

Video Article

Detection of the pH-dependent Activity of *Escherichia coli* Chaperone HdeB *In Vitro* and *In Vivo*

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Abstract

Bacteria are frequently exposed to environmental changes, such as alterations in pH, temperature, redox status, light exposure or mechanical force. Many of these conditions cause protein unfolding in the cell and have detrimental impact on the survival of the organism. A group of unrelated, stress-specific molecular chaperones have been shown to play essential roles in the survival of these stress conditions. While fully folded and chaperone-inactive before stress, these proteins rapidly unfold and become chaperone-active under specific stress conditions. Once activated, these conditionally disordered chaperones bind to a large number of different aggregation-prone proteins, prevent their aggregation and either directly or indirectly facilitate protein refolding upon return to non-stress conditions. The primary approach for gaining a more detailed understanding about the mechanism of their activation and client recognition involves the purification and subsequent characterization of these proteins using *in vitro* chaperone assays. Follow-up *in vivo* stress assays are absolutely essential to independently confirm the obtained *in vitro* results.

This protocol describes *in vitro* and *in vivo* methods to characterize the chaperone activity of *E. coli* HdeB, an acid-activated chaperone. Light scattering measurements were used as a convenient read-out for HdeB's capacity to prevent acid-induced aggregation of an established model client protein, MDH, *in vitro*. Analytical ultracentrifugation experiments were applied to reveal complex formation between HdeB and its client protein LDH, to shed light into the fate of client proteins upon their return to non-stress conditions. Enzymatic activity assays of the client proteins were conducted to monitor the effects of HdeB on pH-induced client inactivation and reactivation. Finally, survival studies were used to monitor the influence of HdeB's chaperone function *in vivo*.

Video Link

The video component of this article can be found at <https://www.jove.com/video/54527/>

Introduction

A common natural environment in which microbial pathogens experience acid-induced protein unfolding conditions is the mammalian stomach (pH range 1-4), whose acidic pH serves as an effective barrier against food-borne pathogens¹. Protein unfolding and aggregation, which is caused by amino acid side chain protonation, affects biological processes, damages cellular structures and eventually causes cell death^{1,2}. Since the pH of the bacterial periplasm equilibrates almost instantaneously with the environmental pH due to the free diffusion of protons through the porous outer membrane, periplasmic and inner membrane proteins of Gram-negative bacteria are the most vulnerable cellular components under acid-stress conditions³. To protect their periplasmic proteome against rapid acid-mediated damage, Gram-negative bacteria utilize the acid-activated periplasmic chaperones HdeA and HdeB. HdeA is a conditionally disordered chaperone^{4,5}. At neutral pH, HdeA is present as a folded, chaperone-inactive dimer. Upon a pH shift below pH 3, HdeA's chaperone function is quickly activated^{6,7}. Activation of HdeA requires profound structural changes, including its dissociation into monomers, and the partial unfolding of the monomers⁶⁻⁸. Once activated, HdeA binds to proteins that unfold under acidic conditions. It effectively prevents their aggregation both during the incubation at low pH as well as upon pH neutralization. Upon return to pH 7.0, HdeA facilitates the refolding of its client proteins in an ATP-independent manner and converts back into its dimeric, chaperone-inactive conformation⁹. Similarly, the homologous chaperone HdeB is also chaperone-inactive at pH 7.0. Unlike HdeA, however, HdeB's chaperone activity reaches its apparent maximum at pH 4.0, conditions under which HdeB is still largely folded and dimeric¹⁰. Moreover, further lowering the pH causes the inactivation of HdeB. These results suggest that despite their extensive homology, HdeA and HdeB differ in their mode of functional activation allowing them to cover a broad pH range with their protective chaperone function. One other chaperone that has been implicated in the acid resistance of *E. coli* is the cytoplasmic Hsp31, which appears to stabilize unfolded client proteins until neutral conditions are restored. The precise mode of Hsp31's action, however, has remained enigmatic¹². Given that other enteropathogenic bacteria such as *Salmonella* lack the *hdeAB* operon, it is very likely that other yet unidentified periplasmic chaperones might exist that are involved in acid resistance of these bacteria¹¹.

