible for the absence of interfacial activation [37]. Obviously,

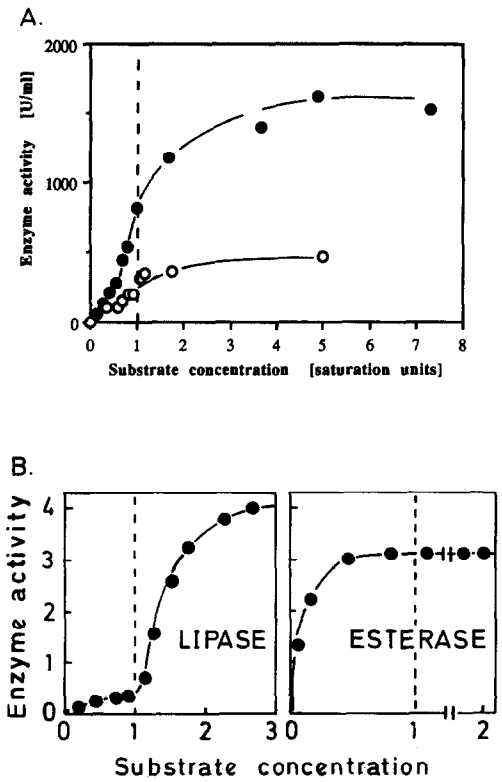

Fig. 6. Activity of *P. aeruginosa* lipase at different substrate concentrations of (A) triacetin (○, saturation concentration = 306 mM) and tripropionin (●, saturation concentration = 15 mM). (B) Classical activity profile of a pancreatic lipase and a horse liver esterase at different substrate concentrations exceeding the saturation point (modified after [1]). The dashed lines indicate the point of substrate saturation.

more crystal structures of lipases need to be solved in order to decide whether the definition of a true lipase (EC 3.1.1.3) should be changed with respect to the property of interfacial activation.

A 3D structural model of the *P. aeruginosa* lipase was built assuming that conserved parts of the amino acid sequences of different lipases represent conserved structural elements. Recently, such conservation was described for residues involved in formation of the active site, the disulfide bridges, salt bridges and some residues forming the protein core [38]. Our structural model fits the α/β hydrolase fold for hydrolytic enzymes [17], which also holds for three lipases with known 3D structures [29]. Based on these findings, and additional information from site-directed mutagenesis experiments of active-site residues in lipases from *P. glumae* [31] and *S. hyicus* [39], we expect a high degree of probability for our model concerning the position and conformation of (i) the catalytic triad, (ii) the disulfide bridge, and (iii) the surface exposition of the region around residue Asp^{38}. On the other hand, we are aware of several uncertainties in our model. The region covering amino acid residues 115–215 may be subject to changes because there is not enough structural information available. Another part which may deviate from reality is the number of β-strands. However, as long as there is no crystal structure available for *P. aeruginosa* lipase we regard the model as a reasonable working hypothesis allowing us to predict biochemical and structural properties which can be experimentally determined. Furthermore, it will be exciting to compare the model structure with a crystal structure of a *Pseudomonas* lipase as soon as the coordinates become available.

Acknowledgements: This work was supported by the EEC-BRIDGE T-project on lipases, contract No. BIOT CT-910272. We thank Robert Verger for critically reading the manuscript. Expert technical assistance of Birgit Forth is gratefully acknowledged.

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