Solubilization of Protein Aggregates by the Acid-Stress Chaperones HdeA and HdeB

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Running title: Protein aggregates solubilization by acid-stress chaperones

The abbreviations used are: DTT: dithiothreitol; Hsp: Heat Shock Protein; bis-ANS: 1,1'-bis(4-anilino)naphtalene-5,5'-disulfonic acid.

SUMMARY

The acid-stress chaperones HdeA and HdeB of Escherichia coli prevent the aggregation of periplasmic proteins at acidic pH. We show in this report that they also form mixed aggregates with proteins that have failed to be solubilized at acidic pH, and allow their subsequent solubilization at neutral pH. HdeA, HdeB, and HdeA and HdeB together, display an increasing efficiency for the solubilization of protein aggregates at pH 3. They are less efficient for the solubilization of aggregates at pH 2, whereas HdeB is the most efficient. Increasing amounts of periplasmic proteins draw increasing amounts of chaperone into pellets, suggesting that chaperones co-aggregate with their substrate proteins. We observed a decrease in the size of protein aggregates in the presence of HdeA and HdeB, from very high molecular weight aggregates to 100-5000 kDa species.

Moreover, a marked decrease in the exposed hydrophobicity of aggregated proteins in the presence of HdeA and HdeB was revealed by bis-ANS binding experiments. In vivo, during the recovery at neutral pH of acid-stressed bacterial cells, HdeA and HdeB allow the solubilization and renaturation of protein aggregates, including those formed by the maltose receptor MalE, the oligopeptide receptor OppA and the histidine receptor HisJ.

Thus, HdeA and HdeB not only help to maintain proteins in a soluble state during acid treatment, as previously reported, but also assist, both in vitro and in vivo, in the solubilization at neutral pH of mixed protein-chaperone aggregates formed at acidic pH, by decreasing the size of protein aggregates and the exposed hydrophobicity of aggregated proteins.
INTRODUCTION

In their natural habitats, bacteria are constantly under assault from a vast array of environmental stresses, including UV irradiation, heat, oxidative, osmotic and pH stresses (1). One of the most frequently encountered is acid stress (2). Enterobacteria, when travelling through the gastrointestinal tract encounter an extremely low pH; facultative intracellular pathogens tolerate episodes of low pH within macrophage phagolysosomes, and fermentative bacteria excrete acidic fermentation products that trigger an endogeneous acid stress (1).

In response to acid stress, bacteria regulate their cytoplasmic pH. Many bacteria possess amino acid decarboxylase systems which consist of cytoplasmic decarboxylases and antiporters that exchange imported amino acids for the cytoplasmic amines produced (1-4), leading to cytoplasmic and periplasmic alkalinization. Helicobacter pylori overexpresses an urease which increases the production of ammonia (5-6). In several bacteria, the proton-translocating F_{i}F_{o} ATPase can export protons as a consequence of ATP hydrolysis (7). Further protection against acid stress is obtained by decreasing the permeability of the inner and outer membranes to protons and by reversing the cytoplasmic membrane potential to an inside-positive potential that slows the proton influx into the cell (2, 7-8). Bacteria can also reorientate their metabolism towards pathways that decrease proton production or increase amine production with a consequent alkalinization (6, 9). Moreover, several protective proteins, such as the DnaK and GroEL chaperone machines (10) and several DNA repair enzymes (11) may be induced upon acid stress.

The bacterial periplasm is probably more vulnerable to acid stress than the cytoplasm, due to the relative permeability of the outer membrane porins to molecules smaller than 600 Da (12, 13). Recently, two periplasmic chaperones, HdeA and HdeB, which support acid resistance in E. coli, were discovered (14-16). They are transcribed from the hdeAB operon which is localized in the acid fitness island, a cluster of 12 genes located at 78.8 min that code for several proteins involved in acid stress resistance (2). HdeA and HdeB each have a molecular weight of around 9 kDa (after processing of their signal sequence), and prevent the acid-induced aggregation of bacterial periplasmic extracts and of several model substrate proteins (15-16). In vitro, HdeA plays a major role in protein solubilization at pH 2, and both proteins are involved in protein solubilization at pH 3 (16). In vivo, however, there seems to be a requirement for both chaperones for optimal resistance to acid stress at either pH 2 or pH 3. HdeA and HdeB expose hydrophobic surfaces at acidic pHs, in accordance with the appearance of their chaperone properties at these pHs. They both dissociate from dimers at neutral pH into monomers at acidic pH, but they do not appear to form heteromultimers (15-16). HdeA exposes more hydrophobic surfaces than HdeB at acidic pH, but, unlike HdeB, it remains partially in its inactive dimeric form at pH 3 (16). These dissimilarities might explain the differences in the chaperone properties of HdeA and HdeB, and the requirement for both chaperones for optimal handling of unfolded proteins.

In this report, we show that HdeA and HdeB not only help to maintain proteins in a soluble state during acid treatment (14-16), but also participate, both in vitro and in vivo, in the solubilization and renaturation at neutral pH of proteins that had aggregated in their presence at acidic pH. Whereas most chaperones, like DnaK (Hsp70) and GroEL (Hsp60), maintain unfolded proteins in a soluble state by preventing their aggregation, other chaperones, like small Hsps, are associated with insoluble proteins during heat stress, and their function is to promote the rapid resolubilization by Hsp70 or Hsp104/Hsp70 of aggregated proteins (17-24). We show in this report that HdeA and HdeB, like small Hsps, belong to the restricted set of chaperones that form mixed aggregates with their substrate proteins and assist in their disaggregation.
EXPERIMENTAL PROCEDURES

Preparation of bacterial extracts, and purification of HdeA and HdeB. HdeA and HdeB were expressed from the overproducing strains BL21 (DE3) pET-21a-hdeA and BL21 (DE3) pET-21a-hdeB, and purified from osmotic shock fluids on DEAE-Sephacel and hydroxyapatite columns as described previously (16). For experiments at acidic pH, HdeA and HdeB were equilibrated in distilled water by gel permeation on a Bio-Gel P10 column (BioRad) equilibrated in water. Periplasmic extracts from the wild-type strain, and from the hdeA mutant were prepared by the osmotic shock procedure described in (16). We performed electrophoresis according to Laemmli, using 16% polyacrylamide gels with Coomassie blue staining (25). We used NIH-Image 1.62 software (rsb.info.nih.gov/nih-image/Default/html) to quantify protein bands on polyacrylamide gels.

