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Role of the calcium ion and the disulfide bond in the *Burkholderia glumae* lipase

Mohamed El Khattabi [a,1], Patrick Van Gelder [b], Wilbert Bitter [a,2], Jan Tommassen [a,*]

[a] *Department of Molecular Microbiology, Institute of Biomembranes, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands*
[b] *Department of Ultrastructure, Vlaams Instituut voor Biotechnologie, Paardestraat 65, 1640 Sint-Genesius Rode, Belgium*

Abstract

The role of the Ca^{2+} ion that is present in the structure of *Burkholderia glumae* lipase was investigated. Previously, we demonstrated that the denatured lipase could be refolded in vitro into an active enzyme in the absence of calcium. Thus, an essential role for the ion in catalytic activity or in protein folding can be excluded. Therefore, a possible role of the Ca^{2+} ion in stabilizing the enzyme was considered. Chelation of the Ca^{2+} ion by EDTA severely reduced the enzyme activity and increased its protease sensitivity, however, only at elevated temperatures. Furthermore, EDTA induced unfolding of the lipase in the presence of urea. From these results, it appeared that the Ca^{2+} ion in *B. glumae* lipase fulfils a structural role by stabilizing the enzyme under denaturing conditions. In contrast, calcium appears to play an additional role in the *Pseudomonas aeruginosa* lipase, since, unlike *B. glumae* lipase, in vitro refolding of this enzyme was strictly dependent on calcium. Besides the role of the Ca^{2+} ion, also the role of the disulfide bond in *B. glumae* lipase was studied. Incubation of the native enzyme with dithiothreitol reduced the enzyme activity and increased its protease sensitivity at elevated temperatures. Therefore, the disulfide bond, like calcium, appears to stabilize the enzyme under detrimental conditions.

Keywords: Lipase; Calcium; Disulfide bond; *Burkholderia glumae*; *Pseudomonas aeruginosa*

1. Introduction

Because of their capacity to hydrolyze a wide variety of triacylglycerols and to synthesize acylglycerol esters, lipases are important in many industrial applications [1]. Especially, lipases of bacterial origin received much attention for their ability to function in extreme environments, such as high temperatures. Therefore, many bacterial lipases, such as those produced by *Pseudomonas* and *Burkholderia* species, have been characterized. The active-site of these lipases is composed of the catalytic triad serine, histidine and an acidic residue, Asp or Glu [2], similarly to lipases from eukaryotic origin [3]. Based on sequence similarities, *Pseudomonas* and *Burkholderia* lipases were classified into three families [4]. Whereas the family III lipases are large (50 kDa) and unrelated to the other lipases, lipases from families I and II show

* Corresponding author. Tel.: +31-30-2532999; fax: +31-30-2513655.
E-mail address: j.p.m.tommassen@bio.uu.nl (J. Tommassen).
[1] Present address: BiOMaDe Technology Foundation, Nijenborgh 4, 9747 AG Groningen, The Netherlands.
[2] Present address: Department of Medical Microbiology, VU Medical Centre, Van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands.

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considerable sequence similarity. Furthermore, the lipases from families I and II are secreted by a two-step secretion pathway, the type II secretion pathway [5,6]. These lipases are synthesized as precursor proteins with a signal sequence, which directs them to the Sec machinery in the inner membrane. After translocation across the inner membrane by the Sec translocase, lipases fold into an active enzyme in the periplasm. Periplasmic folding of the lipases and of other type II-secreted proteins is a prerequisite for their subsequent translocation across the outer membrane. Proteins that fail to fold are not secreted but rapidly degraded in the periplasm [7,8]. The translocation across the outer membrane is mediated by a complex translocation machinery, called Xcp in *Pseudomonas aeruginosa* [9]. The lipases of families I and II are substrates for periplasmic folding catalysts, such as the Dsb system, which is involved in the formation and isomerization of disulfide bonds [10,11]. Furthermore, they depend on a dedicated helper protein, designated Lif (lipase-specific foldase) for obtaining their active conformation. The Lif protein is anchored in the inner membrane by its N-terminal hydrophobic segment, whereas the majority of the protein is located in the periplasm [12]. It functions as a steric chaperone that helps lipase to overcome an energetic barrier in the folding pathway [13].

