HtrA Heat Shock Protease Interacts with Phospholipid Membranes and Undergoes Conformational Changes*

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The HtrA (DegP) protein of *Escherichia coli* is a heat shock serine protease, essential for cell survival only at temperatures above 42 °C. It has been shown by genetic experiments that HtrA is an envelope protease, functioning in the periplasmic space. To clarify the cellular localization of HtrA, *E. coli* cells were fractionated, and HtrA was not detected by the immunoblotting technique in the periplasm or in the fraction of soluble proteins but was found in the inner membrane. The protein could be partially eluted from the total membrane fraction by a high ionic strength solution, whereas solutions affecting protein conformation released HtrA almost completely. These results, taken together with the evidence showing that HtrA functions in the periplasm, indicate that HtrA is a peripheral membrane protein, localized on the periplasmic side of the inner membrane. As the first step toward solving the problem of HtrA-membrane interactions, the structure of HtrA in the presence of phosphatidylglycerol (PG), phosphatidylethanolamine (PE), or cardiolipin (CL) was analyzed by fluorescence and Fourier-transform infrared spectroscopy. The infrared and fluorescence data indicated an interaction of HtrA with PG and CL but not with PE suspensions. Fluorescence spectroscopy revealed that this interaction was at the level of the polar head group of the phospholipid. In the PG/HtrA system, small changes were observed in the HtrA secondary structure and a remarkable decrease of the thermal stability of the protein, which suggested changes in HtrA tertiary structure. This suggestion was supported by fluorescence data that showed a shift of the fluorescence emission spectrum of HtrA tyrosine residues in the presence of PG and a reduced fluorescence intensity, phenomena not observed in the presence of PE or CL suspensions. Infrared data revealed also that the interaction of HtrA with PG leads to a protection of unfolded protein against aggregation at relatively low temperatures. The conformational changes of HtrA in the presence of PG influenced the proteolytic activity of HtrA by increasing it at the temperatures 37–45 °C and inhibiting it at 50–55 °C. CL inhibited HtrA activity at all of the temperatures tested.

The heat shock proteins belong to a larger group of stress proteins, and their synthesis is induced by various environmental insults, such as an abrupt increase of temperature. It is generally accepted that their role is to protect cellular proteins from denaturation, to promote renaturation of reversibly inactivated proteins, and to degrade proteins that have been irreversibly damaged. Thus, heat shock proteins are often proteases (reviewed in Refs. 1–3). HtrA (DegP)¹ serine protease of the bacterium *Escherichia coli* is a 48-kDa protein, induced by heat shock, indispensable for cell survival at temperatures above 42 °C (4, 5). Like other heat shock proteins, it is well conserved in evolution. HtrA homologs have been identified in a variety of bacteria, and some bacterial species were found to have multiple HtrA-like proteins (6, 7). Members of the HtrA family of proteins, as judged by the nucleotide sequences of their genes, have putative proteolytic active sites, suggesting that all of them are serine proteases; the only exception is the HtrA homolog from *Rickettsia* (reviewed in Ref. 6). In the case of the *E. coli* HtrA protease, amino acids of the active proteolytic center have been identified (8). Mutations in *htra* gene may cause susceptibility to heat and oxidative stress, as in *E. coli* (4, 5), *Brucella abortus* (9), and *B. melitensis* (10), or loss of virulence, as in *Salmonella typhimurium* (11).

The proposed physiological role of the *E. coli* HtrA protease, and at least some of the HtrA homologs, is to degrade aberrant proteins arising in the periplasmic space under stress conditions (4, 12, 13). There is a set of data strongly indicating that HtrA is located in the cell envelope (the membranes and/or the periplasm). The HtrA protease is synthesized as an unstable 51-kDa precursor that is processed to a 48-kDa mature form by removal of a 26-amino acid leader peptide, and the cleavage occurs following the consensus target sequence for the *E. coli* leader peptidase enzyme (13). The mutations in the *degP* (*htra*) gene prevent the degradation of certain fusion proteins and mutant proteins, located in the periplasm or inner membrane, but not in the cytoplasm (12). Protein fusions between DegP (HtrA) and alkaline phosphatase have high alkaline phosphatase enzymatic activity (4). The DegP (HtrA) protease was shown to be responsible for a specific cleavage of the acylated precursor form of the colicin A lysis protein, located in the inner membrane (14).

There is also indirect evidence of the envelope localization of HtrA: (i) the *htra* gene is transcribed by RNA polymerase containing α²(α²−1) factor (15), and it has been proposed that α² regulon is involved in processes that occur in extracytoplasmic compartments (16); (ii) *htra* transcription is regulated by the Cpx two-component signal transduction pathway, which responds to changes in cellular envelope (17); and

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The abbreviations used are: DegP, protein identical with HtrA; PG, phosphatidylglycerol; CL, cardiolipin; PE, phosphatidylethanolamine; PAGE, polyacrylamide gel electrophoresis; PL, phospholipid; GP, generalized polarization; amide I, amide I band in a 2H2O medium.

