Role of the lipase-specific foldase of *Burkholderia glumae* as a steric chaperone

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Running Title: Lipase-specific foldase is a steric chaperone
SUMMARY

Most lipases of Gram-negative bacteria require a lipase-specific foldase (Lif) in order to fold in the periplasm into their active, protease-resistant conformation prior to their secretion. The periplasmic domain of the Lif (amino acids 44-353) of *Burkholderia glumae* was purified as a His-tagged protein, and its function in the folding of lipase was studied *in vitro*. Refolding of the denatured lipase into its active conformation was dependent on the presence of the Lif. Circular dichroism revealed that the lipase refolded in the absence of Lif into a form with a native-like conformation, which was more stable against heat-induced denaturation than the native form, but was enzymatically inactive. This form of the protein could be activated by adding Lif after several hours, which demonstrates that the function of this chaperone is to help lipase to overcome an energetic barrier in the productive folding pathway rather than to prevent it from entering a non-productive pathway. The Lif was shown to interact with the native lipase in protease-protection experiments as well as by affinity chromatography, consistent with a role of the Lif late in the folding process. These results demonstrate that the Lif functions in a way analogous to the propeptides of many bacterial proteases and indicate that the amino acid sequence of the lipase does not contain all the information required for the protein to adopt its three-dimensional structure.
INTRODUCTION

Although molecular chaperones are often required to assist protein folding in vivo, it is generally assumed that all the information for a protein to adopt its final three-dimensional structure is confined by its amino acid sequence (1). Molecular chaperones, such as GroEL, function by preventing off-pathway reactions, such as aggregation rather than by providing steric information for newly synthesized proteins to fold correctly (2). However, many bacterial extracellular proteases, including subtilisin and α-lytic protease, are synthesized as precursors with an N-terminal propeptide, which functions as an intramolecular chaperone (for reviews, see 3-5). In contrast to the general chaperones, these propeptides directly catalyze the folding process by lowering a high-energy barrier on the folding pathway. Since these propeptides contribute essential steric information to the mature domain of the protein, they have been termed “steric chaperones” (4). After the folding process, the propeptides are cleaved off by intra- or inter-molecular processing. However, since the propeptides are an integral part of the primary structure of the initial translation product, it can be argued that their existence does not violate the principle of protein self-assembly (6).

Many Gram-negative bacteria, including Burkholderia glumae, Burkholderia cepacia and Pseudomonas aeruginosa, produce extracellular lipases (for a review, 7) that are secreted via the type II or general secretion pathway (8). This secretion pathway involves two separate steps: first, the proteins are transported across the cytoplasmic membrane via the Sec system, and, only after they have folded in the periplasm into their native conformation, they are transported across the outer membrane via the Xcp system (9). The folding of the lipases depends on general periplasmic folding catalysts, such as the disulfide isomerase DsbA (10), and on a dedicated chaperone, which is encoded by a gene located in the same operon as lipA, the lipase structural gene (11-14). These dedicated chaperones have been designated by various names, but the general designation Lif (lipase-specific foldase)\(^1\) has been proposed (7) and will be used in this paper. Lif proteins are
anchored to the inner membrane by an N-terminal hydrophobic segment, whereas the large C-terminal domain is located in the periplasm (11). Lif proteins function in vivo as chaperones that assist in the folding of the lipases into their active, protease-resistant conformation (15). Furthermore, some Lif proteins, including those of P. aeruginosa and B. cepacia, have been shown to be required for the refolding of denatured lipases in vitro (14,16-18). However, the molecular mechanism of the action of the Lifs is not known and was investigated in the present study. We demonstrate that the mode of action of the Lif of B. glumae is very similar to those of the propeptides of the proteases, thus classifying the Lifs as steric chaperones that are not part of the primary translation product.
MATERIALS AND METHODS

Bacterial Strain and Growth Conditions—Escherichia coli K-12 strain DH5α (19) was used for routine cloning and for the expression studies. This strain was grown in Luria Bertani medium (LB) at 37 °C, unless otherwise stated. Ampicillin (100 µg/ml) or kanamycin (25 µg/ml) were added for the maintenance of plasmids.