The three-dimensional structures of some of these lipases have been resolved [14–17]. These structures revealed the presence of a disulfide bond between the two cysteine residues in the sequences and the presence of a bound Ca^{2+} ion. The role of the Ca^{2+} ion is not entirely clear. On one hand, substitution of one of the calcium ligands in the *Burkholderia glumae* or *Burkholderia cepacia* lipases by alanine prevented the formation of active enzymes [14,18], suggesting an important role in catalysis. On the other hand, the distance between the Ca^{2+} ion and the active-site serine [15,16] made a direct role of the Ca^{2+} ion in the catalysis unlikely. The active-site residue His285 in *B. glumae* lipase is located in a loop containing three of the calcium ligands, i.e. Asp287, Gln291, and Val295 [14]. Therefore, binding of calcium may be required to stabilize the architecture of the molecule around the active-site. However, we demonstrated recently that denatured *B. glumae* lipase refolded in vitro into an active conformation in the presence of Lif but without any calcium [13], confirming that calcium is not required for the catalytic activity of the enzyme and demonstrating that it is also not required for the folding of the enzyme. In contrast, the in vitro refolding of lipase from *P. aeruginosa* TE3285 was dependent on calcium [19]. Like *B. glumae* lipase [13], the in vitro refolded lipase from *P. aeruginosa* TE3285 was found to form a complex with its cognate Lif [19], but the formation of this complex was dependent on the presence of calcium [19]. After addition of EDTA, the refolded lipase lost its activity immediately, and this was accompanied by the dissociation of the complex. Thus, calcium appears to play another or an additional role in *P. aeruginosa* lipase as compared to *B. glumae* lipase, although one cannot exclude a priori that the different results were caused by the different experimental conditions.

In this study, we investigated whether the different roles reported for calcium in the folding of lipase from *B. glumae* and from *P. aeruginosa* are related to the different experimental conditions. Therefore, we studied the refolding of lipase from *P. aeruginosa* under the conditions, previously applied for *B. glumae* lipase. Furthermore, we studied the possibility that calcium and the disulfide bond in *B. glumae* lipase fulfil structural roles by stabilizing the protein under conditions that challenge their conformation.

2. Experimental

2.1. Purification of lipase

B. glumae lipase was a generous gift from A.J. Slotboom (Utrecht University). *P. aeruginosa* lipase was purified from the extracellular medium of *lasB* mutant strain PAN10 [20]. For overproduction of the lipase, plasmid pBBL7 [21], a pBBR1MCS derivative [22] containing the structural genes for lipase and the lipase-specific foldase, was introduced into this strain. PAN10 (pBBL7) was grown for 20 h at 37 °C in tryptic soy broth supplemented with 300 μg/ml chloramphenicol for the maintenance of the plasmid. Cells were removed from a 400 ml culture by centrifugation for 10 min at 5000 rpm in SLA-1500 rotor (Sorvall), and the cell-free culture supernatant was concentrated 20-fold in a concentrator unit (Schleicher & Schuell), equipped with a membrane with a molecular weight cut off of 10 kDa. The concentrated supernatant was

washed in 20 mM Tris–HCl (pH 8.0) on the same device, and the lipase was purified after loading the total sample on a Q-sepharose column (Pharmacia) equilibrated with 20 mM Tris–HCl (pH 8.0). The column was washed with the same buffer, and the bound proteins were eluted with a gradient of 0–2 M NaCl in 20 mM Tris–HCl (pH 8.0) in a total volume of 300 ml. The eluted protein fractions containing lipase were concentrated and the solvent was exchanged for 20 mM Tris–HCl (pH 8.0), 100 mM NaCl. Fractions of 1 ml were loaded on a Sephacryl S-300 column (Pharmacia), and the proteins were eluted with the same buffer.

2.2. Unfolding–refolding of the P. aeruginosa lipase

Unfolding–refolding reactions were performed as described previously for *B. glumae* lipase [13], except that dithiothreitol (DTT) was omitted during the unfolding procedure and refolding was carried out in Tris buffer instead of phosphate buffer. These changes in the procedure did not influence the unfolding and refolding efficiencies (unpublished observation). Briefly, 20 μl of lipase stock solution (12 μg) were added to 100 μl of 20 mM Tris–HCl (pH 8.0), 8 M urea, 12.5 mM EDTA, and incubated at 56 °C for 1 h. The refolding was started by diluting 10 μl of unfolded lipase into 1 ml of 20 mM Tris–HCl (pH 8.0), containing or not 10 μg Lif of *P. aeruginosa* and/or 5 mM $CaCl_2$ as described in Section 3. The *P. aeruginosa* Lif used in these experiments (a generous gift from K.-E. Jaeger) is a recombinant Lif, in which the N-terminal membrane anchor was replaced by a poly-histidine tag.

2.3. Lipase assay

Lipase activity was measured by using *para*-nitrophenyl caprylate as the substrate [23]. One lipase unit (U) was defined as the amount of lipase that degrades 1 μmol of the substrate per min.

2.4. Trypsin digestion

Purified *B. glumae* lipase was incubated for 15 min under various conditions (as described in Section 3) in a reaction volume of 20 μl. Subsequently, trypsin was added at a final concentration of 40 μg/ml and incubation was continued for 30 min at room temperature. Addition of 1 mM phenylmethylsulfonyl fluoride stopped trypsin degradation, and the samples were analyzed by SDS-PAGE.