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(iii) two homologs of HtrA, HloA and HloB, suppress mutation in the prc gene, coding for periplasmic Prc protease (6). However, in cells overproducing HtrA from a high-copy-number plasmid, the protease was found both in the membranes and in the fraction of soluble proteins, composed of periplasmic and cytoplasmic proteins (5). It has been proposed that the HtrA (DegP) protease and the Do protease are identical (18). Do has been reported to be a cytosolic enzyme (18), and Yoo et al. (19) showed that it localizes primarily to the cytoplasm, although it could bind to membranes through an ionic interaction.

In this report, we present studies on cellular localization of HtrA, showing that this protein is absent in the periplasmic space and that most of it is bound to the membranes by ionic interactions. Believing that HtrA is a peripheral membrane protein and as such should interact either with integral membrane protein(s) or with phospholipids, we investigated interactions between purified HtrA and artificial membranes formed by phosphatidylglycerol (PG), cardiolipin (CL), or phosphatidylethanolamine (PE), using fluorescence and Fourier-transform infrared spectroscopy.

MATERIALS AND METHODS

Bacterial Strains and Plasmids—The E. coli B178 (W3110 galE sup') strain (5), used for overproduction of the HtrA protein, was from our collection. The plasmid pJS13, carrying the wild-type htrA gene (8), was used for controlled overproduction of the HtrA protein.

Chemicals—Deuterium oxide (99.9% 2H), HCl, and NaO2H were purchased from Aldrich. E. coli cardiolipin (CL), lα-phosphatidyl-α- glycerol (PG), and E. coli lα-phosphatidylethanolamine (PE) were obtained from Sigma. PG was derived from egg yolk phosphatidylcholine, the fatty acid composition of which is described by Menzel and Oloott (20). 2-Dimethylamino-(6-lauroyl)-naphthalene (Laurdan) was purchased from Aldrich. The HtrA protein was overproduced in E. coli B178 bacteria using a T7 promoter/T7 polymerase (Laurdan) was purchased from Molecular Probes (Eugene, OR). Anti-OmpF chased from Molecular Probes (Eugene, OR). Anti-HtrA rabbit antiserum was kindly provided by Krzysztof Kucharczyk. Goat serum was prepared against the gel-purified HtrA protein. Anti-OmpF purchased from Sigma or other chemical companies and were of the purest quality.

Purification of the HtrA Protein—The HtrA protein was overproduced in E. coli B178 bacteria using a T7 promoter/T7 polymerase system, and the htrA gene was cloned in the plasmid pJS13, as described previously (8). Then the protein was purified chromatographically as described by Lipinski et al. (13). The purity of the proteins was estimated to be more than 95%, as proved by SDS-polyacrylamide gel electrophoresis.

Electrophoresis of Proteins—Proteins were analyzed by SDS-PAGE, as described previously (13). Gels containing 12.5% (w/v) acrylamide were used.

Protein Assay—The protein concentration was estimated by staining with Amido Black as described before (13).

Proteolytic Activity Assays—Proteolytic activity assays with β-casein as a substrate were performed in 50 mM Tris-HCl buffer, pH 8.0, as described previously (8).

Immunoblotting Procedures—Following SDS-PAGE, proteins were transferred to nitrocellulose, treated with 3% dried milk in Tris-buffered saline (TBS) (20 mM Tris/HCl, pH 7.5, and 0.5 M NaCl) to block unoccupied protein-binding sites, and exposed to rabbit antiserum against purified HtrA protein or against OmpF protein, diluted in TBS (13). The antigens were detected using goat anti-rabbit biotinylated immunoglobulin G and streptavidin-conjugated alkaline phosphatase as described by Wilchek and Bayer (21).

Preparation of Periplasmic Proteins—The osmotic shock method of Neu and Heppel (22) was used, with minor modifications. The bacteria were grown in Luria broth (LB broth) at 37°C to an A600 of 0.6–0.8, and then the heat was shocked at 46°C for 15 min. Two portions of the culture, each equivalent to 2.4 A600 units were then centrifuged (10 min at 14000 × g) at 4°C, and the cells were washed three times with 1 ml of cold 10 mM Tris-HCl buffer, pH 8.0. Next, the cells were resuspended in 2 ml of 20% sucrose, 30 mM Tris-HCl, pH 8.0, and 20 mM EDTA, incubated for 8 min at room temperature, and centrifuged again (10 min at 5000 × g, 4°C). Both pellets were resuspended in 1 ml of ice-cold distilled water, and one suspension was centrifuged immediately (9000 × g for 7 min at 4°C); the supernatant, containing periplasmic proteins, was collected. The other suspension, containing total cellular proteins, was treated as a reference in further analyses.