Chaperone assays and solubilization of protein aggregates. The chaperone activities of HdeA and HdeB were assayed by incubating periplasmic extracts from the hdeA mutant (deficient in both HdeA and HdeB (16)) for 60 min at 25°C in the presence of HdeA and/or HdeB at acidic pH, and then monitoring the appearance of the proteins (analyzed by SDS-PAGE) in the 15,000 x g pellet or supernatant. In order to test the solubilization of protein aggregates by acid-stress chaperones, we incubated periplasmic extracts from the hdeA mutant at either pH 3 or pH 2 for 60 min at 25°C, either alone or with purified HdeA, HdeB, or both together, followed by centrifugation for 10 min at 15,000 x g. The pH 3 or pH 2 pellets were resuspended and incubated in 100 mM Tris pH 8, 50 mM NaCl for 60 min at 25°C. Samples were centrifuged at 15,000 x g and the pH 8 pellets and supernatants were analyzed by SDS-PAGE. Sulfate (150 mM) was used as the anion permeation on a Bio-Gel P10 column (BioRad) equilibrated in 15,000 x g pellet or supernatant. The pH 3 or pH 2 pellets were centrifuged at 15,000 x g and the pH 8 pellets and supernatants were analyzed by SDS-PAGE. Sulfate (150 mM) was used as the anion permeation on a Bio-Gel P10 column (BioRad) equilibrated in water. Periplasmic extracts from the wild-type strain, and from the hdeA mutant were prepared by the osmotic shock procedure described in (16). We performed electrophoresis according to Laemmli, using 16% polyacrylamide gels with Coomassie blue staining (25). We used NIH-Image 1.62 software (rsb.info.nih.gov/nih-image/Default/html) to quantify protein bands on polyacrylamide gels.

Size of protein aggregates. Periplasmic extracts form the ΔhdeA strain (20 µg) were incubated at pH 3 for various times, ranging from 5 to 90 min, either in the absence or in the presence of HdeA and HdeB (10 µg each), in a total volume of 20 µl, and the samples were loaded onto a Bio-Gel A-15m column (1 ml bed volume, 25 cm height, flow rate 10 µl per min) equilibrated with 150 mM Na2SO4 pH 4.1 (we checked that at this pH, no significant protein aggregation or aggregate solubilization occurs). Proteins were detected by the Bradford assay, and analyzed by SDS-PAGE. Membrane vesicles (V0), Blue dextran (2 MDa), thyroglobuline (670 kDa), catalase (240 kDa), serum albumin (66 kDa), and cytochrome C (12.5 kDa) were used as molecular weight standards.

Bis-ANS labelling. Periplasmic extracts from the ΔhdeA strain (20 µg) were incubated for 60 min at pH 3, either in the absence or in the presence of HdeA and HdeB; 100 µM bis-ANS was then added, the samples were illuminated under UV light for 20 min and centrifuged for 10 min at 15,000 x g (20). Supernatants and pellets were analyzed by SDS PAGE: Fluorescent bands were photographed on a 340 nm transilluminator, and the gel was later stained with Coomassie blue. We used NIH-Image 1.62 software to quantify protein bands on polyacrylamide gels (quantification of fluorescent bands was done on the negative image of the gel). The ratio fluorescence / Coomassie blue staining reflects the exposed hydrophobicity of proteins. We checked, using different quantities of protein pellets without chaperones, that the intensity of fluorescence was proportional to that of the Coomassie blue staining).

Solubilization after acid stress of periplasmic protein aggregates in wild-type and ΔhdeA cells. Cultures of E. coli wild-type and ΔhdeA mutant strains were grown in LB medium at pH 7 to the beginning of the stationary phase, centrifuged and resuspended in LB medium pH 3 for 15 min, followed by centrifugation and resuspension in LB medium pH 7 for 0-60 min (all experiments were done at 37°C under agitation, and, by plating bacteria on LB agar plates after the 15 min acid stress, we checked that it did not affect the viability of cells). Spheroplasts were formed by the lysozyme-EDTA method : 40 ml of cells was centrifuged at 5,000 x g for 5 min, resuspended into 1 ml of 30 mM Tris pH 7.5, 20% sucrose, 10 mM EDTA at 20°C. After 1 min, 200 µl of 10 mg/ml lysozyme was added at once, and the whole was incubated for 5 min at 20°C followed by 20 min at 0°C.
For the global analysis of periplasmic proteins released by the spheroplasting procedure, periplasmic extracts (soluble and aggregated periplasmic proteins) were separated from spheroplasts by a short centrifugation for 1 min at 8,000 x g in a Beckman microfuge, and the supernatant was then centrifuged for 15 min at 15,000 x g. The 15,000 x g supernatants (soluble periplasmic proteins) and pellets (periplasmic protein aggregates) were analyzed by SDS-PAGE followed by Coomassie blue staining. This differential centrifugation procedure was mandatory for the global analysis of periplasmic protein aggregates. It missed, however, a fraction of periplasmic protein aggregates that had already pelleted during the first centrifugation step (unpublished results, and J.M. Betton, personal communication (27)). For the analysis of MalE, OppA, HisJ and MglB, samples were centrifuged for 10 min at 20,000 x g immediately after the spheroplasting procedure, and supernatants containing the periplasmic soluble proteins were withdrawn. Spheroplasts pellets were washed, resuspended in 10 mM Tris pH 7.5, freeze-thawed, ultrasonicated for 15 seconds and centrifuged at 20,000 x g for 15 min. Supernatants were discarded and pellets were washed with 10 mM Tris pH 7.5 and resuspended in the same buffer (this fraction contained the cell membranes and periplasmic protein aggregates) (27).