The protocols presented here allow to monitor the pH-dependent chaperone activity of HdeB *in vitro* and *in vivo*¹⁰ and can be applied to investigate other chaperones such as Hsp31. Alternatively, the complex network of transcription factors that control the expression of *hdeAB* can potentially be investigated by the *in vivo* stress assay. To characterize the chaperone function of proteins *in vivo*, different experimental setups can be applied. One route is to apply protein unfolding stress conditions and phenotypically characterize mutant strains that either overexpress the gene of interest or carry a deletion of the gene. Proteomic studies can be conducted to identify which proteins no longer aggregate under stress conditions when the chaperone is present, or the influence of a chaperone on a specific enzyme can be determined during stress conditions using enzymatic assays¹⁴⁻¹⁶. In this study, we chose to overexpress HdeB in an *rpoH* deletion strain, which lacks the heat shock sigma factor 32. RpoH controls the expression of all major *E. coli* chaperones and its deletion is known to increase sensitivity to environmental stress conditions that cause protein unfolding¹⁵. The *in vivo* chaperone activity of HdeB was determined by monitoring its ability to suppress the pH sensitivity of the $\Delta rpoH$ strain. Altogether, the protocols presented here provide a fast and straightforward approach to characterize the activity of an acid-activated chaperone *in vitro* as well as in the *in vivo* context.

Protocol

1. Expression and Purification of Periplasmic HdeB

NOTE: HdeB was expressed in *E. coli* cells harboring the plasmid pTrc-*hdeB*¹⁰, and purified from the periplasm upon polymyxin lysis.

1. Prepare an overnight culture of *E. coli* cells harboring the plasmid pTrc-*hdeB*¹⁰ in 30 ml LB containing 200 μ g/ml ampicillin (LB_{Amp}). Inoculate four 1 L cultures of LB_{Amp} and grow them at 37 °C and 200 rpm until O.D._{600nm} of 0.7 is reached. Then, add 300 μ M IPTG to induce the expression of HdeB and decrease the growth temperature to 30 °C.
2. After 5 hr of protein expression at 30 °C, harvest the cells by centrifugation at 8,000 x g for 5 min at 4 °C.
3. Wash the cell pellet with 100 ml buffer A (50 mM Tris/HCl, 50 mM NaCl, pH 7.5) and centrifuge the cells again at 8,000 x g for 5 min at 4 °C.
4. Subsequently, resuspend the cell pellet in 80 ml buffer A, containing 1 mg/ml polymyxin sulfate. For efficient disruption of the outer membrane, gently stir the suspension for 1 hr at 4 °C.
5. To remove the cytoplasmic fraction and cell debris, centrifuge the suspension for 20 min at 15,000 x g at 4 °C. This results in ~60 ml supernatant containing the soluble HdeB.
6. Dialyze the supernatant containing the periplasmic extract overnight against 150x volume of buffer B (20 mM Tris/HCl, 0.5 mM EDTA, pH 8.0) using a dialysis membrane with 6 kDa MW cut-off. Concentrate the proteins to 15 ml using centrifugal filter units with a molecular weight cut-off of 3 kDa. Filter the protein solution using a 0.2 μ m pore filter.
7. Apply the protein onto an anion exchange chromatography column (column volume 5 ml) that has been equilibrated with 5 column volumes buffer B with a flow rate of 2.5 ml/min. Once the protein is loaded onto the column, wash the column with buffer B for 10 min at a flow rate of 2.5 ml/min. Elute HdeB with a linear gradient from 0 to 0.5 M NaCl in buffer B over a time period of 50 min with a flow rate of 2.5 ml/min⁶.
8. Identify fractions containing HdeB using a 15% SDS-PAGE. Mix 20 μ l sample with 5 μ l 5x reduced SDS loading buffer. Load 10 μ l onto the gel and run in Tris-glycine buffer (14.4 g/L glycine, 2.9 g/L Tris, 1 g/L sodium dodecyl sulfate, pH 8.3). Run the gel at 150 V until the bromophenol band has migrated close to the bottom of the gel (~45 min).
9. Pool all HdeB-containing fractions, dialyze at 4 °C overnight against 4 L HdeB storage buffer (50 mM Tris/HCl, 200 mM NaCl, pH 8.0), and concentrate the protein to approximately 300 μ M using centrifugal filter units with a molecular weight cut-off of 3 kDa. Determine the concentration of HdeB at 280 nm using the extinction coefficient $\epsilon_{280nm} = 15,595 \text{ M}^{-1}\text{cm}^{-1}$. Prepare 100 μ l aliquots and flash-freeze the aliquots in liquid nitrogen.