Plasmids and DNA manipulations—General DNA manipulations were performed according to Sambrook et al. (20) or according to protocols provided by the manufacturers of the enzymes. A DNA fragment encoding the amino acids 44-353 of B. glumae Lif was amplified by PCR using pMEK13 (21), which contains a partially synthetic lif gene with a lower C + G content than the authentic lif, as template. The oligonucleotide primer corresponding to the 5’ end of the amplified DNA, MK07 (5’TACTGTGGGCAAtGCAGCCAGGCAC 3’), was designed with an NdeI recognition site (underlined; the introduced nucleotide substitutions are represented by small letters). The second oligonucleotide primer used was the M13-universal primer, and the enzyme was Pwo-polymerase (Boehringer Mannheim). The amplified PCR fragment was cloned into the SmaI site of pBluescript SK(-) (Stratagene), and the NdeI-XhoI fragment, containing the truncated lif gene, from the resulting plasmid was subcloned in the same sites of pET16b (Novagen). The resulting plasmid, pMEK52, encodes a truncated, His-tagged Lif, whose expression is under the control of the T7 promoter.

Purification of the His-tagged B. glumae Lif—E. coli DH5α containing the plasmids pMEK52 and pT7-POL23 (22) was grown overnight at 28 °C, diluted 1:50 in 400 ml LB and grown for 3 h at 28 °C. The expression of the His-tagged Lif was induced by incubating the cultures for 1 h at 42 °C, and the cells were allowed to grow for an additional 3 h at 37 °C, unless otherwise stated.
°C. The cells were harvested by centrifugation, and the cell pellet was resuspended in 10 ml lysis buffer (10 mM Tris-HCl, 5 mM EDTA, 100 mM NaCl, pH 8.0). After the addition of 0.2 ml of 50 mg/ml lysozyme and 0.1 ml 20% Triton X-100, the cell suspension was incubated for 10 min at room temperature and subsequently sonicated on ice using a Branson Sonifier 450. Then, 20 ml buffer A (40% sucrose, 10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 8.0) were added, and cell debris was removed by centrifugation at 9000 rpm in an SS34 rotor for 30 min. The supernatant fraction, containing the His-tagged Lif, was extensively dialyzed against column buffer (100 mM NaCl, 50 mM Tris-HCl, pH 8.0) and loaded on a Ni²⁺-nitrilotriacetic acid (NTA) column (Qiagen). The column was washed stepwise with column buffer containing 0, 35 and 50 mM imidazole, and, finally, bound proteins were eluted with column buffer containing 100 mM imidazole. The presence of proteins in the fractions obtained was monitored by UV detection, and the peak fractions were pooled and analyzed by SDS-PAGE (23). The purified His-tagged Lif was dialyzed against 20 mM Tris-HCl, 50 mM NaCl (pH 8.0) and concentrated to 0.5 mg/ml, using Fugisep (Intersep filtration systems, UK). For circular dichroism (CD) studies, an additional purification on a Sephadex G-150 column was applied.