2.5. Circular dichroism spectroscopy

Denaturation of the lipase was monitored by circular dichroism (CD) spectroscopy at 220 nm on an AVIV spectropolarimeter, using a 0.1 mm path cell and a bandwidth of 1 nm. The spectra were obtained by heating a sample of 10 μM lipase in 20 mM sodium-phosphate buffer (pH 7.0), 8 M urea, 45 mM NaCl, containing or not 10 mM EDTA, over a temperature range from 25 to 60 °C. Sampling was done at every degree Celsius with an equilibration time of 6 s and an averaging time of 10 s. Each sampling point was measured three times.

2.6. N-terminal sequencing

Protein fragments were separated by SDS-PAGE with 0.4 mM thioglycolic acid included in the separating gel. After blotting on polyvinylidene difluoride membranes and staining of the blots with Coomassie Brilliant Blue, protein bands were cut from the membranes and used for N-terminal sequencing by Edman degradation, using a protein sequencer model 476A (Perkin-Elmer).

3. Results

3.1. Purification of the P. aeruginosa lipase

The production of lipase by a wild-type *P. aeruginosa* strain was low. However, introduction of a plasmid containing the structural genes for lipase and the lipase-specific foldase resulted in the secretion of high amounts of lipase into the extracellular medium. Therefore, we decided to use this overexpression system to purify the lipase. Plasmid pBBL7 was transferred into the strain PAN10, which is deficient in the production of the major secreted protease elastase, due to a *lasB* mutation. The supernatant of PAN10 (pBBL7), which contained lipase as the major protein (Fig. 1, lane 1), was loaded onto an anion-exchange

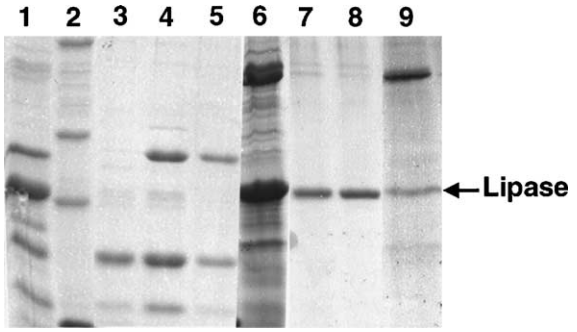

Fig. 1. Coomassie-stained SDS-PAGE gel showing the purification of *P. aeruginosa* lipase. Proteins from the culture supernatant of PAN10 (pBBL7) (lane 1) were loaded on an anion-exchange column. The unbound proteins (lanes 3–5) were washed from the column with 20 mM Tris–HCl (pH 8.0), and the bound proteins were eluted with a 0–2 M NaCl gradient. Peak fractions containing lipase were pooled (lane 6), concentrated and loaded on a gel-filtration column. The protein profiles of the eluted fractions number 5, 15 and 25 are shown in lanes 7–9, respectively. Lane 2 contains molecular weight marker proteins (from top to bottom: 68, 40, 30 and 21.5 kDa). The position of the lipase is indicated with an arrow at the right.

column on which lipase was retained, whereas several other major proteins eluted during the washing with column buffer (Fig. 1, lanes 3–5). The column was subsequently eluted with a salt gradient, and the major lipase-containing fractions were pooled (Fig. 1, lane 6). The lipase was further purified by gel filtration. Remarkably, whereas the molecular weight of lipase is 30 kDa, the enzyme eluted before a 60 kDa protein (Fig. 1, lanes 7–9), indicating that it formed high molecular weight aggregates or that it was associated with other compounds, such as lipopolysaccharides, as described earlier [24]. The peak fractions containing highly pure lipase (fractions 5–20; for example, see Fig. 1, lanes 7 and 8) were pooled, and the purity of the final preparation was over 90%, as estimated from Coomassie Brilliant Blue-stained SDS-PAGE gels.

3.2. *In vitro refolding of the P. aeruginosa lipase*

Previously, we reported that *B. glumae* lipase could efficiently be refolded in the presence of its Lif in the absence of calcium [13]. In contrast, calcium was reported to be essential for efficient folding in vitro of the lipases of *P. aeruginosa* [19] and *B. cepacia* [18]. To exclude the possibility that the different calcium dependency is caused by the different experimental conditions, we decided to study the refolding of *P. aeruginosa* lipase under the same experimental conditions as applied for *B. glumae* lipase. Therefore, purified *P. aeruginosa* lipase was unfolded by incubation for 1 h at 56 °C in 20 mM Tris–HCl (pH 8.0) containing 6.5 M urea and 10 mM EDTA. Refolding of the denatured lipase was initiated by diluting the sample 100-fold in 20 mM Tris–HCl (pH 8.0), containing or not $CaCl_2$ and *P. aeruginosa* Lif, and lipase activity was measured after 2 h incubation at room temperature. In the absence of $CaCl_2$ and Lif, almost no lipase activity was recovered (Fig. 2, bar 1), demonstrating that the lipase was unfolded and did not refold under the conditions used in this experiment. In the presence of Lif but in the absence of $CaCl_2$, similarly low amounts of lipase activity were regained (Fig. 2, bar 2). However, more than 50% of the initial lipase activity (Fig. 2, bar 8) was recovered when the lipase was refolded in the presence of both Lif and $CaCl_2$ (Fig. 2, bar 3), confirming the dependency of *P. aeruginosa* lipase on calcium for regaining its activity. Also Lif was essential for the refolding of the lipase, since almost no lipase activity was recovered when the unfolded lipase was diluted into refolding buffer containing $CaCl_2$ alone (Fig. 2, bar 4).