**Immunoblots.** Proteins from the various fractions were separated by SDS-PAGE. After electrophoresis, proteins were either stained with Coomassie blue or silver stain, or electrotransferred onto nitrocellulose membranes, which were incubated first with rabbit antiserum against MalE, OppA, MglB, and then with horse radish peroxidase-coupled antiserum against rabbit immunoglobulins. The immunoblots were developed with the Supersignal West Pico Trial Kit (Pierce). For quantitative analysis, gels and blots were scanned and quantified with NIH-Image 1.62 software.

**Binding assays of MalE, MglB and HisJ.** MalE and MglB were induced by adding 0.4% maltose and 1 mM fucose to the LB medium. Binding assays were done by filtration on nitrocellulose filters (Millipore HAWP, 0.45 µm) of a mixture containing 50 µl of a dialyzed periplasmic extract and one of the radioactive ligands (4 µM maltose, 320 mCi/mmol, 2 µM galactose, 170 mCi/mmol, 3 µM histidine, 420 mCi/mmol, from Amersham). The mixture was incubated for 5 min at 20°C, and 500 µl of a saturated ammonium sulfate solution at 0°C was added at once to the samples before filtration. Filters were rinsed three times with saturated ammonium sulfate at 0°C and counted for radioactivity (28).

**Reagents.** All chemicals were from Sigma and were reagent grade.
RESULTS

Co-precipitation of HdeA and HdeB with protein aggregates. In a previous report (16), we observed that a large proportion (approximately 90%) of HdeA and HdeB remained in the 15,000 x g supernatants after acid treatment of periplasmic extracts, thus allowing efficient protein solubilization, but that significant amounts (up to 10%) of these chaperones were found in pellets containing aggregated proteins that had escaped solubilization. In the following experiment, we tested whether the aggregation of HdeA and HdeB is dependent on their chaperone activity by analyzing their solubility at different chaperone/substrate stoichiometric ratios. We incubated increasing amounts of periplasmic extracts from the hdeA disrupted mutant (containing neither HdeA nor HdeB (16)), in the presence of a constant amount of either HdeA at pH 2 or HdeB at pH 3. As reported previously (14-16), the presence of these acid-stress chaperones markedly decreases the aggregation of periplasmic extracts: In the absence of any chaperones, the quantity of protein aggregates at pH 2 was 2.4-fold higher than in the presence of HdeA (mean value of the three couples of lanes shown in Figure 1A) and the quantity of protein aggregates at pH 3 was 4.5-fold higher than in the presence of HdeB (mean value of the three couples of lanes shown in Figure 1B). These quantities of aggregated proteins in the absence of chaperones represent up to 60% of the whole amount of periplasmic proteins (not shown), as reported previously (16). Furthermore, HdeA and HdeB solubilize roughly the same proteins, as observed previously (16).

Observation of the HdeA and HdeB bands in Figure 1A and 1B, respectively, shows that increasing amounts of periplasmic extract draw increasing amounts of HdeA and HdeB into the pellet. Whereas in the absence of periplasmic extract, less than 1% of HdeA and HdeB was found in the pellet, this amount rises to 6-8% in the presence of increasing amounts of periplasmic extracts. These amounts were quantified with NIH 1.62 software and plotted as a function of the amount of periplasmic extract added to the incubation mixture; they increased up to six-fold with increasing protein substrate (Figure 1D). We compared the amounts of HdeA and HdeB drawn into the pellet by aggregated proteins with those of the aggregated proteins (we quantified the proteins shown in the second lane in Figure 1A and 1B. HdeA and HdeB represent 8% (w/w) and 6% (w/w), respectively, of the quantities of aggregated substrate proteins. If we estimate, for these aggregated proteins, a mean molecular weight of 60 kDa, the amounts of HdeA (molecular weight, 9 kDa) and HdeB (molecular weight, 9 kDa) drawn into the pellet represent 60% and 40% (mol/mol), respectively, of the amount of aggregated substrate protein. Similar experiments were performed at pH 3 in the presence of both HdeA and HdeB, and increasing amounts of both chaperones were also drawn into the pellets by increasing amounts of aggregated periplasmic extracts (Figure 1C). In this experiment chaperones represented 17% (w/w), i.e. 108% (mol/mol) of the quantity of aggregated substrate proteins. Thus, stoichiometric amounts of HdeA and HdeB are drawn into the pellet by unsolubilized substrate proteins, suggesting that HdeA and HdeB function as chaperones in insoluble protein complexes (see below). It can be noticed that, in vivo, HdeA and HdeB represent up to 8% (w/w) and 5%. (w/w), respectively, of total periplasmic protein (as estimated from the 2D gel analysis of periplasmic extracts from stationary phase cells (not shown).