Unfolding-Refolding of Lipase-Purified B. glumae lipase (a generous gift from A. J. Slotboom) was received at a concentration of 0.8 mg/ml in 5 mM Tris-HCl, 45 mM NaCl, 10 mM CaCl₂ (pH 7.5). The standardized unfolding-refolding reactions used in this study were as follows: 10 µl of the lipase-containing solution were added to 70 µl of 8 M urea, 12.5 mM EDTA (freshly added from a stock solution) in 0.1 M sodium-phosphate buffer (pH 7.4). Subsequently, 8 µl of 100 mM DTT were added, and the lipase was incubated for 1 h at 56 °C. The refolding was started by diluting 10 µl of the denatured lipase in 1 ml of 0.1 M sodium-phosphate buffer (pH 7.4) containing purified, His-tagged Lif. Refolding of lipase into its active conformation was monitored in enzyme assays using para-nitrophenyl
caprylate (Sigma) as a substrate (21). The absorbance at 410 nm was followed spectrophotometrically for 3 min at room temperature. The formation of the disulfide bond in lipase was assessed by making use of the different electrophoretic mobilities of the oxidized and the reduced forms of the protein. During refolding, samples were taken after various time intervals and incubated with 100 mM iodoacetamide (Sigma) for approximately 1 h. The proteins were subsequently precipitated with 5% trichloroacetic acid (TCA) for 1 h on ice, pelleted by centrifugation at maximal speed in a table-top microfuge and resuspended in sample buffer without reducing agent. The electrophoretic mobility of the lipase was analyzed by SDS-PAGE and Western blotting.

For CD-spectroscopy, lipase was unfolded by heating at 56 °C for 1 h in 20 mM sodium-phosphate buffer (pH 7.0), containing 8 M urea, 12 mM EDTA and 45 mM NaCl. Refolding was started by a 100-fold dilution in 20 mM sodium-phosphate buffer (pH 7.0), containing 45 mM NaCl.

CD and Tryptophan Fluorescence Spectroscopy-Samples for CD-spectroscopy contained 10 µM of protein. CD-spectra were recorded on an AVIV spectropolarimeter operating at 25 °C. Five scans were collected for each sample over a wavelength range of 195-270 nm, using a 0.1 mm path cell, a band width of 1 nm and averaging time of 5 s with a sampling each nm. The collected spectra were subjected to background subtraction and are presented without smoothing. The respective dynode Volt output of each run was recorded as well. Denaturation of the proteins was monitored by CD at 220 nm. The spectra were obtained by heating 10 µM of protein over a temperature range from 20-80 °C. Sampling was every degree Celsius with an equilibration time of 6 s and an averaging time of 10 s. Each sampling point was measured three times. Cell path length and slit width were identical to the wavelength scan experiments.
Tryptophan fluorescence was measured at a protein concentration of 1 µM on an SLM Aminco Series 2 fluorometer at 25 °C. The excitation wavelength was 280 nm with a 20 nm/min scan speed over an emission wavelength range of 290-450 nm with a sampling each nm. The slit width for emission and excitation was 5 nm.

Lipase-Lif Interaction—His-tagged Lifs from *B. glumae* (12 µg) and *P. aeruginosa* (12 µg) (a generous gift from K.-E. Jaeger) were coupled to Cu$^{2+}$-charged chelating sepharose beads (Pharmacia) by incubation for 30 min at room temperature on a rotating wheel. After washing of the beads with column buffer containing 0 and 25 mM imidazole, native *B. glumae* lipase (12 µg) was added and incubation was continued for 30 min. The beads were washed twice, once with column buffer and once with column buffer containing 25 mM imidazole. Bound proteins were eluted with column buffer containing 10 mM EDTA. Proteins were precipitated from all fractions with 5% TCA and analyzed by SDS-PAGE.

In protease-protection experiments, 8 µg of the *B. glumae* lipase was added to either 10 µg of the His-tagged *B. glumae* Lif or 16 µg of the His-tagged *P. aeruginosa* Lif. Trypsin was added to a final concentration of 40 µg/ml in a final volume of 20 µl of 0.1 M sodium-phosphate buffer (pH 7.4), and the mixture was incubated for 20 min at 37 °C. As a control, the Lifs were digested with trypsin in the absence of lipase. The protease reactions were stopped by adding 1 mM phenylmethylsulfonyl fluoride and, after TCA precipitation, the protected proteins were analyzed by SDS-PAGE.