β-Galactosidase, Alkaline Phosphatase, and NADH-Oxidase Assays—The β-galactosidase, alkaline phosphatase, and NADH-oxidase activities were measured according to Owen et al. (23).

Preparation of Soluble Proteins and Membrane Fractions—The method of Osborn and Munson (24), as described by Owen et al. (23), was used. The bacteria were grown in LB broth at 37°C to an A600 of 0.6, and then the total membranes and soluble cellular proteins (cytoplasmic and periplasmic) were prepared. The membranes were washed with 10 mM Tris-HCl, pH 8.0, 2 mM MgCl2 buffer and resuspended in the same buffer. Membranes from 100 ml of the initial culture were usually resuspended in 200 μl of the buffer, frozen in liquid nitrogen, and stored at −70°C.

Preparation of Inner and Outer Membranes—The procedure of Osborn and Munson (24) was followed. The total membranes, prepared as described above, were washed with 25% sucrose in 3 mM EDTA, pH 7.5, resuspended in the same solution, and separated into the inner and outer membrane fractions by sucrose-gradient centrifugation. The quality of separation was monitored by: (i) measuring protein concentration; (ii) assaying NADH-oxidase activity according to the method described by Owen et al. (23); and (iii) testing the presence of the OmpF protein by immunoblotting with anti-OmpF rabbit antiserum.

H2/H Exchange and Preparation of Samples for Infrared Measurements—The purified HtrA protein (about 1.5 mg) was transferred into Centricon 30 microconcentrators (Amicon). 300 μl of 20 mM Tris/HCl, 20 mM KCl buffer prepared in H2O or pH 8.0 (pH = pH meter reading + 0.4 (25)) were added, and the sample was centrifuged at 3000 × g and 4°C to a final volume of approximately 50 μl. Then, an additional 300 μl of buffer, prepared in D2O, were added, and the sample was centrifuged again. This procedure was repeated several times to completely replace H2O with D2O. In the final step, the sample was concentrated to approximately 40 μl.

For the experiments in the presence of phospholipids (PLs), the concentrated protein samples were vortexed in Eppendorf tubes on whose caps a proper amount of PL was dried previously. The homogeneous PL/HtrA suspensions were then used for Fourier transform infrared spectroscopy experiments. The PL:HtrA weight ratio that was reported in the figure legends.

Infrared Spectra—The concentrated protein samples were placed in a Graseby-Specac 20500 cell (Graseby-Specac, Ltd., Orpington, Kent, United Kingdom) fitted with CaF2 windows and a 12-mm tin spacer. The cell was maintained at the desired temperature by an external bath circulator. Spectra were obtained by a Perkin-Elmer 1760-x Fourier transform infrared spectrometer at 2 cm−1 resolution and using a normal Beer-Norton apodisation function. For the thermal denaturation experiments, the temperature was raised in 5°C steps. The samples were then maintained at the desired temperature for 6 min before spectrum acquisition. Buffer spectra were collected under the same temperature and scanning conditions. Spectral deconvolution was performed using the Perkin-Elmer ENHANCE function, which is analogous to the method developed by Kaupinen et al. (26). Deconvolution parameters were set with the half-bandwidth at 19 cm−1 and a resolution enhancement factor of 3. Second derivative spectra were calculated over a 9-data-point range (9 cm−1).

Preparation of Samples for Fluorescence Measurements—Labeling of PL multilamellar liposome vesicles with Laurdan was performed as described (27). In particular, the proper amount of PG, CL, or PE, dissolved in chloroform:methanol 3:1 (v/v) was mixed with Laurdan (Laurdan:PL molar ratio, 1.1:1000), and then the solvent was evaporated by a gentle stream of nitrogen. The dried film of PL was rehydrated with 20 mM Tris/HCl, 20 mM KCl buffer, pH 8.0, at 37°C and to 0.23 mM final PL concentration.

Small unilamellar vesicles were prepared by sonication of the rehydrated lipids (0.8 mg/ml) under nitrogen. Labeling of small unilamellar vesicles with Laurdan was carried out by incubation of the fluorescent probe with the vesicles (Laurdan:PL molar ratio, 1:1000) at 30°C up to a constant value of the fluorescence intensity.

HtrA protein (80 μg/ml) was added to Laurdan (Laurdan:PL molar ratio, 1:1000) liposomes (100 μl) in Tris/HCl buffer, pH 8.0, as described above. Then 2 ml of the desired PL suspension was incubated with 0.6 mg of HtrA (PL:HtrA weight ratio, 0.33) for 30 min at 30°C, before fluorescence measurements. HtrA was incubated with unlabeled or Laurdan-labeled PL, depending on the specific fluorescence experiment as described in the next section.

