# Disulfide Bond in *Pseudomonas aeruginosa* Lipase Stabilizes the Structure but Is Not Required for Interaction with Its Foldase

KLAUS LIEBETON, ANNETTE ZACHARIAS, AND KARL-ERICH JAEGER*

*Lehrstuhl für Biologie der Mikroorganismen, Ruhr-Universität Bochum,
D-44780 Bochum, Germany*

*Pseudomonas aeruginosa* secretes a 29-kDa lipase which is dependent for folding on the presence of the lipase-specific foldase Lif. The lipase contains two cysteine residues which form an intramolecular disulfide bond. Variant lipases with either one or both cysteines replaced by serines showed severely reduced levels of extracellular lipase activity, indicating the importance of the disulfide bond for secretion of lipase through the outer membrane. Wild-type and variant lipase genes fused to the signal sequence of pectate lyase from *Erwinia carotovora* were expressed in *Escherichia coli*, denatured by treatment with urea, and subsequently refolded in vitro. Enzymatically active lipase was obtained irrespective of the presence or absence of the disulfide bond, suggesting that the disulfide bond is required neither for correct folding nor for the interaction with the lipase-specific foldase. However, cysteine-to-serine variants were more readily denatured by treatment at elevated temperatures and more susceptible to proteolytic degradation by cell lysates of *P. aeruginosa*. These results indicate a stabilizing function of the disulfide bond for the active conformation of lipase. This conclusion was supported by the finding that the disulfide bond function could partly be substituted by a salt bridge constructed by changing the two cysteine residues to arginine and aspartate, respectively.

The gram-negative bacterium *Pseudomonas aeruginosa* secretes an array of different enzymes via three distinct secretion pathways (40, 42, 43, 46). Most of them are exported in two consecutive steps through the so-called type II secretory pathway into the extracellular medium (11), among them a lipase LipA with a molecular mass of 29 kDa (39). This lipase shows remarkable enzymatic characteristics, e.g., a pronounced stereoselectivity (32) and a broad substrate specificity (23), making it an interesting candidate for biotechnological applications (24).

Enzymes being secreted via the type II secretion pathway contain a N-terminal signal sequence mediating the translocation across the cytoplasmic membrane by the Sec apparatus which has intensively been studied in *Escherichia coli* (8, 31). An additional machinery consisting of at least 12 so-called Xcp proteins is required for the secretion of the periplasmic intermediates through the outer membrane of *P. aeruginosa* (11). Although much effort has been expended to identify a potential recognition signal for the Xcp machinery within the extracellular proteins which distinguishes them from periplasmic proteins, no common linear recognition sequence has so far been identified. There is increasing evidence that extracellular proteins fold into a transport-competent conformation before translocation across the outer membrane (3, 5, 12–15, 30), suggesting that the formation necessary for secretion is a three-dimensional structural element composed of noncontiguous amino acid residues or parts of the primary structure. Many of the proteins secreted via the type II pathway contain a disulfide bond, and their secretion is reduced or abolished when the disulfide bond is broken. Examples include a cellulase from *Erwinia chrysanthemi* (3), the heat-stable enterotoxin II from *E. coli* (27), and the cholera toxin from *Vibrio cholerae* (28). On the other hand, there are also proteins and peptides which are secreted in the absence of intact disulfide bonds, examples being the proaerolysin from *Aeromonas* spp. (14), the lipase-acyltransferase from *Aeromonas hydrophila* (6), the heat-stable enterotoxin I from *E. coli* (47), and the pullulanase from *Klebsiella oxytoca* (35). Thus, it is impossible to ascribe to disulfide bonds in exported proteins a general function for secretion.

Lipases from different pseudomonads have been shown to depend on the presence of a specific additional protein to obtain a stable active conformation (for a review see reference 21). This protein seems to assist the folding process of the lipase in the periplasm, although the exact mechanism is still unknown. In *P. aeruginosa* this foldase is a 33-kDa periplasmic protein named LipH (or Lif) which is anchored to the cytoplasmic membrane (36).

The *P. aeruginosa* lipase is secreted via the type II secretion pathway (22, 25). The enzyme contains two cysteine residues which form a disulfide bond (20). Recently, it has been shown that this lipase is not secreted in a *P. aeruginosa* mutant strain devoid of the oxidoreductase DsbA, indicating the importance of the disulfide bond-forming system to the secretion of this enzyme (41). Here, we show by construction and characterization of cysteine-to-serine variants that the primary function of the disulfide bond in *P. aeruginosa* lipase is to stabilize the protein during and after translocation over the outer membrane.