To study whether the Lif and calcium stimulated sequential events during refolding, the two compounds were added sequentially during refolding of lipase. When $CaCl_2$ and Lif were added together 2 h after initiation of refolding, the same amount of lipase activity was recovered, as when they were present immediately at the initiation of refolding (Fig. 2, bar 5). This result shows that Lif of *P. aeruginosa*, like that of *B. glumae* [13], does not act by preventing an off-pathway reaction during folding, but that it adds steric information necessary for the correct folding of the lipase. When refolding was initiated in the presence of $CaCl_2$, and Lif was added after 2 h, only half the amount of lipase activity was recovered after another 2 h incubation at room temperature (Fig. 2, bar 6). This result suggests that Ca^{2+} ions stimulate an off-pathway folding reaction in the absence of Lif, leading to the irreversible loss of lipase molecules. On the other hand, when the refolding was initiated in the presence of Lif, and $CaCl_2$ was added after 2 h incubation, about 55% of the native lipase activity was

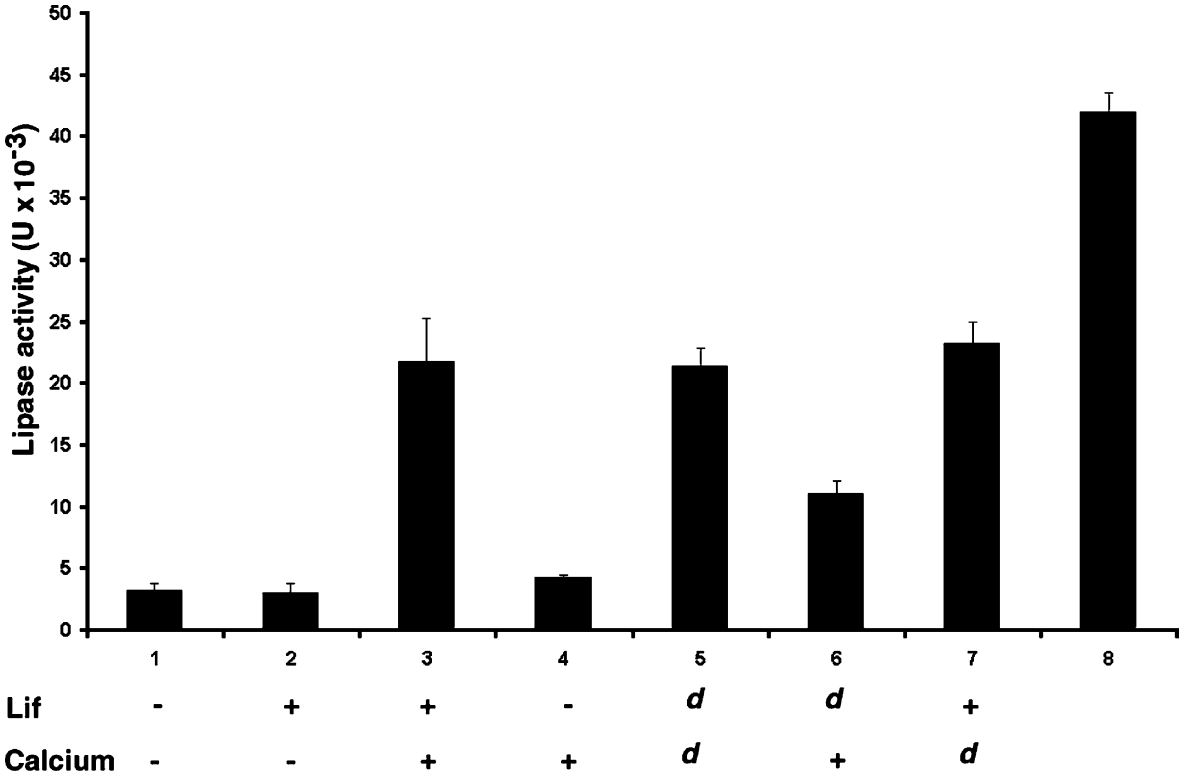

Fig. 2. In vitro refolding of *P. aeruginosa* lipase. Unfolded *P. aeruginosa* lipase (1 μg) was refolded for 2 h at room temperature in 20 mM Tris–HCl (pH 8.0) in the presence (+) or absence (–) of 10 μg His-tagged *P. aeruginosa* Lif and 5 mM $CaCl_2$, and lipase activities were measured. The letter 'd' indicates that the addition of Lif or $CaCl_2$ was delayed, i.e. 2 h after initiation of refolding, in which cases incubation was continued for another 2 h before lipase activities were measured. The lipase activities regained were compared with that of 1 μg native lipase (bar 8). Each value represents the average of three independent experiments, and error bars are indicated.

regained (Fig. 2, bar 7). These results suggest that Lif activity precedes the incorporation of calcium into the lipase.