Fluorescence Spectroscopy—Steady-state fluorescence measure-
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RESULTS

Cellular Localization of the HtrA Protein—To clarify whether the HtrA protein resides in periplasm, membranes, or cytoplasmic space of the E. coli cell, we performed subcellular fractionation of HtrA from the bacteria carrying only a chromosomal htrA gene. The periplasmic fraction was prepared from E. coli cells by the osmotic shock method, periplasmic proteins were precipitated with 10% trichloroacetic acid and resolved by SDS-PAGE (lane 3), together with the total cellular proteins equivalent to 0.8 A_280 units of cells (lane 2); both samples were identical with respect to alkaline phosphatase activity. Lane 1 represents the HtrA standard (0.1 μg). In B, E. coli cells were fractionated into the total membrane (lane 1) and soluble protein (lane 2) fractions; samples of each fraction, equivalent to 0.8 A_280 units of cells, were precipitated in trichloroacetic acid and resolved by SDS-PAGE. C. E. coli outer (lane 1) and inner membrane (lane 2) fractions were resolved by SDS-PAGE together with the soluble proteins (lane 3); each sample contained 10 μg of protein; lane 4, HtrA standard (0.1 μg). The gels were immunoblotted using rabbit anti-HtrA antisemur. The low molecular weight polypeptides, visible mainly in the lanes with HtrA standard, are degradation products of HtrA.

To clarify whether HtrA is a peripheral protein, we fractionated cells into the total membrane and soluble protein fractions, according to the classical method of Osborn and Munson (24) (see “Materials and Methods”), and determined that over 90% of HtrA was present in the membranes (Fig. 1B). The membranes were then fractionated by sucrose gradient centrifugation into the inner (cytoplasmic) membranes and outer membranes. The extent of cross-contamination was checked by assaying the activity of the inner membrane marker enzyme, NADH-oxidase, and by monitoring the presence of the outer membrane marker, OmpF protein, by immunoblotting. The outer membrane fraction had no detectable NADH-oxidase activity, whereas the inner membrane preparation had only trace amounts (less than 1% when compared with the outer membrane) of OmpF (data not shown), proving the good quality of the separation. As judged by immunoblotting, all of the HtrA was localized in the inner membrane fraction (Fig. 1C). The membrane-bound HtrA could be partially removed from the membranes by washing once with 1 M KCl (Fig. 2, lane 2) and almost completely by washing once with 0.1 M Na_2CO_3, pH 11.5, solution (Fig. 2, lane 3) or with 4 M urea (Fig. 2, lane 5). The urea treatment released HtrA more efficiently than the carbonate treatment; therefore, to be able to visualize the HtrA remaining in the membranes, we had to use a double amount of the urea-washed membranes as compared with the carbonate-washed membranes in the immunoblotting procedure (Fig. 2). These results indicate that HtrA is not an integral membrane protein but a peripheral protein bound to the inner membranes.

HtrA Secondary Structure—In a previous study, we analyzed the secondary structure composition of wild-type HtrA heat shock protease and mutant HtrA proteins (30). Our finding that HtrA is a peripheral protein of the inner membrane suggested that it might interact with membrane phospholipids; therefore, we analyzed the effect of PG, PE, and CL on the HtrA conformation. Fig. 3 shows the absorbance, deconvolved, and the second derivative spectra of HtrA proteins. The resonance Raman spectra show the amide I' component bands as well resolved peaks and shoulders. These component bands were previously assigned to particular secondary structural elements (30). In particular, the 1628, 1635, and 1673 cm^{-1} bands were assigned to β-sheets, the 1682 and 1665 cm^{-1} bands to turns, the 1656 cm^{-1} band to α-helix, and the 1648 cm^{-1} band to α-helix and unordered structures. It was also found (30) that the secondary structure composition of wild-type HtrA, which possesses a
serine protease activity, is similar to that of other serine proteases (31).

**Secondary Structure of HtrA in the Presence of PG, PE, or CL**—Information on the secondary structure of HtrA in the presence of PG, PE, or CL suspensions can be obtained by comparison of the corresponding infrared spectra with the spectrum of HtrA in the absence of phospholipids. Fig. 4 shows the absorbance and second derivative spectra of the protein in the presence of PG (A), PE (B), or CL (C). The broad band within approximately 1750–1700 cm\(^{-1}\) in the absorbance spectra is due to the ester C=O stretching vibration of phospholipids (32, 33). In the deconvoluted (data not shown) or second derivative spectra, the 1750–1700 cm\(^{-1}\) band is resolved in two bands due to the sn-1 and sn-2 C=O groups involved in a different hydrogen-bonding with water (32, 33).