## MATERIALS AND METHODS

**Bacterial strains.** *E. coli* JM 109, used for cloning experiments, was grown at 37°C in Luria-Bertani (LB) medium. *E. coli* BL21(DE3) pLysS was used for gene expression experiments which were performed as follows: 10 ml LB medium was inoculated to an optical density at 580 nm ($OD_{580}$) of 0.05 and grown at 37°C to an $OD_{580}$ of 0.6. Gene expression was induced by addition of isopropyl-β-D-

* Corresponding author. Mailing address: Lehrstuhl für Biologie der Mikroorganismen, Ruhr-Universitaet Bochum, Universitätsstrasse 150, D-44780 Bochum, Germany. Phone: (49) 234 322-3101. Fax: (49) 234 321-4425. E-mail: karl-erich.jaeger@ruhr-uni-bochum.de.

thiogalactopyranoside (IPTG) to a concentration of 0.4 mM. Cells were grown for another 3 h and harvested by centrifugation for 15 min at $12,000 \times g$ and 4°C. *P. aeruginosa* PABST7.1 (23), a lipase-deficient mutant of *P. aeruginosa* PAO1, was used as the homologous host for expression of lipase genes. Ten milliliters of 2× LB (20 g of trypton, 10 g of yeast extract, 10 g of NaCl, and 1 liter of distilled water) was inoculated with 50 μl of an overnight culture and grown for 16 h at 30°C. Gene expression was induced by addition of IPTG to a concentration of 0.5 mM. Cells were further grown for 24 h and were separated from the culture supernatant by centrifugation as described for *E. coli*. When appropriate, ampicillin (100 μg/ml) and chloramphenicol (50 μg/ml) were added to the medium for *E. coli* and carbenicillin (200 μg/ml) and tetracycline (50 μg/ml) were added to the medium for *P. aeruginosa*.

**Plasmids.** Wild-type and variant lipase genes were expressed from derivatives of plasmid pUCPKS (44) constructed as follows: a *Bam*HI/*Hin*dIII restriction fragment from pMALip4A, a pMa6 derivative (38) containing a recombinant lipase operon with a unique *Apa*I site between the genes *lipA* and *lipH* was ligated to pUCPKS opened with the same restriction enzymes. To eliminate a second *Apa*I site, this plasmid was digested with *Afl*III, deleting a 515-bp fragment, and the ends were blunted with T4 DNA polymerase and self-ligated, yielding plasmid pUCPL6A. For expression in *E. coli* the lipase gene encoding the mature protein was fused to the *pelB* signal sequence from *Erwinia carotovora* by ligating an *Eco*RI/*Eco*72I fragment from pUCPL6A and its cysteine variant derivatives to pELpelB-IR-O (S. Rösmann, unpublished), a pET22b derivative containing the lipase operon (Novagen, Bad Soden, Germany), which was cut with the same restriction enzymes.

**Site-directed mutagenesis.** The cysteines in *P. aeruginosa* lipase, C183 and C235, were replaced by serines or by arginine and aspartate, respectively, by PCR mutagenesis using the method of Barettino et al. (2). An *Eco*RI/*Apa*I fragment of the gene *lipA* was ligated to pBluescript II KS (Stratagene, Heidelberg, Germany), yielding plasmid pMut3. This plasmid was digested with *Afl*III/*Kpn*I and the ends were blunted with T4 DNA polymerase and self-ligated, yielding plasmid pMut4. These plasmids were used as the template DNA in the first and second PCR reactions respectively. For screening purposes, an endonuclease restriction site was either introduced or eliminated in each mutagenic primer in addition to the corresponding mutation. The following oligonucleotides were purchased from Eurogentec (Seraing, Belgium) (mutagenic base substitutions in bold and additional restriction site base substitutions in italics): C183S (TCGG CC**A**GCGGCGAAGGCGCGTACAAGGTC; Δ*Kas*I), C235C (CTGGTCGGC ACC*A*GCAGTTCGCACCTG; Δ*Pst*I) C183R (CCCACCTCGG*C*GCGGC GAAGGCGCC; *Bss*HI), C235D (CTGGTCGGCACC*GA*CAGTTCGCACC TG; Δ*Pst*I), universal primer A (GCGCAATTAACCCTCACTAAAGGGAAC AAA), and universal primer B (GCGTAATACGACTCACTATAGGGCAA). Amplification was performed using 1 ng of template DNA, 10 pmol each of mutagenic primer and universal primer A in the first PCR reaction and universal primers A and B in the second PCR reaction, and 2 U of Goldstar-*Taq* polymerase (Eurogentec) in a 50-μl reaction mixture volume containing 75 mM Tris-HCl (pH 9.0 at 25°C), 20 mM (NH₄)₂SO₄, 0.01% (wt/vol) Tween 20, 1.5 mM MgCl₂, 10% (vol/vol) dimethyl sulfoxide, and a 200 μM concentration of each deoxynucleoside triphosphate. To the second PCR reaction mixture 1 to 10 ng of the amplicon from the first PCR reaction was added as a megaprimer which was purified by agarose gel electrophoresis and subsequent gel elution using the Nucleospin extraction kit (Macherey & Nagel, Düren, Germany). Reaction conditions began with 2 min at 98°C, followed by the addition of the *Taq* polymerase and 30 cycles of 1 min at 94°C, 2 min at 64°C, and 1 min at 72°C, as well as a final incubation for 7 min at 72°C on a Robocycler 40 (Stratagene). After purification as described above, PCR products were digested with *Apa*I/*Eco*RI and ligated to pBluescript II KS (Stratagene), yielding pKSC183S, pKSC235S, pKSC183R, and pKSC235D. The identity of all PCR-amplified fragments was confirmed by DNA sequencing (34). The double variants C183S-C235S and C183R-C235D were constructed by replacement of the *Hin*dIII/*Apa*I fragments from pKSC183S and pKSC183R with the corresponding fragments obtained from pKSC235S and pKSC235D, respectively. Expression vectors containing variant lipase genes were constructed by ligating the corresponding *Eco*RI/*Apa*I fragment from the corresponding pKS derivatives to pUCPL6A.