Efficient HdeA- and HdeB-assisted solubilization at neutral pH of pellets formed in their presence at pH 3. First, periplasmic extracts from the hdeA mutant (deficient in both HdeA and HdeB) were incubated at pH 3 for 60 min, either alone or in the presence of HdeA, HdeB, or both together. These extracts were centrifuged at 15,000 x g and pH 3 pellets were resuspended and incubated in 30 mM Tris pH 8, 50 mM NaCl for 60 min; samples were then centrifuged at 15,000 x g, and pH 8 pellets and supernatants were analyzed by SDS-PAGE (Figure 2A). In the absence of any chaperone, a large number of periplasmic proteins were found in the pH 3 pellet (lane 0), as reported previously, and 58% of these aggregated proteins remained insoluble (42% were solubilized) after the neutralization step (pH 8 pellet, lane 0). In contrast, when the acid treatment was carried out in the presence of HdeA (lanes A), HdeB (lanes B) or both together (lanes AB), not only were there fewer proteins in the pH 3 pellet (as reported previously (16)), but also the majority (66%, 64% and 92%, respectively) of the aggregated proteins could be solubilized at pH 8, and were found in the pH 8 supernatant. The combination of HdeA and HdeB together was the most efficient for this solubilization. Similar results were obtained using a pH 7 (instead of pH 8) resolubilization buffer (not shown), and no improvement in the solubilization of
protein aggregates was observed in the presence of 1mM ATP, 5 mM MgCl₂ (not shown).

We also studied the solubilization at neutral pH of pellets formed in the presence of a constant amount of periplasmic extract (25 μg) and increasing amounts of HdeA and HdeB both together (0, 4, 8, 12 μg each). As shown in Figure 2B, 2C, large amounts of both chaperones (10-15 μg each) are required for an efficient solubilization of protein aggregates. The necessity for such large amounts of chaperones for the solubilization of protein aggregates can be explained by the fact that the majority of these chaperones are committed to the solubilization of the proteins that remain in the pH 3 supernatant. Thus, HdeA, HdeB, and HdeA and HdeB together not only maintained proteins in a soluble form at pH 3 (by acting as soluble chaperones), but also helped in the solubilization at neutral pH of proteins that had aggregated in their presence at pH 3 (acting as chaperones in insoluble protein complexes).

**Inability of HdeA and HdeB to solubilize protein aggregates when added after the aggregation step.** We checked whether HdeA and HdeB could solubilize at neutral pH, protein aggregates formed in their absence at acidic pH. Several identical samples of a periplasmic extract from the hdeA mutant were incubated at pH 3 for 60 min. They were centrifuged at 15,000 x g and the pH 3 pellets were resuspended for 60 min at pH 3, either alone or in the presence of HdeA, HdeB, or both together. The samples were centrifuged, the 15,000 x g pellets were resuspended in 100 mM Tris pH 8, 50 mM NaCl, incubated for 60 min at room temperature, centrifuged at 15,000 x g, and the pH 8 pellets were analyzed by SDS-PAGE. The quantities of protein remaining in the pH 8 pellets were similar independently of the presence of HdeA or/and HdeB (not shown). Similar negative results were obtained when the chaperones were added during the pH 8 resuspension step (not shown). Thus, HdeA and HdeB have to co-aggregate with their substrate proteins at acidic pH in order to function as chaperones for the disruption of these aggregates at neutral pH.

**Limited HdeA- and HdeB-assisted solubilization at neutral pH of pellets formed in their presence at pH 2.** Similar experiments were done after treatment of periplasmic extracts at pH 2. In the absence of any chaperone, a large number of periplasmic proteins aggregated, as reported previously (15, 16), and the majority (76%) of these proteins remained insoluble (pH 8 pellet) after the neutralization step (Figure 3A). In the presence of HdeA or HdeB, fewer proteins aggregated at pH 2, as previously reported (16), but only a small amount of aggregated protein could be solubilized at neutral pH (20% and 30%, respectively) (Figure 3A). HdeB did not significantly prevent protein aggregation at pH 2, as previously reported (16). It did allow, however, an improved solubilization at neutral pH of several proteins that had aggregated in its presence at pH 2; the corresponding pH 8 supernatant (supernatant B) contains a higher amount of protein (40% of the pH 2 pellet) than the control supernatant (supernatant 0) which contains only 20% of the pH 2 pellet (Figure 3). These results show that only HdeB helps, albeit moderately in the solubilization of pH 2 pellets. The negligible efficiency of HdeA, and moderate efficiency of HdeB in the solubilization of pellets that had formed in their presence at pH 2 contrasts with their efficiency in the solubilization of pH 3 pellets.

**Decrease in the size of protein aggregates formed in the presence of HdeA and HdeB.** When periplasmic extracts were incubated at pH 3 (5 to 90 min) in the absence of chaperone and then analyzed by filtration on a gel permeation A-15m column (exclusion limit 15,000 kDa) equilibrated at pH 4.1 (a pH at which no significant protein aggregation or aggregate solubilization occurs), less than 30% of the proteins eluted in the low molecular weight fractions centered around 35 kDa, representing proteins that remain soluble at pH 3 (Figure 4A). We did not observe any intermediate molecular weight complex (in the range 200-15,000 kDa), and most of the proteins remained at the top of the column in the form of high molecular weight aggregates that could not enter the gel (these aggregates from the 60 min experiment were visualized on polyacrylamide gels by collecting resin from the top of the column and boiling it in 1% SDS containing buffer (22) (Figure 4B, lane 4)). Thus, in the absence of chaperones, only small quantities of periplasmic proteins remain soluble for more than 1 hour, while the majority form high molecular weight aggregates that do not enter the gel.