3.3. Structural role of the Ca^{2+} ion in the *B. glumae* lipase

The *B. glumae* lipase could be refolded efficiently in vitro in the presence of the steric chaperone Lif [13]. The kinetics of folding appeared to be very fast, either in the presence or absence of calcium. Thus, the Ca^{2+} ion, which was found in the structure of the lipase, is neither required for the catalytic activity nor essential for the proper folding of the enzyme. Hence, we considered the possibility that the Ca^{2+} ion contributes to the stability of the protein. To test this possibility, the purified native enzyme was incubated for 15 min with either 10 mM EDTA or 10 mM $CaCl_2$ at various temperatures, and the enzyme activity was subsequently measured at room temperature. The preincubation with EDTA at temperatures up to 50 °C did not affect the lipase activity (Fig. 3A, horizontally hatched bars), consistent with the notion that the Ca^{2+} ion is not essential for enzymatic activity. However, preincubation with EDTA at 70 °C drastically affected the enzyme activity (Fig. 3A), indicating that calcium has a structural role and prevents the lipase from loosing its active conformation at high temperatures. This notion was supported when the protease sensitivity of the enzyme was assessed (Fig. 4). When preincubated with $CaCl_2$, the lipase remained resistant to trypsin independently of the preincubation temperature applied. However, whereas the enzyme remained resistant to the protease

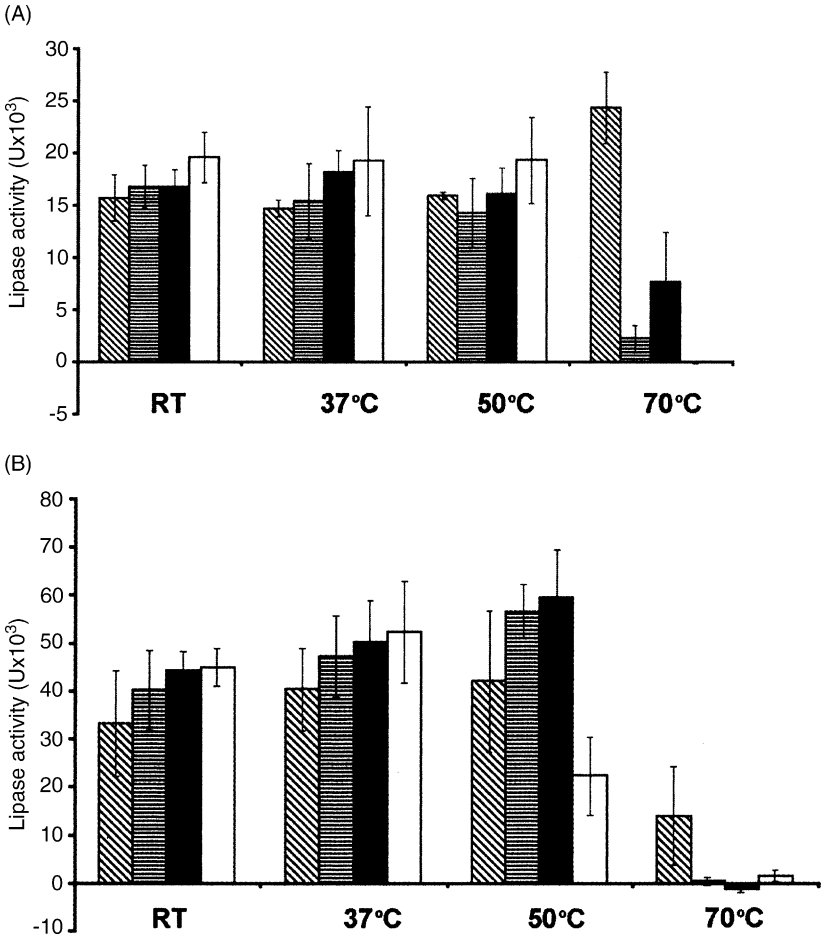

Fig. 3. Activity of *B. glumae* and *P. aeruginosa* lipases after preincubation in the presence of EDTA or DTT. Native *B. glumae* lipase (1 μg) (A) or *P. aeruginosa* lipase (1.5 μg) (B) were dissolved in 20 mM Tris–HCl (pH 8.0) containing 10 mM $CaCl_2$ (oblique hatched bars), 10 mM EDTA (horizontally hatched bars), 10 mM DTT (black bars) or 10 mM DTT and 10 mM EDTA (white bars) and incubated at the temperatures indicated for 15 min. RT: room temperature. Subsequently, lipase activities were measured at room temperature. Each value represents the average of three independent experiments, and error bars are indicated.

after preincubation with EDTA at room temperature, it was degraded after preincubation with EDTA at the higher temperatures (Fig. 4). Finally, we determined by CD measurements whether the Ca^{2+} ion stabilizes the enzyme in the presence of a denaturant. For this purpose, the enzyme was incubated in 8 M urea in the presence or absence of 10 mM EDTA over a temperature range from 25 to 60 °C, and the ellipticity at 220 nm was determined. In the presence of EDTA, the lipase had lost its structure already at 25 °C, whereas the protein was stable up to approximately 43 °C in the absence of EDTA (Fig. 5). Together, these results demonstrate that the Ca^{2+} ion stabilizes the lipase and, hence, that calcium plays a structural role.