**In vitro refolding.** The in vitro refolding procedure will be described elsewhere. Briefly, cell lysates were obtained by suspension of cells in lysis buffer (50 mM Tris-HCl [pH 8.0], 2 mM EDTA) to an OD₅₈₀ of 20. After freezing and thawing, 600 μl of the suspension was treated with ultrasonication (10 min, 60% duty cycle, 50% power output) with a Branson Sonifier 250 equipped with a microtip. Subsequently, solid urea was added to a final concentration of 8 M and samples were incubated for 1 h at 37°C with agitation. Finally, samples were diluted in refolding buffer (50 mM Tris-HCl [pH 8.0], 3.5 mM CaCl₂, 45%

[vol/vol] glycerol, 0.7 mM laurylmaltoside, 0.5 mM oxidized glutathione, 10 ng of purified LipH per ml) and incubated for 3 h at 30°C with agitation.

**Enzyme activity assay.** The photometric assay with *p*-nitrophenylpalmitate as the substrate has been described elsewhere (45). An ΔOD₄₁₀ of 1.0 is equivalent to 0.161 nkat.

**Immunoblotting.** Proteins from culture supernatants and cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% T–3% C separation gel and blotted onto a polyvinylidene difluoride membrane (Bio-Rad, München, Germany) using carbonate buffer (10 mM NaHCO₃, 3 mM Na₂CO₃ [pH 9.9]) containing 20% methanol (9). Lipase expressed in *E. coli* was detected using a polyclonal antiserum raised against the whole denatured *P. aeruginosa* lipase which was diluted 1:250,000 in Tris-buffered saline containing 0.2% (vol/vol) Tween 80 (TBST). Lipase expressed in *P. aeruginosa* was detected with a polyclonal serum diluted 1:80,000 in TBST raised against a peptide corresponding to amino acids 48 to 60 of the mature lipase. Horseradish peroxidase-labeled goat anti-rabbit antibody (Bio-Rad) was diluted 1:5,000 in TBST and used as second antibody. Detection was performed using the ECL system (Amersham, Braunschweig, Germany). Samples were prepared as follows: Aliquots of culture supernatant from *P. aeruginosa* corresponding to an OD₅₈₀ of the culture of 0.05 were dried under vacuum and suspended in sample buffer containing 4% SDS. Cell lysates were prepared by boiling cells of *P. aeruginosa* cultures or concentrated and ultrasonified *E. coli* cultures in sample buffer. Aliquots of cultures corresponding to an OD₅₈₀ of 0.15 were used. All samples were boiled for 10 min at 95°C. Densitometric analysis of immunoblots was performed with a video imager (MWG, Ebersberg, Germany) and the TINA 2.09 software (Raytest Isotopenmessgeräte GmbH, Straubenhardt, Germany).

**Heat inactivation of lipase.** Aliquots of in vitro refolded lipase or samples from *P. aeruginosa* culture supernatant were incubated for the indicated time periods and temperatures in a temperature-controlled water bath and subsequently stored in an ice-water bath until determination of enzyme activity. Initial activities were chosen to yield a ΔOD₄₁₀/15 min of 1 to 2.

**Protease susceptibility.** To release intracellular proteases a culture of *P. aeruginosa* PABST7.1(pUCPKS) was adjusted to an OD₅₈₀ of 20. Cells were sonified in 10 mM Tris-HCl (pH 8.0) as described above for *E. coli*. Samples of in vitro refolded lipase were mixed with *P. aeruginosa* cell lysate to give a protein mass ratio of 1:25, respectively, as determined by the Bradford assay (4), with bovine serum albumin as the standard. These samples were incubated at 37°C for the indicated time periods and kept in an ice-water bath until determination of enzyme activity.