The second derivative spectra of HtrA in the absence and in the presence of PG suspensions (PG:HtrA weight ratio, 1.8) show only small differences, especially in the 1648–1655 cm\(^{-1}\) region, indicating that PG induces small changes in the secondary structure of the protein. PG also induces a broadening of the amide I' band, and the effect was not observed in the case of PE (Fig. 4B) or CL (Fig. 4C). The same results were obtained with a PG:HtrA weight ratio of 1 or 3.5. The second derivative spectrum of HtrA in the presence of PE is almost indistinguishable from the control spectrum, indicating that PE does not significantly affect the secondary structure of the protein. A similar effect is also observed in the case of the HtrA/CL system (Fig. 4C).

**Thermal Denaturation of HtrA in the Absence and in the Presence of PG, PE, or CL**—To gain more information on the HtrA structure, we collected infrared spectra as a function of the temperature. The thermal denaturation of a protein may be followed by monitoring different parameters of the infrared spectrum including the amide I' bandwidth (34, 35). Fig. 5 shows the temperature-dependent changes of the amide I' bandwidth (width at one-half height) for HtrA in the absence and in the presence of PE (A), PG (B), or CL (C). Although PE does not affect the thermal denaturation behavior of HtrA, PG markedly modifies the thermal denaturation curve of the protein. In particular, two transitions at about 55 and 80 °C are present in the samples corresponding to a PG:HtrA weight ratio of 1.8 or 3.5, whereas apparently a unique transition at about 55 °C is present in the sample with a PG:HtrA weight ratio of 1. Fig. 5B also shows that, below 55 °C, the value of the amide I' bandwidth is affected by the presence of different amounts of PG. HtrA in the presence of different amounts of CL (Fig. 5C) shows almost the same temperature of denaturation (Tm) as the control (−65 °C), but above Tm, the amide I' bandwidth is much more affected by the presence of CL than below Tm or at Tm.

These data suggest an interaction of PG with HtrA at all the temperatures tested. A significant interaction of HtrA with CL is also suggested to occur at temperatures above Tm, whereas below Tm or at Tm, the interactions seem to occur to a lower extent, at least as compared with the HtrA/PG system. No significant interaction of PE with the protein seems to occur at any of the temperatures tested.

The meaning of the two transitions observed in the presence of PG can be obtained from difference spectra (Fig. 6). Difference spectra have been obtained by making the difference between two original absorbance spectra collected at different temperatures, and they represent a powerful method to monitor the temperature-dependent protein conformational changes (35). For instance, the negative 1632 cm\(^{-1}\) peak (Fig. 6A, spec-
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Influence of Phospholipids on Proteolytic Activity of HtrA—Having found that in vitro HtrA interacts with PG and CL but not with PE, we monitored proteolytic activity of HtrA in the presence of these phospholipids. Because HtrA is a heat shock protein and its activity rapidly increases with temperature (30), we tested the activity at temperatures ranging from 37 to 55 °C. We incubated HtrA with β-casein as a substrate, in the presence of a phospholipid, and the reaction products were resolved by SDS-PAGE. The gels were analyzed densitometrically, as described previously (30), and a relative amount of undegraded substrate at a given time point was calculated. The results, presented in Fig. 7, showed that whereas PE had no effect on HtrA activity, there was a significant influence of PG, dependent on temperature. At 37, 40, and 45 °C (Fig. 7, A and B, and results not shown) PG stimulated the activity, whereas at a range from 50 to 55 °C (Fig. 7, C and D), PG had a well pronounced inhibitory effect. CL inhibited the HtrA activity at all of the temperatures tested (Fig. 7).

Fluorescence Experiments—Laurdan is a fluorescent probe sensitive to the polarity of the microenvironment in which it is located (36). It localizes at the hydrophobic-hydrophilic interface of the lipid bilayer at the glycero backbone level (28), and thus it can give information on modifications that have occurred at this level and/or at the polar head group of the PL assemblies. Parasassi et al. (29) introduced the parameter GP, related to the dynamics of the solvent molecules surrounding the probe. The GP value depends on the polarity of the microenvironment, and in liposomes, it decreases with the increase of temperature (27, 29).

Fluorescence experiments were carried out on multilamellar vesicles as well as small unilamellar vesicles. The results of experiments performed with small unilamellar vesicles (data not shown) were similar to those obtained with multilamellar vesicles as shown in Fig. 8 for Laurdan-labeled PG, PE, or CL multilamellar vesicles, both in the absence and in the presence of HtrA. The GP_{340} values are the same at all temperatures in PE and PE/HtrA samples, indicating that the protein does not interact with PE. GP_{340} values for Laurdan embedded in PG suspensions decrease with the increase of temperature, but they remain almost constant in the PG/HtrA sample, indicating that the protein interacted with PG at the level of the PL polar head group because Laurdan is a probe for this region. The GP_{340} values for CL suspensions decrease in both samples with the increase in the temperature. The decrease is slightly but significantly lower in the CL/HtrA sample as compared with the control at a temperature up to −42 °C, indicating a small modification of the lipid microenvironment, where Laurdan is located, induced by the protein. This effect is much more marked above 42 °C, where the GP_{340} values for CL/HtrA remain almost constant.