*N-terminal sequencing*—Proteins 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).
RESULTS

Purification of His-tagged Lif-Since we anticipated that the purification of the native *B. glumae* Lif could cause problems because of its hydrophobic N-terminal membrane-anchor, and since this membrane-anchor is dispensable for Lif functioning *in vivo* (21), we decided to purify only the periplasmic domain of the Lif. The DNA segment encoding the N-terminal 43 amino acid residues of the Lif, which include the transmembrane segment, were replaced by a DNA segment encoding a poly-histidine tag, and the recombinant gene was placed under the control of the T7 promoter (see Experimental Procedures). The recombinant Lif was produced in *E. coli* DH5α and found to be soluble, implying that it could be purified under non-denaturing conditions by affinity chromatography on a Ni²⁺-NTA column. The column was eluted stepwise with column buffer containing increasing amounts of imidazole. The His-tagged Lif eluted at 100 mM imidazole (Fig. 1, lane 4). This purification procedure resulted in a highly pure His-tagged Lif, after a single affinity chromatography step. Occasionally, a minor contaminating band of 21 kDa was observed (Fig. 1, lane 4), which also reacted with the antiserum against the Lif (results not shown), implying that it represents a degradation product.

Unfolding-Refolding of Lipase-To study the function of the His-tagged Lif in the refolding of the lipase, we first established conditions to reversibly unfold the *B. glumae* lipase. Such conditions were met by incubating the enzyme at 56 °C for 1 h in the presence of the denaturant urea, of DTT to reduce the internal disulfide bond and of EDTA to chelate the Ca²⁺-ion, known to be present in the structure of the enzyme (24). The CD-spectrum demonstrated that the protein lost its secondary structure under these conditions (Fig. 2A). The incubation temperature for unfolding had to be carefully chosen, since incubation at 37 °C did not result in the unfolding of the lipase and incubation at 75 °C or higher resulted in a lipase that could not be refolded in subsequent refolding experiments, possibly by
covalent modification of the protein by urea (25). Refolding of the denatured lipase was initiated by diluting the sample 100-fold in 0.1 M sodium-phosphate buffer (pH 7.4). In the absence of the His-tagged Lif, no lipase activity was detected after refolding (Fig. 2B). Furthermore, in contrast to the native lipase, the refolded lipase was sensitive to trypsin digestion (result not shown), even though CD-analysis showed a spectrum, which was similar to that of the native lipase (Fig. 2A). The difference observed at wavelengths below 200 nm is due to buffer effects as could be judged from the Dynode Volt output (result not shown). Furthermore, the Tryptophan fluorescence of the native and the refolded lipase were indistinguishable (results not shown). These data imply that the lipase acquired a near-native structure after refolding in the absence of the Lif, but the lipase did not adopt the active conformation.

High lipase activity was regained after incubation for 2 h at 37 °C in the presence of the His-tagged Lif (Fig. 2B). Refolding of the lipase was induced specifically by the cognate Lif, and not by similar amounts of His-tagged Lif of P. aeruginosa or of bovine serum albumin (results not shown). The refolding efficiency was dependent on the amounts of Lif. Addition of 0.5 µg of the Lif to 0.9 µg unfolded lipase (i.e., a Lif : lipase molar ratio of 1 : 2) resulted in refolding of approximately 40% of the lipase, suggesting that the Lif does not act catalytically and that each Lif molecule can refold one lipase molecule. Up to about 65% of the lipase activity could be regained when the amount of Lif was increased to 5 µg (i.e., a Lif : lipase molar ratio of 5 : 1) (Fig. 2B). Even at higher Lif concentrations, the refolding efficiency did not reach 100%, suggesting that about 35% of the lipase molecules were irreversibly lost during the unfolding-refolding procedure. Alternatively, the presence of high concentrations of Lif could inhibit the enzymatic activity of the lipase to some extent. To test this possibility, native lipase was incubated with the His-tagged Lif, and lipase activity was measured (Fig. 2C). Only at very high Lif concentrations, a slight
reduction of the lipase activity was noticed, demonstrating that the Lif does not act as an inhibitor of lipase.