**Reduction of disulfide bond in wild-type lipase.** Samples of culture supernatant from *P. aeruginosa* PABST7.1(pUCPL6A) were first preincubated for 1 h at room temperature (RT) in presence of 100 mM dithiothreitol (DTT) (Sigma, Deisenhofen, Germany) or in the presence of 100 mM DTT and 100 mM iodoacetamide (IAA) (Sigma Aldrich) and then for 1 h at RT in the presence of 0.5% (wt/vol) SDS (Biomol, Hamburg, Germany) or for 1 h at 50°C. Subsequently, lipase activity was determined and compared to an untreated sample. When indicated, DTT and excess IAA were removed by gel permeation chromatography on PD 10 columns (Pharmacia, Freiburg, Germany) with 3.5 ml of 20 mM Tris-HCl buffer (pH 8.0) as the mobile phase. Fractions of 0.5 ml were collected. Lipase eluted in fractions 5 to 7 of which fraction 5 was used for further experiments.

## RESULTS

**Secretion by *P. aeruginosa* of wild-type and cysteine variant lipases.** The genes of *P. aeruginosa* lipase (PAL) and of variant proteins C183S, C235S, and C183S-C235S were expressed in *P. aeruginosa* using a T7 RNA polymerase-based expression system (23). High lipolytic activity was obtained only in culture supernatants containing the wild-type protein (Table 1). Activities in supernatants from strains expressing the serine-for-cysteine variants were less than 0.05% of the wild-type activity. The variants with single cysteine-to-serine substitutions showed only one-third of the activity of the double variant. Immunoblotting of culture supernatants and cell lysates revealed that the reduced activities were due to a reduced amount of extracellular lipase protein, indicating that the disulfide bond is necessary for secretion (Fig. 1).

TABLE 1. Extracellular lipolytic activity in culture supernatants of *P. aeruginosa* PABST7.1 expressing wild-type or cysteine variant lipase

| Lipase | Extracellular lipolytic activity[a] (nkat/ml of culture supernatant) | Relative activity (%) |
|---|---|---|
| Wild-type | 3,571 | 100 |
| C183S | 0.49 | 0.01 |
| C235S | 0.51 | 0.01 |
| C183SC235S | 1.61 | 0.05 |
| C183RC235D | 4.34 | 0.12 |
| Control | 0 | 0 |

[a] Activity was assayed with *p*-nitrophenolpalmitate as the substrate and normalized to a cell density corresponding to an $OD_{580}$ of 1.

**Enzymatic activities of cysteine variants.** PAL can be refolded in vitro by its foldase after expression in *E. coli* and subsequent urea denaturation (37). We thus expressed the genes encoding PAL and the cysteine variants in *E. coli* and subjected the cell lysates to denaturation and in vitro refolding.
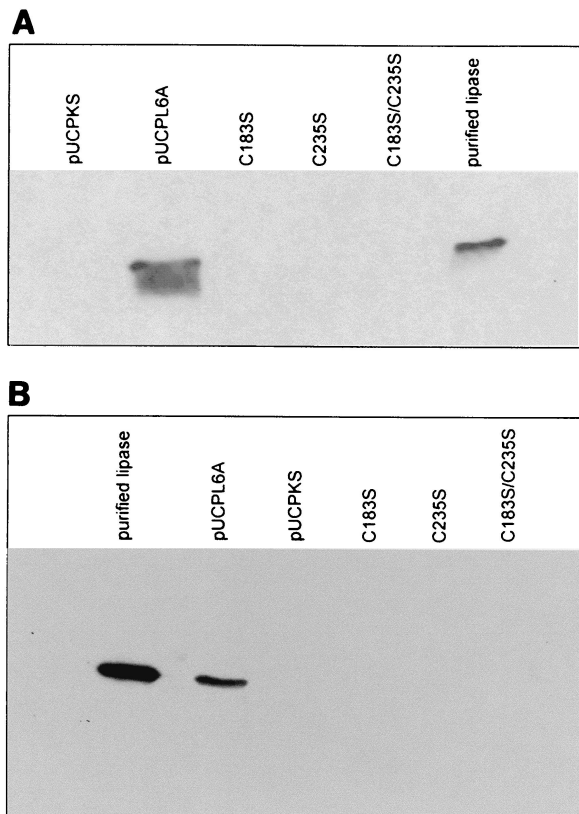