In contrast, when periplasmic extracts were incubated at pH 3 in the presence of HdeA and HdeB, intermediate size protein complexes were detectable (100-5000 kDa) (Figure 4A). For short incubation times (5 min), most of the proteins eluted in the peak centered around 35 kDa. At increased
incubation times (60 and 90 min), most of the proteins eluted as complexes of 100-3000 kDa, and the size of these complexes increased with time, up to 2.5 MDa for a 60 min incubation time and 4-5 MDa for a 90 min incubation time. These complexes contained both substrate proteins and chaperones: the complexes from the 90 min experiments are shown in Figure 4B, lane 1 (tubes 7-10, 90 min incubation time), lane 2 (tubes 13-14, 90 min incubation time) (we quantified the proteins shown in the first lane in Figure 4B. HdeA and HdeB represent 35% (w/w), i.e. around 210% (mol/mol) of the quantity of aggregated substrate proteins; moreover, a run of the sample displayed in lane 1 on a 18% polyacrylamide gel (not shown) showed that both HdeA and HdeB (which migrates slightly more slowly than HdeA) are present in this fraction in a ratio HdeA/HdeB of around 65% / 35% (the expression ratio (measured by transcript levels) of HdeA and HdeB in E. coli cells grown in minimal medium with glycerol as carbon source is 82%/18% (Echobase, http://www.york.ac.uk/res/thomas/)). A small amount of proteins (around 15%) formed high molecular weight aggregates that did not enter the gel, and contained a small amount of chaperone (Figure 4B, lane 3).

We also performed these gel permeation experiments with a constant amount of periplasmic extract (20 μg as above), and different amounts of both chaperones (from 10 μg each as above, to 5 μg and 2 μg each). Large amounts of both chaperones (10 μg each) were required for the efficient solubilization of protein aggregates. With lower amounts, the intermediate molecular weight peaks (240-4,500 kDa) decreased dramatically (not shown).

Thus, HdeA and HdeB decrease the size of the protein aggregates formed at acidic pH, and allow the formation of intermediate size protein complexes (100-5000 kDa) that contain both chaperones and substrate proteins.

Decrease of Bis-ANS labelling of protein aggregates containing acid stress chaperones. The fluorescent probe 1,1’-bis(4-anilino)napthalene-5,5’-disulfonic acid (bis-ANS) has been used extensively to demonstrate the presence of hydrophobic sites on the surface of proteins. In a previous study, bis-ANS was shown to incorporate covalently into the hydrophobic domain of the molecular chaperone GroEL when exposed to UV light (31). Using bis-ANS labelling, we evaluated the exposed hydrophobicity of the acid-stress chaperones and of their protein substrates, both in 15,000 x g pellets and supernatants.

Periplasmic extracts were incubated for 60 min at pH 3, either in the absence or in the presence of HdeA and HdeB; 100 μM bis-ANS was then added, and the samples were illuminated under UV light for 20 min. They were centrifuged for 10 min at 15,000 x g, and supernatants and pellets were analyzed by SDS PAGE: Fluorescent bands were photographed on a 340 nm transilluminator, and the gel was later stained with Coomassie blue. The ratio fluorescence / Coomassie blue staining reflects the exposed hydrophobicity of proteins.

Coomassie blue staining of periplasmic extracts exposed to pH 3 either alone or in the presence of HdeA and HdeB (Figure 5A) shows that the presence of chaperones decreases the amount of protein in the pellet, and increases the amount of protein in the supernatant, as previously reported (16). This reflects the known chaperone properties of HdeA and HdeB at acidic pHs (14-16, 30).

Interestingly, the fluorescence of the HdeA/HdeB-containing pellet is much less intense than that of the pellet without chaperones (Figure 5B, negative image of ANS fluorescence). Quantification of protein bands and fluorescence intensities in pellets show that chaperone-containing pellets display 62% of the amount of protein of control pellets and exhibit 32% of their fluorescence intensity (Figure 5A, 5B). This suggests that acid-stress chaperones significantly decrease the exposed hydrophobicity of aggregated periplasmic proteins (a faint fluorescent band could be seen at the position of the HdeAB chaperones in the AB pellet lane (arrow), which became bright if the gels were overexposed (not shown)).

The fluorescence of the supernatants was far less intense than that of the pellets, and we had to overexpose the supernatants with regard to the pellets displayed in Figure 5B (under the same exposition as that used for the pellets, the chaperone band was the sole detectable band (not shown)). This suggests that soluble periplasmic proteins expose fewer hydrophobic surfaces than aggregated ones (protein aggregation is generally believed to result from an interaction between exposed hydrophobic surfaces).

We performed these bis-ANS labelling experiments in the presence of a constant amount of periplasmic extract (20 μg) and different amounts of both chaperones (10, 5, 2 μg, each), and to obtain an efficient decrease in the hydrophobicity of pellet proteins, large amounts of each chaperone were necessary (10 μg each) (not shown).
The decreased hydrophobicity of the chaperone-containing pellet described above likely explains the efficient solubilization of protein aggregates at neutral pH.

**In vivo solubilization of protein aggregates by the acid stress chaperones.** To obtain evidence that HdeA and HdeB also facilitate the solubilization of periplasmic protein aggregates in vivo, we monitored the kinetics of periplasmic protein disaggregation at neutral pH after acid stress treatment of *E. coli* wild-type cells, and ΔhdeA cells that lacked both HdeA and HdeB. Early stationary phase cells grown in LB medium at pH 7 were incubated at pH 3 for 15 min (a sub-lethal acid stress), followed by centrifugation, resuspension and incubation in LB medium pH 7 for various times in the presence of 20 µg/ml chloramphenicol. Periplasmic proteins were prepared as a lysozyme-EDTA spheroplast supernatant (spheroplasts were centrifuged for 1 min at 8,000 x g), which was then centrifuged for 10 min at 15,000 x g in order to separate aggregates and soluble proteins. There was a greater quantity (1.3-fold more) of periplasmic protein aggregates in the chaperone-deficient strain, reflecting the chaperone activities of HdeA and HdeB in vivo (Figure 6A). Furthermore, there was little protein disaggregation at neutral pH in the chaperone-deficient strain for up to 60 min, whereas, in wild-type cells, the amounts of several aggregated proteins (especially bands a, b, c) decreased with a half-time of around 20 min (Figure 6A, 6B). This decrease correlated with an increase in the amount of these proteins in the supernatant, whereas no such increase was observed in the chaperone-deficient mutant (Figure 6C, 6D). Since this experiment was done in the presence of chloramphenicol, it likely reflects the HdeA-, HdeB-dependent solubilization of aggregated proteins after acid stress.