**Disulfide-bond formation during refolding** - The native lipase of *B. glumae* contains one disulfide bond (24). Surprisingly, the presence of an oxidizing agent was not required during the refolding reactions described in the previous paragraph, suggesting that either the disulfide bond is not essential for the formation of the active enzyme, or the disulfide bond was formed by spontaneous oxidation. To discriminate between these possibilities, the redox state of the cysteines after refolding was assessed by making use of the different electrophoretic mobilities of the reduced and the oxidized form of the lipase (Fig. 3). Directly after the initiation of refolding, the enzyme was present in the reduced form (Fig. 3, lanes 2 and 3). After 2 h of refolding in the presence of Lif, a minor proportion of the lipase was in the oxidized form (Fig. 3, lane 5), whereas at this time point 65% of the activity was regained. Furthermore, after 2 h of refolding in the absence of Lif, an approximately equal amount of the lipase was oxidized (Fig. 3, lane 4), which was, however, inactive. These results indicate that the disulfide bond is not essential for enzymatic activity of the lipase.

**Kinetics of Refolding** - The kinetics of the refolding were studied at a Lif : lipase molar ratio of 5 : 1. At 37 °C and in the presence of the Lif, more than 50% of the native lipase activity was regained within 10 min, whereas the maximum activity of 70% was regained within 2 h under these conditions (Fig. 4). The amount of refolded lipase did not increase after longer refolding periods of up to 20 h (results not shown). In the absence of the Lif, no lipase activity was regained within 3 h (Fig. 4), even though a considerable degree of secondary structure was formed (Fig. 2A).
To investigate whether the lipase that was refolded in the absence of Lif was trapped as a folding intermediate that is stable in time, His-tagged Lif was added after 3 h incubation at 37 °C. Refolding was efficiently induced under these conditions and 65% of the native lipase activity was regained after 2 h incubation (Fig. 4). This result demonstrates that the lipase accumulates as an intermediate in the correct folding pathway in the absence of Lif and that the Lif is probably required to overcome an energy barrier during the folding process.

**Stability of the folding intermediate**—Although the CD-spectrum of the lipase refolded in the absence of Lif was indistinguishable from that of the native lipase (Fig. 2A), this folding intermediate was not active. To determine the stability of the folding intermediate, its heat-induced denaturation was monitored by CD spectroscopy at 220 nm (Fig. 5). While denaturation of the native lipase followed a sigmoidal curve with a transition at 50 °C, the folding intermediate denatured at 58 °C (Fig. 5), indicating that the folding intermediate is more stable than the native lipase to heat-induced denaturation.

**Lipase-Lif Interaction**—To study whether Lif can interact with native lipase, affinity chromatography was applied. His-tagged Lif was immobilized to Cu²⁺-charged chelating sepharose beads. Subsequently, these beads were incubated with purified lipase. The beads were washed with column buffer containing 0 mM and 25 mM imidazole and the bound proteins were eluted with 10 mM EDTA. Most of the lipase was retained on the beads and was eluted together with the His-tagged Lif by EDTA (Fig. 6, lanes 1-3). When the beads were not charged with His-tagged Lif or charged with the His-tagged Lif of *P. aeruginosa*, the lipase was not retained on the beads (Fig. 6, lanes 4-9). The interaction between the *B. glumae* lipase and its cognate Lif was further demonstrated in protease-protection experiments. Whereas the native lipase is resistant to trypsin (15),
His-tagged Lif appeared to be sensitive to trypsin digestion (Fig. 7, lane 3). However, when the His-tagged Lif was digested with trypsin in the presence of lipase, a 26 kDa protected fragment was observed (Fig. 7, lane 1). Sequencing of the N terminus of this fragment by Edman-degradation revealed the sequence Ala-Met-Pro-Leu-Pro, which corresponds to amino acids 72 to 76 of the native Lif. This result indicates that the lipase interacts with the cognate Lif and protects it from trypsin degradation. No protected fragment was detected when the *P. aeruginosa* Lif was incubated with *B. glumae* lipase (Fig. 7, lane 4), which is consistent with the specificity of the interaction.