FIG. 1. Immunoblotting analysis of culture supernatants (A) and cell lysates (B) from *P. aeruginosa* PABST7.1 expressing wild-type or cysteine variant lipases. Aliquots of culture supernatants of *P. aeruginosa* PABST7.1 expressing wild-type (bearing plasmid pUCPL6A) or cysteine-to-serine variants of lipase were subjected to SDS-PAGE and immunoblotting. Cell lysates were prepared by boiling aliquots of the cultures corresponding to an $OD_{580}$ of 0.15 in sample buffer. Samples were subjected to SDS-PAGE and immunoblotting. A polyclonal antiserum raised against a peptide consisting of amino acids 48 to 60 in mature *P. aeruginosa* lipase was used to detect lipase. *P. aeruginosa* PABST7.1(pUCPKS) and purified lipase served as negative and positive controls, respectively.
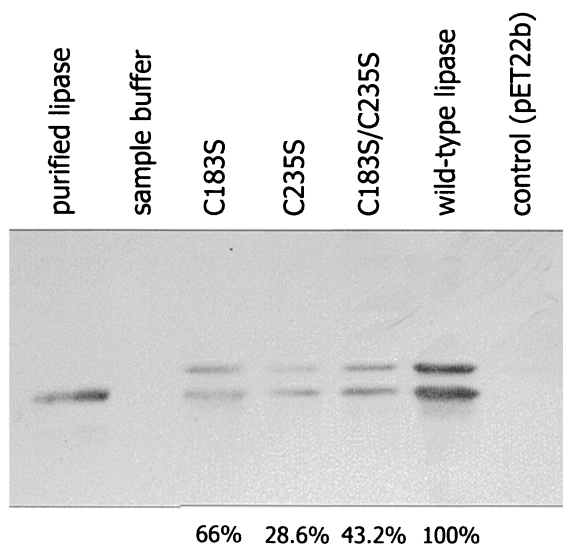


FIG. 2. Expression of wild-type and cysteine variant lipases in *E. coli*. Samples of cell lysates from *E. coli* BL21(DE3)(pLysS) at an $OD_{580}$ of 0.15 expressing wild-type or cysteine variant lipases were urea denatured and subjected to SDS-PAGE and immunoblotting. Lipase protein was identified with an antiserum raised against purified denatured lipase from *P. aeruginosa*. The relative intensities of immunoblot signals of mature lipase in these cell lysates were determined by densitometric analysis and are given below the corresponding lanes.

Lipolytic activity could only be detected after fusing the genes encoding wild-type and variant lipases to the PelB signal sequence from *E. carotovora* pectate lyase. The expression of the fusion genes yielded a high amount of processed lipase in the periplasm (33). Both the signal sequence-containing 32-kDa precursor protein and the processed 29-kDa lipase were identified by immunoblotting in all strains tested (Fig. 2).

Lipolytic activity was detected with wild-type and all variant lipases. Therefore, an intact disulfide bond is required neither for the interaction of lipase with its foldase nor for reaching and maintaining an enzymatically active conformation. Although cell lysates from strains expressing wild-type and variant lipase genes differed in absolute lipolytic activity after refolding of the lipase (Fig. 3), the activities were correlated with the amount of lipase protein present in the cell lysates (Fig. 2) as determined by densitometric analysis of lipase signals on immunoblots.

**Cysteine-to-serine replacements affect lipase stability.** The relationship between activity and stability was investigated by recording heat denaturation profiles at different temperatures for the C183S-C235S variant lipase and comparing them to those of wild-type lipase. As shown in Fig. 4, variant lipase displayed a marked temperature sensitivity, with half-life values of 11.5 min and 2.6 min at 50°C and 60°C, respectively, whereas the corresponding values for the wild-type lipase were 29.1 min and 6.4 min. These results demonstrate that the disulfide bond stabilizes the active conformation of the enzyme.

Cysteine variant lipases unable to form a disulfide bond could not be detected in cell lysates of *P. aeruginosa* (Fig. 1B), suggesting a rapid degradation of these proteins. This hypothesis was tested by incubating in the presence of *P. aeruginosa*
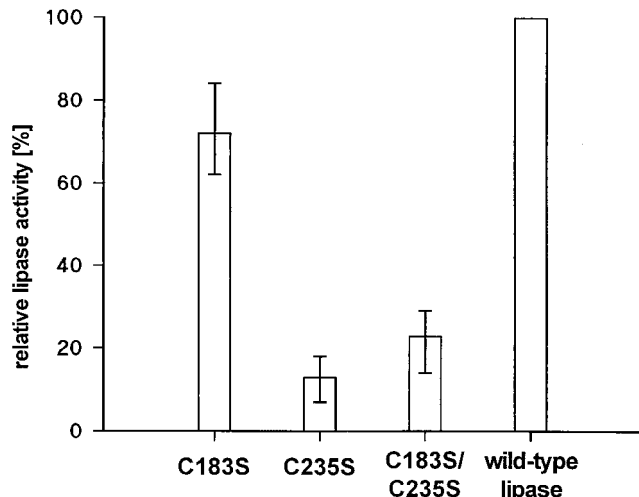
FIG. 3. Relative lipolytic activities of wild-type and variant lipases after heterologous expression in *E. coli* and *in vitro* refolding. Lipases from cell lysates of samples for which results are presented in Fig. 2 were urea denatured and in vitro refolded. Activities of the cysteine variants were compared to those of the wild-type enzyme. The results shown are means ± standard deviations (error bars) and were obtained from six different experiments.

cell lysates wild-type and C183S-C235S variant lipases obtained by in vitro refolding. As shown in Fig. 4, inactivation of the variant protein proceeded much faster than that of the wild-type protein. Although both lipases lost their activity to nearly the same extent during the first 10 min of incubation with the *P. aeruginosa* cell lysate, the activity of wild-type lipase further decreased by only 16% (from 76 to 60%) of inital activity during the next 3 h, whereas the cysteine variant showed a residual activity of only 8% under the same experimental conditions (Fig. 4B).