We also analyzed, during the recovery of cells from acid stress, the behaviour of several periplasmic proteins including the oligopeptide receptor OppA, the maltose receptor MalE, the galactose receptor MglB and the histidine receptor HisJ. These proteins were detected by immunoblotting (OppA, MalE and MglB) and/or ligand binding (MalE, MglB and HisJ). Thanks to the immunodetection of these periplasmic proteins, we were able to avoid the differential centrifugations described above and we directly centrifuged spheroplasts for 10 min at 20,000 x g. Supernatants contained soluble periplasmic proteins, and pellets which contained both spheroplasts and aggregated periplasmic protein were treated as described under « Experimental Procedures » (27). As shown in Figure 7A, 7B and 7C smaller fractions of MalE and OppA aggregated in the wild-type strain (30% and 42% of the whole, respectively) than in the HdeAB deficient strain (50% and 85%, respectively). Furthermore, in the wild-type strain, MalE and OppA aggregates disappeared with half-lives of around 20 min and 30 min, respectively, and there was a corresponding increase in the amounts of soluble OppA and MalE (these experiments were carried out in the presence of chloramphenicol). This suggests that OppA and MalE aggregates are solubilized rather than degraded during the recovery stage at neutral pH. In contrast, in the hdeA strain, aggregates decreased only slightly after transfer of bacteria to neutral pH (with half times of several hours). Furthermore, there was no significant increase in the amounts of soluble OppA or MalE. In contrast with OppA and MalE, the galactose receptor MglB did not significantly aggregate during acid stress (not shown).

We also measured the ligand binding activities of the maltose receptor MalE and of the histidine receptor HisJ in periplasmic supernatants from the hdeA mutant and from the control strain as a function of the recovery time after acid stress. As shown in Figure 7D, the maltose and histidine binding activities of the wild-type strain periplasm increased as a function of the recovery time whereas those of the mutant strain periplasm remained constant and low. These experiments suggest that the MalE and HisJ receptors of the HdeA-, HdeB-containing strain are not only solubilized, but also renatured during the recovery step at neutral pH. These experiments suggest that HdeA and HdeB are efficient in vivo for the solubilization and renaturation of protein aggregates after acid stress.
**DISCUSSION**

In this report, we show that HdeA and HdeB not only help to maintain proteins in a soluble state during acid treatment, as previously reported (14-16), but also promote the solubilization at neutral pH of proteins that had aggregated in their presence at acidic pHs. Whereas the major fraction of HdeA and HdeB remains in a soluble form at acidic pH, increasing amounts of these chaperones co-aggregate with increasing amounts of substrate protein, thereby promoting the subsequent resolubilization of the latter at neutral pH. Although the chaperone / substrate protein ratios (w/w) in the pellets were low (7-35%), the stoichiometric ratios (mol/mol) ranged from 0.5 to 2 (since acid stress chaperones have a molecular weight of 9 kDa, compared to a mean molecular weight of around 60 kDa for aggregated proteins). These values are in the range of the chaperone/substrate ratios for the soluble chaperones Hsp70, Hsp31 (31-32) and HdeA (HdeA efficiently prevents alcohol dehydrogenase aggregation at a chaperone/substrate ratio of 0.4 (15)), and of chaperone/substrate ratios in mixed protein aggregates containing small Hsps (the molar ratios of Hsp25/citrate synthase subunits reported in ref. 24 range from 1 to 4), and the Hsp18/malate dehydrogenase ratios reported in ref. 20 range from 1.6 to 3).

In addition to their role as soluble chaperone (14-16), HdeA and HdeB form mixed aggregates with proteins that have failed to be solubilized. Small Hsps from eukaryotes (Hsp16, Hsp18 and Hsp 26 (17, 21) and prokaryotes (IbpA and IbpB both together (22)) form mixed aggregates with thermally aggregated proteins and stabilize them in a disaggregation and refolding competent state, allowing disaggregation of these small Hsp-containing aggregates by the bichaperone Hsp70-Hsp100 system (21).

In the presence of HdeA and HdeB, we observed a decrease in the size of protein aggregates formed by periplasmic extracts at pH 3 with the formation of intermediate size (100-5000 kDa) protein complexes. In some cases (21, 22), but not in all (20, 21) small Hsps have been shown to decrease the size of protein aggregates that form a high temperatures; Hsp16 and Hsp18 decrease malate dehydrogenase aggregates formed at 47°C, from 24,000 kDa to 2,100 kDa (21). The small size of protein aggregates is a positive factor for their solubilization (at least since they have a higher surface/volume ratio), and small protein aggregates are more easily refolded by the DnaK chaperone (33). The exposed hydrophobicity of periplasmic proteins in aggregates is decreased by the presence of HdeA and HdeB, as shown by bis-ANS labelling experiments. Since protein aggregation relies mainly on hydrophobic forces, such a decrease should be favourable for the solubilization of protein aggregates at neutral pH. It has often been reported (20-21) that the hydrophobicity of thermally aggregated substrate proteins is not decreased by the presence of sHsps (but see 33 for a positive report of experiments not shown). It is generally suggested that small Hsps assist in a faster solubilization of protein aggregates by the Hsp100-Hsp70 bichaperone machine, by maintaining aggregated proteins in a (poorly defined) folding competent state (20-21). In our study, the decreased exposed hydrophobicity of aggregated proteins in the presence of chaperones likely corresponds to a solubilization competent state.