The HtrA protein contains five tyrosines but not tryptophan residues (37). HtrA in the absence or in the presence of PE or

trum g, 70–65 °C) indicates a lower content of secondary structural elements in the sample at 70 °C with respect to the protein at 65 °C, and in turn, indicates unfolding (denaturation) of the protein induced by the increased temperature. The higher the peak, the higher the extent of denaturation. The positive 1617 cm⁻¹ band represents protein intermolecular interactions (aggregation) brought on by protein denaturation (35), and the broad negative bands at about 1550 cm⁻¹ observed in the spectra concomitantly to protein denaturation is due to an additional H/H exchange in the samples at a higher temperature (35). As Fig. 6 shows, denaturation and aggregation occur concomitantly in the HtrA and PE/HtrA samples, and the maximum of the events occurs between 65 and 70 °C (spectra g). This is reflected in Fig. 5 as a unique transition (A). In Fig. 6C (PG/HtrA weight ratio, 3.5), the protein first unfolds (maximum of unfolding between 50 and 55 °C) and then aggregates (maximum aggregation at 75 and 80 °C). This is reflected in Fig. 5B as two transitions, the first of which is related to protein denaturation and the second one to protein aggregation. Fig. 6B reflects an intermediate situation between that of Fig. 6, A and C. In fact, when the PG:HtrA weight ratio = 1, denaturation of the protein occurs almost concomitantly (at 50–55 °C) with the aggregation event, which is shifted slightly to higher temperatures. This is seen in Fig. 5 as a transition at about 55 °C, followed by a continuous increase in the “W 1/2 H” value.

These data further support an interaction of HtrA with PG, which seems to protect the unfolded protein against aggregation at relatively low temperatures. The difference spectra of the CL/HtrA system indicate a denaturation and aggregation occurring concomitantly at the same temperature (data not shown).

Influence of Phospholipids on Proteolytic Activity of HtrA—Having found that in vitro HtrA interacts with PG and CL but not with PE, we monitored proteolytic activity of HtrA in the presence of these phospholipids. Because HtrA is a heat shock protein and its activity rapidly increases with temperature (30), we tested the activity at temperatures ranging from 37 to 55 °C. We incubated HtrA with β-casein as a substrate, in the presence of a phospholipid, and the reaction products were resolved by SDS-PAGE. The gels were analyzed densitometrically, as described previously (30), and a relative amount of undegraded substrate at a given time point was calculated. The results, presented in Fig. 7, showed that whereas PE had no effect on HtrA activity, there was a significant influence of PG, dependent on temperature. At 37, 40, and 45 °C (Fig. 7, A and B, and results not shown) PG stimulated the activity, whereas at a range from 50 to 55 °C (Fig. 7, C and D), PG had a well pronounced inhibitory effect. CL inhibited the HtrA activity at all of the temperatures tested (Fig. 7).

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Cl. HtrA in the presence of PG, PG:HtrA weight ratio of 3.5.

Fraction (5) (data not shown). This data suggested that HtrA from a plasmid, localizes to a high extent in the soluble protein ally, HtrA is a hydrophilic protein and, when overproduced which may cause release of some peripheral proteins. Addition-

not shown). This method uses disruption of cells by sonication,

of tyrosine fluorescence to environmental changes and/or to weak interac-
tions inducing HtrA structural changes. The lack of a shift in the tyrosine fluorescence emission spectrum in the CL/HtrA system could be due to the low sensitivity of tyrosine fluorescence to environmental changes and/or to weak interac-
tions of HtrA with CL.

**DISCUSSION**

We found that there was no HtrA present in the periplasm of either heat-shocked or non-heat-shocked cells (Fig. 1 and data not shown). This shows that under both physiological and stress conditions, when the protease is necessary for survival of the cell, it is not released to the periplasmic space. We used periplasm prepared by a mild osmotic shock because, in our results, HhoA may be a true periplasmic protease, or sim-
ilarly to HtrA, may be localized in the periplasm only when

overexpressed in a cell.