NOTE: HdeB can be stored at -70 °C for at least 6 months.

2. Chaperone Activity Assay Using Thermally Unfolding Malate Dehydrogenase (MDH)

NOTE: The influence of purified HdeB on the aggregation of thermally unfolding porcine mitochondrial malate dehydrogenase (MDH) at different pH values was monitored as described below. All listed protein concentrations refer to the monomer concentration.

1. To prepare MDH, dialyze MDH at 4 °C overnight against 4 L buffer C (50 mM potassium phosphate, 50 mM NaCl, pH 7.5) and concentrate the protein to approximately 100 μ M using centrifugal filter units with a molecular weight cut-off of 30 kDa.
NOTE: Careful dialysis of MDH is required as MDH is delivered as ammonium sulfate solution.
2. To remove aggregates, centrifuge the protein for 20 min at 20,000 x g at 4 °C. Determine MDH concentration by absorbance at 280 nm ($\epsilon_{280nm} = 7,950 \text{ M}^{-1}\text{cm}^{-1}$). Prepare 50 μ l aliquots of MDH and flash-freeze the aliquots for storage.
3. Place 1 ml quartz cuvette into a fluorescence spectrophotometer equipped with temperature controlled sample holders and stirrer. Set $\lambda_{ex/em}$ to 350 nm.
4. Add appropriate volumes of pre-warmed (43 °C) buffer D (150 mM potassium phosphate, 150 mM NaCl) at the desired pH values (here: pH 2.0, pH 3.0, pH 4.0, and pH 5.0) to the cuvette and set the temperature in the cuvette holder to 43 °C. The total volume is 1,000 μ l.
5. Add 12.5 μ M HdeB (or alternatively the same volume of HdeB storage buffer for the buffer control) to the buffer, followed by the addition of 0.5 μ M MDH. Begin monitoring light scattering. Incubate the reaction for 360 sec to allow sufficient unfolding of MDH.
6. Raise the pH to 7 by adding 0.16-0.34 volume of 2 M unbuffered K_2HPO_4 and continue recording light scattering for another 440 sec.
7. Set the extent of MDH aggregation that is recorded in the absence of the chaperone at a defined time point after neutralization (here after 500 sec, when maximal light scattering of MDH was observed) to 100%. Normalize HdeB's activity to the light scattering signal of MDH in the absence of HdeB at each indicated pH value.

3. Detection of HdeB-LDH Complex Formation by Analytical Ultracentrifugation (aUC)

NOTE: Sedimentation velocity experiments of HdeB alone or in complex with thermally unfolding lactate dehydrogenase (LDH) were performed using an analytical ultracentrifuge.