Further evidence for a stabilizing function of the disulfide bond was obtained by construction of the double variant C183R-C235D, which allows for the formation of a salt bridge. Strains producing this variant exhibited a threefold increase in extracellular lipolytic activity as compared to the strain producing the variant C183S-C235S (Table 1).

**Reduction of disulfide bond in wild-type lipase requires additional destabilizing factors.** Wild-type lipase was treated with DTT, resulting in inactivation of the enzyme only in the presence of 0.5 % (wt/vol) SDS (Fig. 5A), which itself had no inactivating effect (data not shown). At this detergent concentration the activity of DTT-treated lipase was markedly reduced within 1 h of incubation at room temperature. Removal of DTT by gel permeation chromatography followed by SDS treatment had no inactivating effect, indicating that the disulfide bond is accessible to DTT only in the presence of the detergent. The addition of IAA during the DTT treatment did not alter this result.

We also investigated the effect of elevated temperatures on DTT-treated lipase. Again, the lipolytic activity was lost only when DTT exerted its inactivating effect in the presence of another destabilizing factor, e.g., elevated temperature (Fig. 5B). Removal of DTT by gel permeation chromatography after 1 h of incubation at RT and subsequent incubation of the lipase at 50°C had no inactivating effect, indicating that the

disulfide bond is not accessible to DTT under these conditions. The addition of IAA did not alter this result.

## DISCUSSION

The function of the disulfide bond in *P. aeruginosa* lipase was investigated by replacing each of the two cysteine residues with a serine. A cysteine-to-serine double variant was also con-
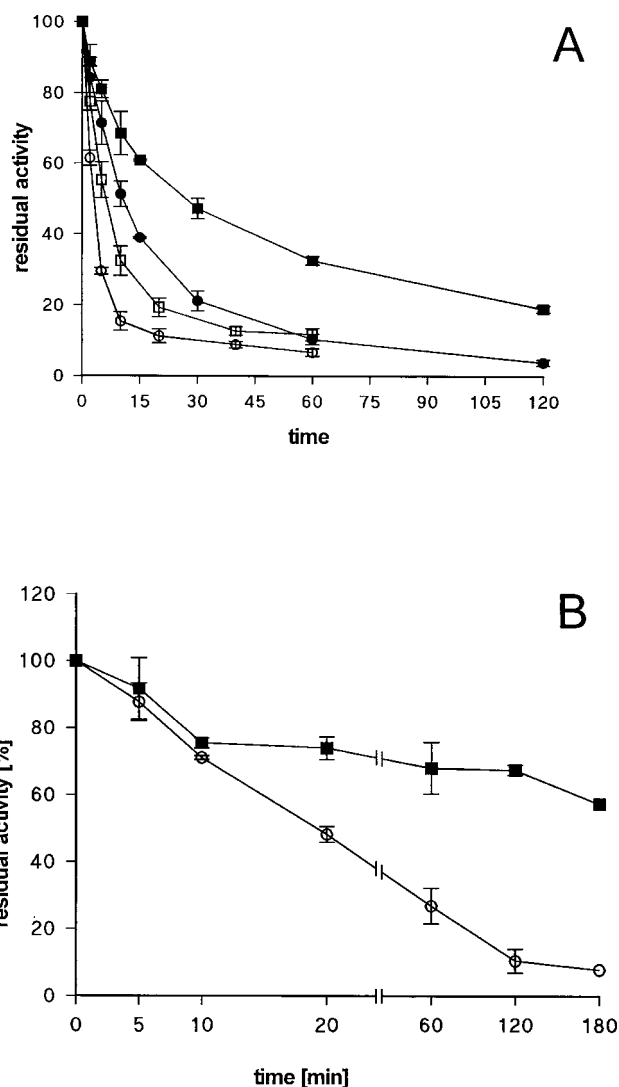