CD-spectroscopy was applied to determine whether the interaction between lipase and Lif results in any conformational changes (Fig. 8). Whereas the CD-spectrum of a solution containing the *B. glumae* lipase and *P. aeruginosa* Lif was equal to the sum of the two separate spectra (Fig. 8A), the CD-spectrum of a solution of the lipase and its cognate Lif was distinct from the sum of the two separate spectra (Fig. 8B). This result confirms that the lipase interacts with the cognate Lif and demonstrates that the interaction results in a conformational change in either one or both of the proteins.
DISCUSSION

In this work, we have studied the role of the Lif in the folding of the lipase of *B. glumae* in *vitro*. Under the standardized conditions, the Lif was indispensable for acquiring an active lipase. Remarkably, the presence of Ca\(^{2+}\)-ions and the formation of the disulfide bond were not required for obtaining an active enzyme. Consistently, the enzymatic activity of the related lipase of *Pseudomonas* sp. strain KWI-56 was not inhibited by DTT or EDTA (26), suggesting that neither the disulfide bond, nor the Ca\(^{2+}\)-ion are essential for the catalytic mechanism of this enzyme. However, in contrast to our results, the formation of the disulfide bond appeared to be required during the folding of this *Pseudomonas* lipase after its *in vitro* synthesis in an *E. coli* lysate (26). We demonstrated that the denatured lipase refolds in the absence of Lif into a near-native conformation, which is heat-stable but enzymatically inactive. This lipase conformation was sensitive to proteases similar to the lipase produced *in vivo* in the absence of the Lif (15). This form of the protein is a folding intermediate, since it can be converted into the active form upon addition of the Lif. Hence, it appears that the function of the Lif is not to prevent off-pathway folding reactions but to catalyze the folding of an energetically-trapped folding intermediate. Thus, the Lif functions similarly as the propeptides of extracellular proteases, such as α-lytic protease and subtilisin (27,28), which have been designated “steric chaperones” (4), since they apparently add steric information, which is lacking in the mature domains of these proteins. Even though these propeptides have been shown to be able to function in *trans* as separate polypeptides (29,30), they are normally an integral part of the primary structure during the folding process. Therefore, their existence does not violate the principle of protein self-assembly (6). However, the Lifs are synthesized as separate polypeptides, and therefore, the primary structure of the lipases does not seem to contain all the information that is required for folding.
The Lif was shown to form a stable complex with the native lipase, which is consistent with a role late in the folding process. The affinity of the Lif for the native lipase opens the possibility to study the interaction between the proteins at the atomic level by crystallization and X-ray diffraction. Interestingly, also the propeptide of the α-lytic protease has been shown to interact with the native protein, thereby forming a large complementary interface (31). Since lipase protected a large 26 kDa fragment of the Lif from tryptic digestion, the interface in the lipase-Lif complex is probably large as well. The association of the propeptides of bacterial proteases to the native enzymes may be related to a second function of these chaperones, i.e. they act as inhibitors, which prevent proteolytic activity before the enzymes are secreted into the extracellular medium (32,33). Similarly, an inhibitor function of the Lifs has been suggested (34). However, the His-tagged Lif did not inhibit the enzymatic activity of the lipase in the assay described in this study. Probably, an inhibitor function is not required in the case of lipases, since the cell membranes consist of phospholipids and lipopolysaccharides, towards which the lipases have no activity.