1. To prepare LDH, dialyze LDH at 4 °C overnight against 4 L buffer C (50 mM potassium phosphate, 50 mM NaCl, pH 7.5) and concentrate the protein to approximately 200 μ M using centrifugal filter units with a molecular weight cut-off of 30 kDa.
NOTE: Careful dialysis of LDH is required as LDH is delivered as ammonium sulfate solution.
2. To remove aggregates, centrifuge LDH for 20 min at 20,000 $\times$ g at 4 °C. Determine LDH concentration by absorbance at 280 nm ($\epsilon_{280\text{ nm}} = 43,680\text{ M}^{-1}\text{ cm}^{-1}$). Prepare 50 μ l aliquots of LDH and flash-freeze the aliquots for storage.
3. Incubate 3 μ M LDH in the presence and absence of 30 μ M HdeB in buffer D (pH 4 and 7, respectively) for 15 min at 41 °C.
NOTE: Incubation of LDH at higher temperatures results in its complete aggregation, and no chaperone effect of HdeB can be observed.
4. Let the samples cool down to room temperature. Then, load samples into cells containing standard sector shaped 2-channel centerpieces with 1.2 cm path length. Load the cells into the ultracentrifuge and equilibrate to 22 °C for at least 1 hr prior to sedimentation.
5. Spin samples at 22 °C and 167,000 $\times$ g in the respective rotor for 12 hr, and monitor the sedimentation of the protein continuously at 280 nm. As previously demonstrated, the signal to noise ratio is improved when the transmitted light intensity of each channel is measured rather than absorbance. This also improves the quality of subsequent data fitting.
6. Conduct data analysis with SEDFIT (version 15.01b, December 2015), using the continuous c(s) distribution model¹⁷. A tutorial describing how to use SEDFIT can be found in reference¹⁸.
 1. Set the confidence level for the ME (Maximum Entropy) regularization to 0.7.
7. Calculate buffer density as well as viscosity using SEDNTERP¹⁹. To estimate the amount of aggregated client protein, compare the integrals of sedimented LDH in pH 4 to pH 7 as a reference.
NOTE: Integration of the sedimentation distribution plots can be done directly in SEDFIT. Alternative software to analyze sedimentation velocity data can be found in a recent review²⁰.

4. Monitoring MDH Inactivation and Reactivation in the Presence of HdeB

NOTE: The influence of purified HdeB on the refolding of pH-unfolded MDH was determined by monitoring MDH activity upon neutralization.

1. Incubate 1 μ M MDH in buffer D at the desired pH values (here: pH 2.0, pH 3.0, pH 4.0, and pH 5.0) for 1 hr at 37 °C in the absence or presence of 25 μ M HdeB. Then, shift the temperature to 20 °C for 10 min.
NOTE: No MDH refolding was observed even in the presence of HdeB when MDH was incubated at temperatures higher than 37 °C.
2. To initiate refolding of acid denatured MDH, neutralize the samples to pH 7 by addition of 0.13-0.42 volume of 0.5 M sodium phosphate, pH 8.0.
3. After incubation for 2 hr at 20 °C, determine MDH activity by monitoring the decrease of NADH at 340 nm⁹.
NOTE: MDH catalyzes the NADH-dependent reduction of oxaloacetate into L-malate.
 1. Mix 50 μ l of the incubation reaction with 950 μ l of assay buffer (50 mM sodium phosphate, pH 8.0, 1 mM oxaloacetate, and 150 μ M NADH).
NOTE: The final concentration of MDH in the assay buffer should be 44 nM.
 2. Monitor the change in absorbance using a spectrophotometer, equipped with a Peltier temperature control block set to 20 °C.
 3. Report the MDH activity relative to 44 nM native MDH that has been kept at pH 7.0.

5. Effect of HdeB Overexpression on *E. coli* Survival under Acid Stress

NOTE: *E. coli* MG1655 genomic DNA was isolated using a published protocol²¹.