FIG. 4. Stability of in vitro refolded wild-type and variant lipases. (A) Heat denaturation profiles. Cell lysates of *E. coli* BL21(DE3) (pLysS) expressing wild-type or C183S-C235S variant lipases were urea denatured and in vitro refolded. Samples were incubated for the indicated time periods at 50 and 60°C. The activity is expressed as residual activity relative to that of an untreated sample. ■, wild-type lipase at 50°C; ●, C183S-C235S lipase at 50°C; □, wild-type lipase at 60°C; ○, C183S-C235S lipase at 60°C. (B) Protease treatment. Cell lysates of *E. coli* BL21(DE3)(pLysS) expressing wild-type (■) or C183S-C235S variant lipases (○) were urea denatured and in vitro refolded. Samples were mixed with *P. aeruginosa* PABST7.1(pUCPKS) cell lysate at a ratio of 1:25 based on mass of protein. These mixtures were incubated at 37°C for the indicated time periods. Lipolytic activity is expressed as residual activity relative to that of an untreated sample. Activities were corrected for the activity determined for the *P. aeruginosa* cell lysate.
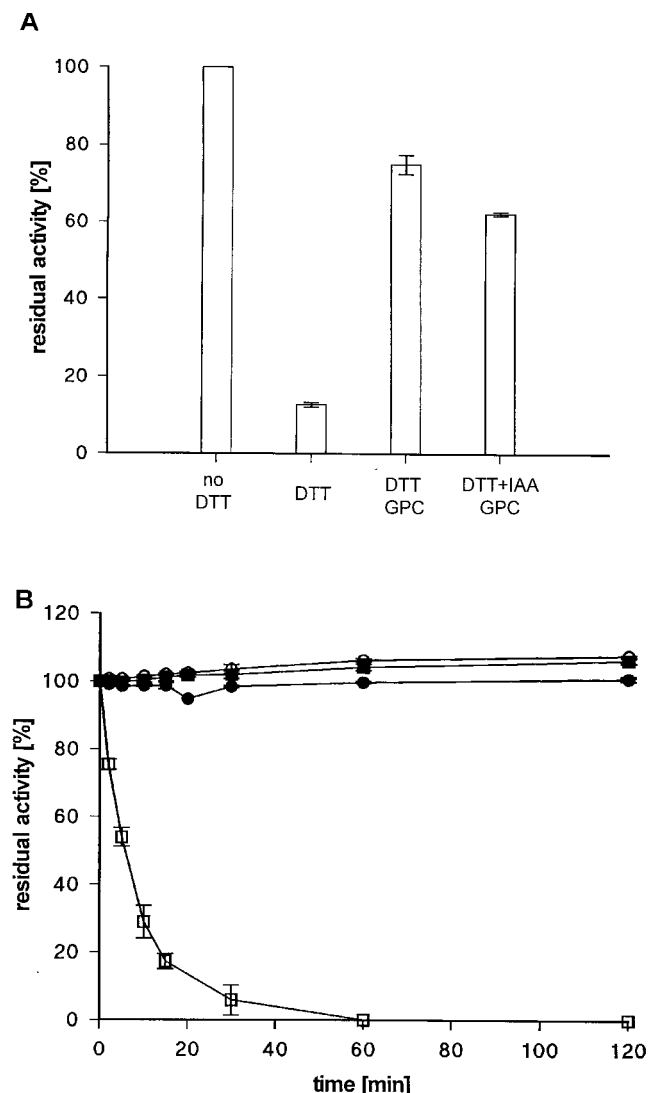
**A**



**B**



FIG. 5. Effect of DTT treatment on activity of extracellular *P. aeruginosa* lipase. Samples of lipase from *P. aeruginosa* PABST7.1 (pUCPL6A) were preincubated with 100 mM DTT or 100 mM DTT and 100 mM IAA for 1 h at room temperature and subsequently incubated for 1 h at RT in the presence of 0.5% (wt/vol) SDS (A) or at 50°C (B) for the indicated time periods. Where indicated, DTT was removed by gel permeation chromatography (GPC) prior to SDS or heat treatment. Lipase activity is expressed as residual activity relative to that of an untreated sample.

structed since we expected intermolecular disulfide bond formation during folding in the periplasm of single cysteine variants. Such incorrectly formed disulfide bonds might then hinder transport across the outer membrane. Indeed, the activity of the double variant was almost three times higher than that of the single variants although the absolute lipase activity in culture supernatants of all cysteine-to-serine variants was low.

Lipase protein was detectable by immunoblotting neither in cell lysates nor in culture supernatants from strains expressing any of the cysteine variants. This result indicates that an intact disulfide bond is important to ensure the transport of the enzyme across the outer membrane into the culture supernatant.

When the *P. aeruginosa* lipase fused to the *E. carotovora* PelB signal sequence was expressed in *E. coli*, the lipase partly accumulated in the periplasm (33). This lipase can be refolded in vitro in the presence of its chaperone LipH after urea denaturation (36, 37). We have used this in vitro denaturation and refolding procedure to isolate and characterize the cysteine variants of lipase.