HdeA and HdeB also facilitate the solubilization of periplasmic protein aggregates in vivo, since we observed an impaired solubilization of periplasmic protein aggregates in the chaperone-deficient mutant after its transfer to neutral pH. We were able to demonstrate that, for several periplasmic proteins (the maltose receptor MalE, the oligopeptide receptor OppA, the histidine receptor HisJ), the decrease in the amount of protein aggregates during the recovery step after acid stress correlates with an increase in the amount of the soluble and active form of the receptors, and indeed represents an aggregate solubilization process, especially since our recovery experiments were carried out in the presence of chloramphenicol to inhibit protein synthesis. In the case of the Hsp100/Hsp70/small Hsp chaperone network, data from both in vitro and in vivo experiments argue in favour of its role in protein solubilization after thermal stress (17-24).

For mixed protein aggregates containing small Hsps, no significant protein disaggregation occurs in the absence of the Hsp100-Hsp70 bichaperone system, and disaggregation requires at least Hsp100 or Hsp70 (17, 21, 24). Small Hsps, Hsp100 and Hsp70 form a triad for the resolubilization and renaturation of protein
aggregates, with small Hsps involved in the weakening of protein aggregates, Hsp100 in active disaggregation and Hsp70 in disaggregation and refolding (17, 21, 24). Mixed protein aggregates containing HdeA and HdeB, however, can be solubilized at neutral pH in vitro without any help from ATP-dependent chaperones (HdeA and HdeB function in the bacterial periplasm which is devoid of any ATP-dependent chaperone). Thus, the presence of HdeA and HdeB in mixed protein aggregates weakens these aggregates to such an extent that their solubilization at neutral pH occurs in the absence of any additional chaperone. It remains possible, however, that in vivo, other periplasmic chaperones, disulfide isomerases or/and peptidyl prolylisomerases (34) help in the solubilization and renaturation of aggregated proteins after acid stress.

ACKNOWLEDGEMENTS. The authors thank Dr. Hirotada Mori (Nara Institute of Sciences and Technology, Nara, Japan) for the construction of the hdeA-disrupted strain, Dr. Jean-michel Betton (Unité de Biochimie Structurale, Institut Pasteur, Paris) for helpful advices concerning periplasmic protein aggregates, Antonia Kropfinger for correction of the English language, and Myriam Barre for her help in preparation of the manuscript.

REFERENCES


LEGENDS TO FIGURES

**Figure 1. Substrate-dependent HdeA and HdeB aggregation.** Increasing amounts of periplasmic extracts (12, 24, 36 μg) (0 μg in the first lanes) from the *hdeA*-deficient strain (which expresses neither HdeA nor HdeB) were incubated for 60 min, A) at pH 2 in the absence or in the presence of a constant amount of HdeA (15 μg), B) at pH 3 in the absence or in the presence of a constant amount of HdeB (15 μg), C) at pH 3 in the absence or in the presence of a constant amount of HdeA and HdeB (10 μg each), and 15,000 x g pellets were analyzed on SDS polyacrylamide gels. D) The amounts of HdeA (circles), HdeB (squares) or HdeA and HdeB together (triangles) in pellets were quantified using NIH 1.62 software, and plotted against the amount of periplasmic extract added to the incubation mixture.

**Figure 2. HdeA- and HdeB-assisted solublization at neutral pH of pellets formed in their presence at pH 3.** A) Periplasmic extracts (25 μg) from the *hdeA* mutant were incubated at pH 3 for 60 min, either alone or with HdeA (15 μg), HdeB (15 μg), or HdeA and HdeB together (7.5 μg each). The 15,000 pellets (pH 3 pellets) were isolated, resuspended and incubated for 60 min in pH 8 buffer, and samples were centrifuged at 15,000 x g, giving pH 8 pellets and pH 8 supernatants. B) Periplasmic extracts (25 μg) from the *hdeA* mutant were incubated at pH 3 for 60 min, in the presence of increasing amounts of HdeA and HdeB (0, 4, 8, 12 μg each). The 15,000 x g pellets (pH 3 pellets) were isolated, resuspended and incubated for 60 min in pH 8 buffer, and samples were centrifuged at 15,000 x g, giving pH 8 pellets and pH 8 supernatants. C) We plotted the ratio of the amount of protein found in the pH 8 supernatant to that found in the pH 8 pellet as a function of the relative amount of acid stress chaperone added in the experiment described in Figure 2B. D) Inability of HdeA and HdeB to solubilize at neutral pH the protein pellets formed in their absence at pH 3. Four samples of a periplasmic extract from the *hdeA* mutant (25 μg each) were incubated at pH 3 for 60 min. The 15,000 x g pellets (pH 3 pellets) were isolated, resuspended and incubated for 60 min at pH 3, either alone or with HdeA (15 μg), HdeB (15 μg), or HdeA and HdeB together (7.5 μg each). The 15,000 x g pellets were isolated and resuspended in 100 mM Tris pH 8, 50 mM NaCl. Samples were incubated for 60 min, centrifuged at 15,000 x g, and pH 8 pellets were analyzed by SDS-PAGE.