1. Amplify *hdeB* from *E. coli* MG1655 by PCR using primers *hdeB-BamHI*-rev GGT GGT CTG **GGA TCC** TTA ATT CGG CAA GTC ATT and *hdeB-EcoRI*-fw GGT GCC **GAA TTC** AGG AGG CGC ATG AAT ATT TCA TCT CTC C.
2. Set up the PCR reaction in 50 μ l as follows: 10 μ l 5 $\times$ polymerase buffer, 200 μ M dNTPs, 0.5 μ M primer JUD2, 0.5 μ M primer JUD5, 150 ng genomic DNA MG1655, 0.5 μ l DNA polymerase, add ddH₂O to 50 μ l.
3. Perform amplification of *hdeB* as follows: Step 1: 5 min at 95 °C, 1 cycle; step 2: 30 sec at 95 °C, 30 sec at 55 °C, 30 sec at 72 °C, 40 cycles; Step 3: 10 min at 72 °C.
4. Clone resulting PCR fragment into the *EcoRI* and *BamHI* sites of plasmid pBAD18 using standard methods for restriction site cloning. Purify the plasmid using a plasmid purification kit according to manufacturer's instructions. Verify the resulting plasmid by sequencing¹⁰.
5. Transform the plasmid expressing HdeB or the empty vector control pBAD18 into strain BB7224 (Δ *rpoH*) (genotype: F⁻, λ ⁻, e14⁻, [*araD139*]_{B/r} Δ (*argF-lac*)169 *flhD5301* Δ (*fruK-yeiR*)725(*fruA25*) *relA1* *rpsL150*(Sm^R) *rbsR22* Δ (*fimB-fimE*)632(::IS1) *ptsF25* *zhf::Tn10*(Tc^S) *suhX401* *deoC1* *araD*⁺ *rpoH::kan*⁺,¹⁶) using chemically competent cells.
NOTE: This strain is temperature-sensitive.
6. After 45 sec heat-shock at 42 °C and prior to the plating, incubate cells at 30 °C and 200 rpm. Perform single colony streak-outs of the positive clones and incubate overnight at 30 °C. Prepare an overnight culture in 50 ml LB_{Amp} and cultivate the cells at 200 rpm and 30 °C.
7. Dilute overnight cultures 40-fold into 25 ml LB_{Amp} and grow the bacteria in the presence of 0.5% arabinose (Ara) at 30 °C and 200 rpm to an O.D._{600nm} = 1.0 to induce HdeB protein expression.
8. For the pH shift experiments, use LB_{Amp+Ara} to dilute the cells to O.D._{600nm} of 0.5 and adjust to the respective pH values (here: pH 2.0, pH 3.0, and pH 4.0) by adding appropriate volumes of 5 M HCl.
9. After the indicated time points (pH 2, 1 min; pH 3, 2.5 min; pH 4, 30 min), neutralize the cultures by addition of the appropriate volumes of 5 M NaOH.

10. Monitor the growth of the neutralized cultures in liquid culture for 12 hr at 30 °C using O.D. measurements.

Representative Results

HdeA and HdeB are homologous *E. coli* proteins, known to protect periplasmic proteins against acid stress conditions¹⁰. Our work revealed that similar to HdeA, HdeB also functions as an acid activated molecular chaperone. However, in contrast to HdeA, HdeB functions at a pH that is still potentially bactericidal, but significantly higher than the pH optimum of HdeA^{6,9,10,22}. To investigate the pH optimum of HdeB's chaperone activity *in vitro*, native MDH was diluted into pre-warmed (43 °C) buffer of the indicated pH in the presence or absence of HdeB. After 360 sec of incubation, the incubation reaction was neutralized. This neutralization triggers aggregation of MDH at 43 °C⁹. Representative results show that the light-scattering signal of MDH in the absence of HdeB increases dramatically upon neutralization due to aggregation of MDH (**Figure 1**, black line at each pH). In the presence of HdeB, the light-scattering signal is significantly decreased upon neutralization from pH 4 or pH 5 indicating that HdeB prevents the aggregation of MDH (**Figure 1**, pH 4 and pH 5). In contrast, however, upon neutralization from pH 2 and pH 3, MDH rapidly aggregates to the same extent independent of the presence or absence of HdeB (**Figure 1**, pH 2 and pH 3), indicating that the pH optimum for HdeB's chaperone activity is between pH 4 and 5. The HdeB storage buffer had no effect on MDH aggregation (**Figure 1**, buffer control), indicating that the reduced light-scattering signal of MDH in the presence of HdeB at pH 4 is due to its chaperone function. HdeA is chaperone active in its monomeric and unfolded form at pH 2-3 but shows no activity at or above pH 4¹⁰. These results suggest that HdeB has its optimal chaperone activity around pH 4¹⁰.