Since, in vivo, only the lipase is secreted, while the Lif remains associated with the inner membrane, the tight association between the lipase and Lif observed in vitro raises the question how the lipase is released from this complex. The interaction with components of the Xcp secretion machinery might be involved in this process. Similarly, it was recently reported that the activation of elastase of *P. aeruginosa* requires secretion via the Xcp machinery (35). In the absence of a functional secretion apparatus, elastase could be released into the extracellular medium if the outer membrane was leaky due to structural defects in the lipopolysaccharides. However, the elastase that was released in this way remained inactive, probably because the dissociation of the propeptide, which acts as an inhibitor of the enzyme (33), requires interaction with a functional Xcp machinery (35).
Interestingly, there are more similarities between the folding pathways of the α-lytic protease and lipase. The intermediate folding stage of the α-lytic protease, which is obtained in the absence of the propeptide, has been reported to be more stable than the native state (36). Similarly, the folding intermediate of lipase generated in the absence of Lif was more stable to denaturation than the native enzyme. Thus, the native states both of α-lytic protease and of lipase are not at the minimum free energy, and their apparent stability must derive from a large unfolding barrier.

The propeptide of the α-lytic protease binds more tightly to the folding transition state than to the native state of the protease (36,37). The crystal structure of the propeptide-native protease complex revealed the presence of regions of extensive hydration in the interface, which could explain this observation (31). It was proposed that in the transition state of the propeptide-catalyzed folding reaction, a domain of the α-lytic protease is distorted into a conformation more complementary to the propeptide, expelling the bound water molecules. Thus, stabilization could be achieved by increasing the surface area of interaction and reducing the entropic costs by freeing bound waters. Interestingly, conditions have been reported to refold the B. glumae lipase in vitro in the absence of the Lif (15). Most notably, these refolding experiments were performed in a solution containing 40% glycerol. Consistently, in our refolding conditions, the Lif was dispensable if 40% glycerol was included in the refolding buffer (unpublished observation). Apparently, glycerol, which is thought to create a nonaqueous environment that stimulates hydrophobic interactions within an unfolded protein (38), mimics the function of the Lif. Possibly, the presence of bound water molecules stabilizes the folding intermediate of both lipase and α-lytic protease, and the exclusion of water by interaction with the steric chaperones lowers the energy barrier that has to be overcome to reach the final conformation. It will be interesting to determine whether glycerol can also substitute for the steric chaperones in the case of the propeptide-dependent proteases.
It has been argued that proteins that must survive in the presence of proteases need to have a highly cooperative unfolding barrier to suppress transient unfolding, which would render them susceptible to proteolysis (39). A consequence of a large unfolding barrier is a large barrier for folding as well. The co-evolution of a foldase with the barrier provides an irreversible pathway to a kinetically-stabilized native state (31). Obviously, this is the case for bacterial extracellular proteases, but it also applies for other enzymes that are secreted by the same organisms, such as lipase. In this respect, it is consistent to find that the lipases of various bacteria, including Burkholderia spp. and Pseudomonas spp., are dependent for their folding on a chaperone that functions in the same way as the propeptides of the proteases. The synthesis of the lipase and the Lif as separate polypeptides may be related to the incapability of the mature enzyme of autoproteolytic processing.

**Acknowledgements**-We thank A. J. Slotboom for the B. glumae lipase, K.-E. Jaeger for the His-tagged P. aeruginosa Lif and F. van der Lecq for N-terminal sequencing of the proteins at the Sequencing Centre of the Centre for Biomembranes and Lipid Enzymology at Utrecht University. This work was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for Scientific Research (NWO), and by the European Community, E.U. grant bio4-CT960119.
REFERENCES


Footnotes

1 The abbreviations used are: Lif, lipase-specific foldase; LB, Luria Bertani medium; NTA, nitrolotriacetic acid; PAGE, polyacrylamide gel electrophoresis; CD, circular dichroism; DTT, dithiothreitol; TCA, trichloroacetic acid.
LEGENDS TO THE FIGURES

Fig. 1: **Purification of His-tagged Lif.** Coomassie-stained SDS-PAGE gel showing the fractions obtained after Ni\textsuperscript{2+}-affinity chromatography. Soluble proteins of *E. coli* expressing the His-tagged Lif were applied to the column and the column was eluted with imidazole. Lane 1, unbound proteins; lane 2, proteins eluted during wash with column buffer; lane 3, proteins eluted with 35 mM imidazole; lane 4, proteins eluted with 100 mM imidazole. The position of the His-tagged Lif is indicated at the right. MW, molecular weight marker proteins indicated in kDa at the left.