Several reports on lipases from pseudomonads belonging to groups I and II of family I (1) emphasize the dependence of these enzymes on the presence of intermolecular chaperones for correct folding and/or secretion (7, 12, 16, 18, 19, 26). A distinct specificity must exist for this interaction, since the chaperones of different species are not interchangeable, although the lipases have very similar three-dimensional structures (10). The mechanism by which these foldases recognize their cognate lipase and exert their function is still unknown. It is therefore conceivable that the disulfide bond may be part of such a recognition signal or that it stabilizes an essential folding intermediate, making it indispensable to the folding process. An important role during the folding process was suggested for the cysteine residues of *Burkholderia cepacia* lipase, which could not be refolded in the presence of DTT or β-mercaptoethanol (17). Such a folding function was recently confirmed by investigation of cysteine-to-serine variants of the *B. cepacia* lipase for which only low activities were obtained in an in vitro transcription-translation system (48). Also the wild-type lipase from *P. aeruginosa* was refolded with lower efficiency in the presence of β-mercaptoethanol (37). However, by refolding the disulfide bond variants of *P. aeruginosa* lipase expressed in *E. coli* we have demonstrated that this lipase can adopt an active conformation even in the absence of the disulfide bond. Thus, the disulfide bond is necessary neither for the recognition of lipase by its foldase nor for the interaction of both proteins. The effect of DTT on the refolding of the wild-type lipase must therefore be different from the reduction of cysteines. Furthermore, the formation of the disulfide bond is essential neither to the folding process nor to gaining enzymatic activity. Although we were unable to purify refolded wild-type and cysteine variant lipases and exactly determine specific activities, it seems likely that the absence of the disulfide bond did not change the activity markedly. The amount of lipase protein subjected to refolding was determined by densitometric analysis of immunoblots. Assuming that wild-type and variant lipases could be refolded with the same efficiency, we found that the amount of lipase protein correlated with the enzyme activity obtained after refolding.

Increasing experimental evidence suggests that secreted proteins must adopt a folded conformation to be released from the cell (3, 5, 13–15, 30). Since the cysteine variants were capable of folding into an active conformation, we conclude that it is not incorrect folding that blocked secretion. The fact that we could identify wild-type but no variant lipase in cell lysates of *P. aeruginosa*, even under overexpressing conditions, pointed to a rapid degradation of the cysteine variants. As extracellular lipase was found to be protease resistant, presumably due to a tight association of the protein with lipopolysaccharide (LPS) (39), we assume that proteolytic degradation of the lipase takes place in the periplasm. The enhanced susceptibility of the variant to proteolytic degradation by cell lysates of *P. aeruginosa* underscores this hypothesis. Therefore, the markedly re-

duced amount of secreted cysteine variant proteins is probably caused by lipase instability leading to rapid degradation by periplasmic proteases. Furthermore, the rapid inactivation of the cysteine double variant at elevated temperatures confirmed the stabilizing function of the disulfide bond.

Interestingly, attempts to chemically reduce the disulfide bond in wild-type lipase failed although the three-dimensional structure of the lipase revealed a surface-exposed (and thus accessible) location of the disulfide bond (26a). However, the simultaneous treatment of lipase with DTT and SDS or at elevated temperatures led to the inactivation of the enzyme, which was not observed when DTT was removed before incubation with SDS or exposure to elevated temperatures. This result indicates that additional destabilizing factors are necessary to render the disulfide bond accessible to the reductant. Brumlik et al. (6) found that the disulfide bond of the lipase-acyltransferase of *A. hydrophila* was reduced by DTT only in the presence of SDS. These authors suggested a buried location of the disulfide bond which became accessible to DTT only by the denaturing effect of SDS. We believe that the tight association of the *P. aeruginosa* lipase with LPS (39) might stabilize the enzyme and protect it from reduction by DTT. Such a stabilization could be weakened by the solubilizing effect of SDS. LPS association may also explain why lipase isolated from *P. aeruginosa* culture supernatant remained stable for at least 2 h at 50°C, which is unusual for an enzyme produced by a mesophilic organism. On the other hand, urea denatured and in vitro refolded wild-type lipase isolated from cell lysates of *E. coli* was inactivated at this temperature with a half-life of 29.1 min. In this case, an association with heterologous LPS might be less effective or even missing, leading to an increased susceptibility to heat denaturation.

The introduction of a salt bridge instead of a disulfide bond by substitution of the cysteines for arginine and aspartic acid did not result in a marked stabilization of the protein. Due to the very different pK$_a$ values of arginine (pK$_a$ = ~12) and aspartic acid (pK$_a$ = 3.9 to 4.4) only a very small fraction of these amino acids may be present in a charged form under physiological conditions and thus capable of forming a salt bridge. Apparently, a change of the pK$_a$ values of the free amino acids caused by the local protein environment which has been reported for other enzymes (29) had not occured. Nevertheless, the lipase activity of this variant was about three times the activity of the cysteine-to-serine double variant, indicating that the salt bridge can at least partly compensate for the loss of the disulfide bond. This result again emphasizes that the only function of the disulfide bond is to stabilize the structure of the enzyme.

In conclusion, we have demonstrated here by replacing cysteine residues that the disulfide bond in *P. aeruginosa* lipase is not required for correct folding and interaction of lipase with its foldase. However, it confers to this enzyme a more stable conformation which is important not only in the bacterial culture supernatant but also during passage of the enzyme through the periplasm.

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