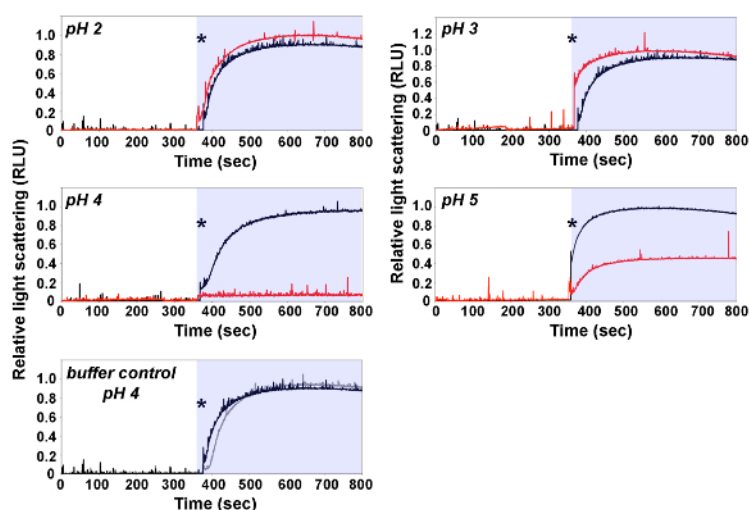


Figure 1: Chaperone activity of HdeB at acidic pH. 0.5 μ M MDH was incubated in pre-warmed buffer D at the indicated pH in the absence or presence of 12.5 μ M HdeB for 360 sec at 43 °C. The pH of the samples was then raised to pH 7 (as indicated by the asterisk) by addition of 0.16-0.34 volume 2 M unbuffered K_2HPO_4 . MDH aggregation was measured for an additional 440 sec by monitoring light scattering at 350 nm at neutral pH (blue background). Figure is modified from Dahl *et al.*¹⁰. This research was originally published in the Journal of Biological Chemistry. Dahl JU, Koldewey P, Salmon L, Horowitz S, Bardwell JC, Jakob U. HdeB functions as an acid-protective chaperone in bacteria. J Biol Chem. 2015, 290(1):65-75. doi: 10.1074/jbc.M114.612986. Copyright the American Society for Biochemistry and Molecular Biology. [Please click here to view a larger version of this figure.](#)

To address whether HdeB forms stable complexes also with other client proteins, 3 μ M LDH was thermally unfolded at 41 °C in the presence or absence of 30 μ M HdeB at different pH conditions. Analysis of the sedimentation behavior of these samples by analytical ultracentrifugation was conducted at 22 °C. When LDH and HdeB were incubated together at pH 7 and 41 °C, LDH remained exclusively tetrameric (molecular weight of 120 kDa) and HdeB remained dimeric. These results indicated that the proteins do not form stable complexes at pH 7, and LDH does not undergo any irreversible changes in oligomerization upon a 20 min incubation at 41 °C (**Figure 2**, green line). Similarly, HdeB remained dimeric upon incubation at 41 °C and pH 4.0, indicating that low pH incubation of HdeB does not affect its oligomerization state even under heat shock conditions (**Figure 2**, red line). LDH when incubated at pH 4 and 41 °C in the absence of HdeB rapidly aggregated as indicated by the fact that 40% of LDH sedimented before the first scan was recorded (**Figure 2**, stated in the upper right corner). The remaining LDH appeared to sediment predominantly as monomer (**Figure 2**, blue line). In contrast, incubation of LDH and HdeB at pH 4 and 41 °C caused a large proportion of the two proteins to co-sediment (HdeB-LDH_C), forming a new species with a molecular weight of 134 kDa. This species likely represents a complex between HdeB dimers and thermally unfolding LDH. Moreover, in the presence of HdeB, no significant LDH aggregation prior to the sedimentation was observed, which is consistent with our *in vitro* aggregation measurements. These results show that HdeB exhibits chaperone activity in its dimeric form at pH 4.0. This is in stark contrast to HdeA, which is chaperone active in its monomeric form. The very dynamic nature of HdeB that allows it to undergo structural rearrangements between pH 4 and pH 7 is likely sufficient for the activation of HdeB's chaperone function¹⁰.