**Figure 3. HdeA- and HdeB-assisted solublization at neutral pH of pellets formed in their presence at pH 2.** Periplasmic extracts (25 μg) from the *hdeA* mutant were incubated at pH 2 for 60 min, either alone or with HdeA (15 μg), HdeB (15 μg), or HdeA and HdeB together (7.5 μg each). The 15,000 x g pellets (pH 2 pellets) were isolated, resuspended and incubated for 60 min in pH 8 buffer, and samples were centrifuged at 15,000 x g, giving pH 8 pellets and pH 8 supernatants.

**Figure 4. Size of protein aggregates.** Periplasmic extracts from the *hdeA* mutant (20 μg) were incubated at pH 3 for 5 min (circles), 60 min (squares) or 90 min (triangles) in the absence (empty symbols) or in the presence (filled symbols) of HdeA and HdeB (10 μg each), in a total volume of 20 μl. Samples were adjusted to pH 4.1 and loaded onto a Biogel A 15-m column (1 ml bed volume, flow rate, 10 μl per min) equilibrated with 150 mM Na₂SO₄, pH 4.1. A) Elution profile of proteins detected by the Bradford test. Membrane vesicles (Vₐ), Blue dextran (2 MDa), thyroglobulin (670 kDa), catalase (240 kDa), serum albumin (66 kDa), and cytochrome C (12,5 kDa) were used as molecular weight standards. HdeA and
HdeB, either alone or both together, migrate in fractions 16-19 after incubation at pH 3 for 60 min, (not shown). B) SDS-PAGE analysis of column fractions: fractions 7-10 (lane 1), fractions 13-14 (lane 2) from periplasmic extracts incubated for 90 min in the presence of HdeA and HdeB; aggregates extracted by boiling in 1% SDS the top of a column loaded with 20 μg of a periplasmic extract incubated for 90 min in the presence of chaperones (10 μg each) (lane 3) or with 20 μg of a periplasmic extract incubated in the absence of chaperones (lane 4). The gel was silver stained.

Figure 5. Bis-ANS labelling. Periplasmic extracts from the hdeA mutant (20 μg) were incubated for 60 min at pH 3, either in the absence or in the presence of HdeA and HdeB (10 μg each); 100 μM bis-ANS was then added, the samples were illuminated under UV light for 20 min and centrifuged for 10 min at 15,000 x g. Supernatants and pellets were analyzed by SDS-PAGE. Fluorescent bands were photographed on a 340 nm transilluminator, and the negative image of ANS fluorescence is shown in (B); the gel, which was then stained with Coomassie blue, is displayed in (A). We quantified the protein bands of the polyacrylamide gel using NIH-Image 1.62 software and display the whole amount of protein and of ANS fluorescence below each pellet. The Bis-ANS labelling experiment was repeated three times and gave similar results ±8%. In contrast with OppA and MalE, the galactose receptor MglB did not significantly aggregate during acid stress (not shown). The fluorescent image of the supernatants is overexposed compared with that of the pellets.

Figure 6. Impaired resolubilization after acid stress of periplasmic protein aggregates in ΔhdeA cells. Wild-type and ΔhdeA strains were grown in LB medium at pH 7. Bacteria were centrifuged and resuspended in LB medium pH 3 for 15 min, followed by centrifugation and resuspension in LB medium pH 7 containing 20 μg/ml chloramphenicol for 0-60 min. Bacteria were transferred on ice at the indicated times, and converted to spheroplasts as described under « Experimental Procedures ». The spheroplast supernatants (obtained after centrifugation of spheroplasts for 1 min at 8,000 x g) are supposed to contain both the soluble periplasmic proteins and a fraction of the aggregated periplasmic proteins. Aggregated (A) and soluble (C) periplasmic proteins were separated by centrifugation (15,000 x g for 10 min) and analyzed by SDS-PAGE followed by silver staining. B) The amount of protein in bands a, b, and c of Figure 6A was quantified with NIH-Image 1.62 software, and plotted as a function of the recovery time at pH 7. D) The amount of protein in bands b, c and d of Figure 6C was quantified with NIH-Image 1.62 software, and plotted as a function of the recovery time at pH 7.

Figure 7. Impaired resolubilization after acid stress of MalE, OppA and HisJ aggregates in ΔhdeA cells. Wild-type and ΔhdeA strains were grown in LB medium at pH 7. Bacteria were centrifuged and resuspended in LB medium pH 3 for 15 min, followed by centrifugation and resuspension in LB medium pH 7 containing 20 μg/ml chloramphenicol for 0-90 min. Bacteria were transferred on ice at the indicated times, and converted to spheroplasts as described under « Experimental Procedures ». The spheroplast supernatants (obtained after centrifugation of spheroplasts for 10 min at 20,000 x g) contained soluble periplasmic proteins. Aggregated periplasmic proteins were recovered from the spheroplast pellet as described under « Experimental Procedures ». A) Soluble and aggregated periplasmic proteins were separated by SDS-PAGE, electrotransferred onto nitrocellulose membranes and detected with antisera against OppA and MalE. B) OppA and C) MalE in the supernatants (S) and pellets (P) of Figure 7A were quantified as described under « Experimental Procedures », and their amounts are shown as a function of the recovery time at pH 7. All the pellets and supernatants represent identical amounts of bacteria. D) The periplasmic supernatants displayed in Figure 7A were tested for maltose (squares) and histidine (circles) binding activities as described under « Experimental Procedures ». The binding activities of the supernatants from the wild type (filled symbols) and ΔhdeA (empty symbols) strains are shown as a function of the recovery time at pH 7.
Figure 1
Figure 2
% of the pH 2 pellet proteins in the pH 8 pellet or supernatant

Figure 3
Figure 4
Figure 5
Figure 6
Figure 7