3.4. Role of the disulfide bonds in B. glumae lipase

The disulfide bond in *B. glumae* lipase is not required for enzymatic activity [13]. However, it might still contribute to the stability of the enzyme. To test this possibility, the native lipase was incubated for 15 min at various temperatures with 10 mM DTT,

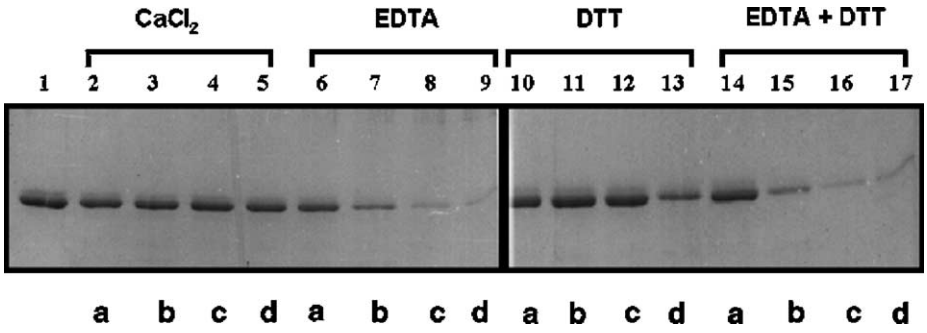

Fig. 4. Protease resistance of *B. glumae* lipase. Native *B. glumae* lipase (3 μg) was dissolved in 20 mM Tris–HCl (pH 8.0) containing 10 mM $CaCl_2$, 10 mM EDTA, and/or 10 mM DTT as indicated and incubated for 15 min at room temperature (a), 37 °C (b), 50 °C (c) or 70 °C (d). Samples were subsequently digested with trypsin for 30 min at room temperature and analyzed by SDS-PAGE and Coomassie staining. Lane 1 contains the undigested lipase.

and lipase activity was subsequently measured at room temperature (Fig. 3A). The lipase activity did not decrease by preincubation with DTT, except at 70 °C, when about 50% of the lipase activity was lost (Fig. 3A, black bars). Furthermore, also the protease sensitivity of lipase changed after preincubation at these high temperatures in the presence of DTT (Fig. 4). This result indicates that the disulfide bond, like the Ca^{2+} ion, fulfils a structural role in *B. glumae* lipase.

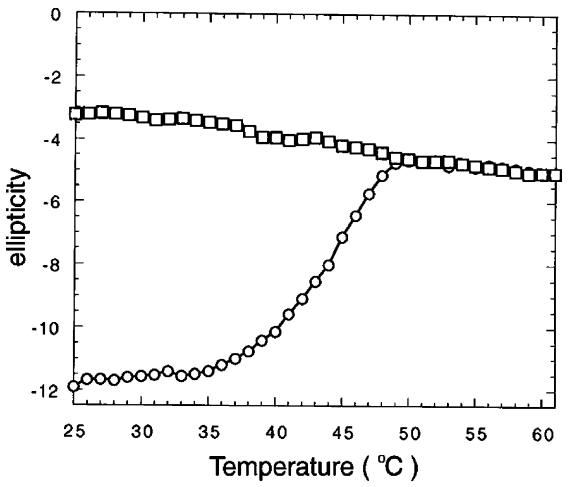

Fig. 5. Loss of the secondary structure of *B. glumae* lipase in urea in the presence or absence of EDTA. Native *B. glumae* lipase (10 μM), contained in 20 mM sodium-phosphate buffer (pH 7.0), 8 M urea, 45 mM NaCl and either 10 mM EDTA (□) or no EDTA (○), was heated over a temperature range from 25 to 60 °C, and the ellipticity at 220 nm was recorded on a spectropolarimeter.

3.5. Effect of EDTA and DTT on *P. aeruginosa* lipase activity

The effect of preincubation at different temperatures with EDTA and DTT on the activity of *P. aeruginosa* lipase was also investigated. Similar results as described for *B. glumae* lipase were obtained with *P. aeruginosa* lipase, except that this lipase was less stable at 70 °C even in the presence of Ca^{2+}. The activity of the enzyme was even further reduced after preincubation at 70 °C in the presence of DTT, EDTA or both (Fig. 3B). Furthermore, its activity was severely reduced at 50 °C in the presence of both EDTA and DTT (Fig. 3B, white bars). These results indicate that, like in *B. glumae* lipase, both the disulfide bond and the Ca^{2+} ion fulfil a structural role in *P. aeruginosa* lipase.