Peripheral membrane proteins are normally bound to membranes by electrostatic forces that can be disrupted by high ionic strength solutions (42). Our finding that treatment with 1 M KCl caused significant dissociation of HtrA from the membranes indicates that indeed HtrA is a peripheral membrane protein. However, approximately 50% of HtrA remained in the membranes after the elution with 1 M KCl (Fig. 2 and results not shown), suggesting that HtrA is not loosely bound to the membranes via weak electrostatic interactions. In contrast, treatment with either 4 M urea or 0.1 M Na2CO3, pH 11.5, released HtrA from the membranes almost completely (Fig. 2). Both Na2CO3, pH 11.5, and urea primarily act to denature protein structure without disrupting the organization of the membrane bilayer and, thus, often dissociate peripheral pro-

teins (43–45). Because both reagents perturb protein structure, the observed efficient release of HtrA suggests that conformation of HtrA may be important in mediating its interaction with the membrane. Taking into consideration the fact that HtrA functions in the periplasmic space (12), we propose that HtrA is bound to the inner membrane on the periplasmic side. This proposal is not, in fact, contradictory to the generally held belief that HtrA is a periplasmic protease. This belief is based on the experiments showing that HtrA degrades periplasmic and inner membrane proteins (12) and that protein fusions between DegP (HtrA) and alkaline phosphatase have high alkaline phosphatase activity (4). Localization on the periplasmic side of the inner membrane is in agreement with these facts.

Recently, two homologs of HtrA, named HhoA and HhoB, have been identified in *E. coli* (6, 46). They were shown to suppress the conditional lethal phenotype of a strain mutated in the *prc* gene coding for the Prc (Tsp) periplasmic protease, and the HhoA protease, when overproduced from a plasmid, was found in the periplasmic fraction of the cell (6). In view of our results, HhoA may be a true periplasmic protease, or similarly to HtrA, may be localized in the periplasm only when overexpressed in a cell.
It has been proposed that the Do protease is identical with the HtrA protein (18), but there is a difference concerning cellular localization of these proteins; the envelope localization of HtrA is a well established fact, whereas Do is believed to be a cytoplasmic protease, able to bind to membranes through an ionic interaction (19). However, Yoo et al. (19), who found that Do was localized in the cytoplasmic fraction, performed fractionation of cellular material using sonication of osmotically shocked cells. Our unpublished results indicate that such treatment causes partial release of HtrA from the membranes. In conclusion, it seems that, if HtrA and Do are the same proteins, the discrepancy concerning their localization can be explained by different methods used for fractionation.

Finding that HtrA is a peripheral membrane protein poses a question of whether it is bound to the membrane via protein-protein interactions, protein-phospholipid interactions, or both. As the first step toward solving this problem, we investigated conformation of HtrA in the presence of suspensions of three phospholipids, PG, PE, and CL, using infrared and fluorescence spectroscopy. PG, PE, and CL are three major phospholipids present in E. coli membranes; the inner membranes contain roughly equal amounts of zwitterionic PE and two acidic lipids, PG and CL (47). Both infrared and fluorescence data indicated an interaction between HtrA and PG but not with PE. There was an interaction between HtrA and CL, however, less pronounced than in the case of PG. In addition, fluorescence data indicated a remarkable reduction of the thermal stability of HtrA.

FIG. 7. Effect of phospholipids on HtrA proteolytic activity. The HtrA protein (0.1 μg) was incubated with 10 μg of β-casein in 10 mM Tris-HCl, pH 8.0, buffer without a phospholipid (○), with PG (■), CL (×), or PE (▲), in the final volume of 25 μl; the weight ratio of PL to HtrA was 2.0. The reaction was stopped at the times indicated in the graphs by the addition of an equal amount of twice-concentrated lysis buffer for electrophoresis and immediate placing of the sample at 100 °C for 5 min. The samples were then resolved by SDS-PAGE, stained with Coomassie Brilliant Blue, and analyzed densitometrically as described previously (30). The amount of the undegraded casein, expressed as a percentage of absorption, is shown in graphs A–D (100% absorption was the absorption of the casein band at the beginning of the reaction). The temperature of each reaction is specified on the graph.

FIG. 8. Laurdan GP (GP340) for PG, PE, or CL suspensions in the absence and in the presence of HtrA at different temperatures. ▲, ■, and ○, control PE, CL, and PG vesicles, respectively. △, □, and ◆, HtrA in the presence of PE, CL, and PG, respectively.

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only slightly affected by PG. Reduced thermal stability and no or minor changes in the secondary structure of proteins induced by lipids were also observed by other authors who associated the events with alteration in the tertiary structure of the polypeptides (34, 48). Changes in the tertiary structure of HtrA were further supported by the tyrosine fluorescence emission spectra, which were slightly but significantly modified in the presence of PG. As judged by Fourier transform infrared spectroscopy, CL did not alter the secondary structure of HtrA and did not affect significantly the thermal denaturation of HtrA, suggesting no or minor changes in its tertiary structure. This could be due to a low and/or different interaction of HtrA with CL as compared to PG.