Fig. 2: **Unfolding and refolding of the lipase.** A: CD-spectra of the lipase at different folding stages. O, native lipase; ◊, unfolded lipase; Δ, lipase refolded in the absence of the Lif. B: Unfolded lipase (0.9 µg) was refolded in the presence of the indicated amounts of the His-tagged Lif at 37 °C for 2 h, and lipase activity was measured. Lipase activity of the same amount of the native lipase is indicated at the right. C: Enzymatic activity of the native lipase in the presence of the indicated amounts of the His-tagged Lif. In panels B and C, each value represents the mean of three independent experiments and error bars are indicated.

Fig. 3: **Formation of the disulfide bond during refolding of lipase.** Denatured lipase (0.9 µg) was refolded in the presence or absence of Lif (5 µg) at 37 °C for 0 min (lanes 2 and 3) or 120 min (lanes 4 and 5). Subsequently, 100 mM iodoacetamide were added and the lipase was analyzed by non-reducing SDS-PAGE and Western blotting. Lane 1 shows the position of the native oxidized lipase.

Fig. 4: **Kinetics of the refolding of the lipase.** The unfolded lipase (0.9 µg) was refolded in the presence of 5 µg (♦) or in the absence (●) of the His-tagged Lif at 37 °C. In the
latter case, 5 µg of the Lif was added after 3 h and incubation was continued. The lipase activity regained was measured at the time points indicated. Each value represents the mean of three independent experiments and error bars are indicated.

Fig. 5: **Heat-induced denaturation of the native lipase and of the folding intermediate.**
CD-spectra of the native lipase (Ο) and the folding intermediate (∇) were recorded at 220 nm at the temperatures indicated. Both proteins were at a concentration of 10 µM in 20 mM sodium phosphate buffer (pH 7.0), containing 90 mM urea, 12 mM EDTA and 45 mM NaCl.

Fig. 6: **Interaction between the His-tagged *B. glumae* Lif and native lipase.** Cu$^{2+}$-ions were coupled to chelating sepharose beads. These beads were then charged with His-tagged Lif proteins of *B. glumae* or *P. aeruginosa* or uncharged (-) and incubated with the native *B. glumae* lipase. The proteins in the fractions obtained after the wash with column buffer (lanes 1, 4 and 7), the wash with 25 mM imidazole (lanes 2, 5 and 8) and the elution with EDTA (lanes 3, 6 and 9) were precipitated with TCA and analyzed by SDS-PAGE. The positions of the different proteins are indicated at the right. *P. a.*, *P. aeruginosa*; *B. g.*, *B. glumae*. MW, molecular weight marker proteins in kDa are indicated at the left.

Fig. 7: **Protection of the His-tagged *B. glumae* Lif by the cognate lipase from tryptic digestion.** His-tagged Lifs of *B. glumae* (*B. g.*) and of *P. aeruginosa* (*P. a.*) were digested for 30 min with trypsin in the presence or absence of the *B. glumae* lipase as indicated. Protected proteins were analyzed by SDS-PAGE. Positions of the lipase and the 26 kDa fragment of the Lif are indicated at the left.
Fig. 8: **Conformational changes upon interaction between the His-tagged *B. glumae** Lif and native lipase. The CD-spectra of the lipase (■) and the His-tagged Lifs (○) from *P. aeruginosa* (A) or *B. glumae* (B) were measured separately. The CD-spectrum of a solution containing both the lipase and Lif (◊) was also measured and compared to the theoretical sum of the spectra of the lipase and of the His-tagged Lif separately (▼).
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J. Biol. Chem. published online June 19, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M003258200

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