3.6. Protease sensitivity of lipase in the presence of Lif

It was previously reported that *P. aeruginosa* lipase, refolded by and associated with its cognate Lif, was more sensitive to Ca^{2+} chelation by EDTA than was the native enzyme [19]. This result suggests that Ca^{2+} ions are essential for complex formation between the Lif and its cognate lipase, or alternatively, that lipase is in a different conformation when associated with the Lif. Since its cognate Lif could refold unfolded *B.*

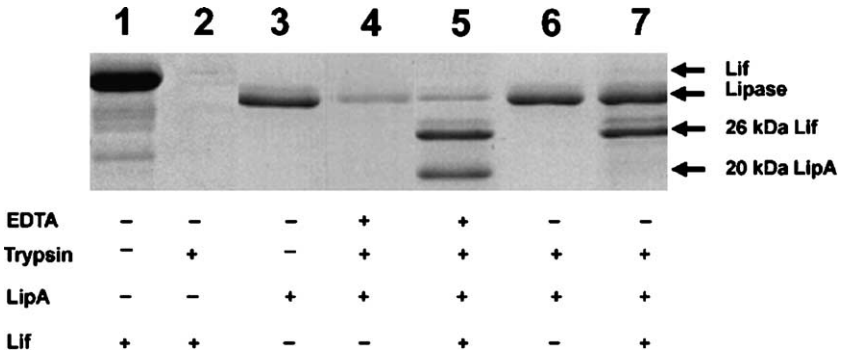

Fig. 6. Stability of the *B. glumae* lipase in the presence of EDTA and *B. glumae* Lif. *B. glumae* lipase was digested with trypsin (40 μg/ml) at 37 °C in the presence of EDTA (lane 4) and in the presence of EDTA and *B. glumae* Lif (lane 5) and compared to trypsin digestion of the lipase in the absence of EDTA and Lif (lane 6) or in the absence of EDTA but in the presence of Lif (lane 7). Lane 1 contains untreated Lif. Lane 2 contains Lif, that was digested with trypsin (40 μg/ml) at 37 °C. Lane 3 contains untreated lipase. In each reaction, 3 μg of lipase and 6 μg of Lif were used. EDTA was used at 10 mM. Protected proteins were analyzed by SDS-PAGE and Coomassie staining. The positions of the Lif, the lipase, the 26 kDa Lif fragment and the 20 kDa lipase fragment are indicated at the right.

glumae lipase even in the absence of calcium, it was of interest to study whether the Lif affects the conformation of lipase in the presence or absence of calcium. Therefore, native *B. glumae* lipase was incubated with trypsin in the presence or absence of *B. glumae* Lif and EDTA. In the absence of EDTA and Lif, the lipase was resistant to trypsin digestion at 37 °C (Fig. 6, lane 6), whereas the 37 kDa Lif (Fig. 6, lane 1) is completely degraded by trypsin in the absence of lipase (Fig. 6, lane 2). Trypsin treatment of the lipase in the presence of the Lif resulted in the appearance of a 26 kDa fragment of Lif (Fig. 6, lane 7), as reported previously [13], whereas the lipase was not degraded. In the presence of EDTA at 37 °C, the lipase was largely degraded by trypsin, leaving only a small proportion of the undigested full-length lipase (Fig. 6, lane 4). When both the Lif and EDTA were present, the mature form of lipase almost disappeared, whereas a fragment of approximately 20 kDa appeared in addition to the 26 kDa Lif fragment (Fig. 6, lane 5). The N-terminal sequence of this 20 kDa fragment was Ala-Asp-Thr-Tyr-Ala, which corresponds to amino acids 1–5 of the mature lipase. Thus, whereas the lipase is sensitive to trypsin when calcium is chelated by EDTA, an N-terminal 20 kDa fragment of the enzyme is protected under these conditions by the presence of the Lif. This result indicates that the lipase undergoes a conformational change by its interaction with Lif in the presence of EDTA.

4. Discussion

Folding of lipase is a complex event requiring several steps. Before secretion, secondary and tertiary structure is acquired, a disulfide bridge and a *cis*-peptide bond are formed, and a Ca^{2+} ion is integrated into the structure. Various chaperones and folding catalysts, such as Lif [13,19] and DsbA [25], are involved in these events. Also, a peptidyl–peptidyl *cis–trans* isomerase might be involved, since the peptide bond between Gln291 and Leu292 in *B. glumae* lipase is in the *cis*-configuration. This *cis*-peptide bond was found in all lipases from bacterial origin with resolved three-dimensional structures [15,17,26]. However, in vitro refolding studies demonstrated that only the Lif protein is essential for the refolding of *B. glumae* lipase [13]. Calcium appeared to be dispensable in these studies, demonstrating that it is required neither for the catalytic activity nor for the appropriate folding of this lipase. In contrast, it has been reported that calcium is required for the in vitro refolding of *P. aeruginosa* lipase [19]. To exclude the possibility that the different requirement for calcium is due to different experimental conditions, we have now refolded *P. aeruginosa* lipase under the same experimental conditions as applied for *B. glumae* lipase. Our results confirmed that calcium is required for folding of this enzyme. Apparently, the Ca^{2+} ion fulfils different roles in these two related lipases. In *P.*