The interaction of HtrA with PG and CL but not with PE may find an explanation in the acidic and basic nature of the phospholipids and of the protein (47, 5), respectively. It may be postulated that upon binding to PG or CL membranes, there is competition between electrostatic interactions within the protein (involving oppositely charged amino acid residues) and the interactions between basic protein residues and the negatively charged PG and CL head group. These interactions may weaken the internal stabilizing forces of HtrA, leading to a loosening and a destabilization of the tertiary structure of the protein.

The formation of less stable HtrA on the surface of PG assemblies has a direct influence upon the proteolytic activity of HtrA. While at the temperatures 37–45 °C we observed a significant activating effect of PG on HtrA (Fig. 7, A and B), at the temperatures ranging from 50 to 55 °C the phospholipid caused a decrease of proteolytic activity (Fig. 7, C and D). The switch from activation to inhibition of HtrA, occurring between 45 and 50 °C, corresponds very well to the unfolding of the HtrA structure, which in the presence of PG starts between 45 and 50 °C (Fig. 5B and Fig. 6C). Apart from the biological significance of this correlation, it is another example of the remarkable agreement between the infrared spectroscopy data characterizing conformation of a protein and the biological activity of the protein.

It is generally believed that the function of HtrA is to degrade aberrant proteins accumulating in the periplasmic space under denaturing conditions of a high temperature (4, 12, 13). Our finding that PG stimulates HtrA activity under mild heat shock conditions (up to 45 °C) (Fig. 7) is in agreement with the postulated function of HtrA as well as with the idea that HtrA may be bound to the inner membrane via the acidic polar head group of PG. However, the inhibitory effect of PG on HtrA activity observed at the extreme temperatures of 50–55 °C (Fig. 7, C and D) seems to contradict the idea of a HtrA-PG bond existing in vivo. On the other hand, it is possible that in vivo HtrA interacts not only with PG but also with a membrane or periplasmic protein (or proteins), which modulates the destabilizing effect of PG at the extreme temperatures.

The observation that PG destabilizes the tertiary structure of HtrA may also have a more general biological significance. In particular, the destabilization of HtrA on PG membranes may represent a general property of a larger class of polar proteins, the binding of which to PL membranes is governed by electrostatic interactions. The PL-induced destabilization could represent a necessary factor for a mechanism of protein translocation across biological membranes as proposed by Endo et al. (49). Involvement of the acidic phospholipids in protein translocation across the E. coli inner membrane has been shown clearly (50, 51). In the particular case of HtrA, the unfolded protein could also be protected by PG against self-aggregation. In fact, the thermal denaturation experiments, besides modification in the tertiary structure of the protein, indicated a protection effect of PG against the aggregation of a thermally unfolded protein. At the PG:HtrA weight ratio of 1, the aggregation process occurred almost concomitantly with the protein denaturation, but when the PG:HtrA weight ratio was increased to 1.8 or higher values, the aggregation process was separated from protein denaturation and occurred at higher temperatures (80 °C).

The biological significance of the strong inhibitory effect of CL on HtrA activity, observed at all temperatures, is difficult to interpret at this stage of our experiments. It can be speculated that if HtrA interacts with CL in vivo, this phospholipid may have a modulating influence on HtrA proteolytic activity. It is also possible that the CL molecules do not interact in vivo with HtrA but in vitro bind nonspecifically to the protease, masking its active site. Different effects of various acidic phospholipids on one protein have been described before. Cardiolipin specifically inhibits the binding of SV40 T antigen to the replication origin of SV40 DNA, whereas PG, phosphatidylserine and phosphatidylinositol do not affect the binding activity (52). Protein kinase C is very specifically and cooperatively activated by phosphatidylserine but not other acidic PLs (53).

Our finding that the acidic PLs affect the structure and activity of HtrA adds to the increasing amount of data showing that acidic PLs regulate the functions of not only integral membrane proteins but also those of peripheral and cytosolic enzymes. These enzymes include Sec A protein (54, 55), DnaA protein (56), DNA topoisomerase I (52), cytochrome c (53), calcineurin (57), phosphatidylinositol-4-phosphate kinase (58), sphingosine kinase (59), glycerol-3-phosphate acyltransferase (60), and the already mentioned protein kinase C and SV40 T antigen.

Summarizing our experiments on HtrA-PL interactions, we conclude that in vitro there is a definite interaction between HtrA and acidic phospholipids, PG and CL, influencing the conformation and activity of HtrA, the former especially in the case of PG. These results encourage the pursuit of the question of whether in vivo HtrA is bound to the inner membrane via the phospholipids and/or integral protein and whether the temperature-induced changes in physical properties of phospholipids play a role in regulating the activity of HtrA, an element of the heat shock response.

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