aeruginosa lipase, the Ca^{2+} ion might be involved in the correct positioning of the histidine residue of the catalytic triad, since three of the Ca^{2+} ion ligands are contained in a loop together with the histidine residue [26]. However, in spite of the similar architecture of *B. glumae* lipase [14], this enzyme refolded in the absence of calcium, suggesting that the active-site histidine is correctly positioned even without the help of calcium. Subsequently, we investigated whether calcium has a stabilizing role in the case of *B. glumae* lipase. Indeed, calcium was found to stabilize this enzyme under denaturing conditions, such as the presence of urea or high temperatures. Previously, it has been demonstrated that the substitution of one of the calcium ligands of *B. glumae* lipase, Asp241 [14], by Ala abolished the production of active lipase in vivo [2]. This result suggested that the Asp241 residue, and therefore the Ca^{2+}-binding site, is important for catalytic activity. However, it was also noticed that this mutation resulted in a drastically decreased amount of lipase produced [2], suggesting that the enzyme is less stable when it cannot bind calcium and is therefore degraded by proteases produced in the bacteria. Consistently, in our studies, the enzyme became sensitive to trypsin when the Ca^{2+} ions were chelated with EDTA at 37 °C, i.e. at a physiological temperature.

In the absence of its Lif, the folding competence of the unfolded *P. aeruginosa* lipase was lost more rapidly in the presence of calcium. This suggests that either the Lif-mediated folding step should occur before calcium can be incorporated into the enzyme or that Lif is directly involved in the productive interaction between lipase and calcium, similarly as in the case of the propeptide of subtilisin, which was suggested to regulate the binding of calcium to the mature protease [27]. Consistently with the latter possibility, calcium was found to be required for the interaction of *Pseudomonas* sp. 109 lipase with the cognate Lif and formation of the lipase–Lif complex was important for regaining enzymatic activity [19]. However, the incorporation of calcium cannot be the primary role of Lif, since refolding of *B. glumae* lipase, which is independent of calcium, was still dependent on its Lif. Furthermore, interaction of *B. glumae* and *P. aeruginosa* lipases with their cognate Lif's occurred independently of calcium ([13] and results not shown). In protease protection experiments, the protease-resistant native *B. glumae* lipase protected a large 26 kDa fragment of its cognate Lif, lacking the N-terminal 71 amino acid residues [13], from trypsin degradation. This protected fragment probably contains the entire domain of the Lif required for chaperone activity, since a slightly longer fragment of *P. aeruginosa* Lif, lacking the N-terminal 61 residues, has been reported to retain its chaperone activity [28]. On the other hand, in the presence of EDTA, which renders *B. glumae* lipase sensitive to trypsin degradation, the cognate Lif protected a 20 kDa fragment of lipase from trypsin digestion. This result suggests a physical interaction between the proteins, which leads to conformational changes in both proteins, consistent with the function of the Lif as a steric chaperone.

In vitro refolding of *B. glumae* lipase into its active conformation occurred in the absence of an oxidizing agent, and we confirmed that the disulfide bridge between the two cysteines was not formed under these conditions. Apparently, the disulfide bond is important neither in catalysis nor in the folding of the lipase into its active conformation. Furthermore, *P. aeruginosa* lipase variants, in which one or both cysteine residues were replaced by a serine residue could still be folded in vitro into an active enzyme, indicating that the disulfide bond is not required for the activity of this lipase [25]. However, when the formation of the disulfide bond was prevented in vivo, either by substitution of the cysteines [25] or by inactivation of the *dsbA* gene [11], the amount of lipase produced was drastically decreased. Therefore, the enzyme might be unstable in the absence of the disulfide bond and degraded by cellular proteases. Alternatively, disulfide bond formation might increase the kinetics of folding, thereby reducing the time of exposure of the unfolded or partially folded lipase to detrimental proteases. We show in this study that disruption of the disulfide bond decreased the enzyme activity and stability of the *B. glumae* lipase at high temperatures. Although the destabilizing effect of DTT on *B. glumae* lipase was relatively small, it might be possible that the disulfide bond is only accessible to the reducing agent in the presence of a second denaturant, such as SDS or high temperature, as suggested previously in the case of *P. aeruginosa* lipase [25]. In conclusion, both the Ca^{2+} ion and the disulfide bond are not essential for the enzymatic activity of *B. glumae* lipase, but they have a stabilizing role under detrimental conditions,

whereas the Ca^{2+} ion in *P. aeruginosa* lipase has an additional role, possibly in the correct folding of the active center of the enzyme.

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