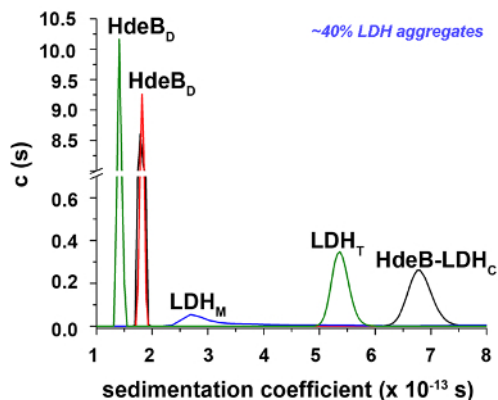


Figure 2: Detection of complex formation between HdeB and unfolded LDH at pH 4 by analytical ultracentrifugation. 3 μ M LDH was incubated in the presence of a 10-molar excess HdeB in buffer D (150 mM KHPO_4 , 150 mM NaCl) for 15 min at 41 $^\circ\text{C}$ at either pH 7 (green line) or pH 4 (black line). For comparison, LDH alone (blue line) or HdeB alone (red line) were incubated for 15 min at 41 $^\circ\text{C}$ at pH 4. Analytical ultracentrifugation sedimentation velocity was used to determine the stoichiometry of HdeB, LDH, and the complex formed between HdeB and LDH at different pH conditions. Note that ~40% LDH aggregated prior to the first scan when incubated at pH 4 and in the absence of HdeB (as noted in the upper right corner). Shown is a sedimentation coefficient distribution plot ($c(s)$) analyzed using the program SEDFIT. Letters indicate the respective oligomeric state of LDH or HdeB, respectively: HdeB_D, HdeB dimer; LDH_M, LDH monomer, LDH_T, LDH tetramer; HdeB-LDH_C, HdeB-LDH complex. Figure is modified from Dahl *et al.*¹⁰. This research was originally published in the Journal of Biological Chemistry. Dahl JU, Koldewey P, Salmon L, Horowitz S, Bardwell JC, Jakob U. HdeB functions as an acid-protective chaperone in bacteria. J Biol Chem. 2015, 290(1):65-75. doi: 10.1074/jbc.M114.612986. Copyright the American Society for Biochemistry and Molecular Biology. [Please click here to view a larger version of this figure.](#)

To test whether HdeB supports the refolding of client proteins upon neutralization, HdeB's influence on the refolding of pH-denatured MDH was analyzed. Thermally denatured MDH was incubated at different pH values in the presence or absence of HdeB. After low pH incubation, the pH was neutralized (which initiates MDH refolding), and MDH activity was determined after 2 hr. As shown in **Figure 3**, significant reactivation of MDH was achieved upon neutralization from pH 4 in the presence of HdeB. No MDH activity was determined when HdeB was absent from the low pH incubation.

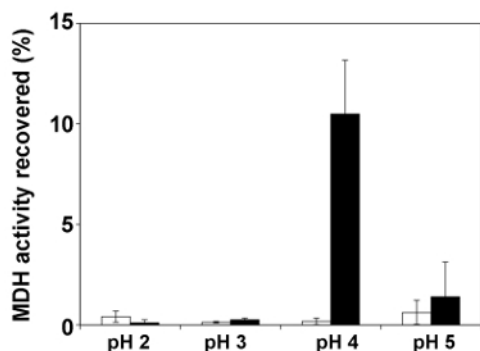


Figure 3: HdeB facilitates the refolding of acid denatured MDH to an enzymatically active state. 1 μ M MDH was incubated in buffer D at the indicated pH for 1 hr at 37 $^\circ\text{C}$ in the absence or presence of 25 μ M HdeB. Then, the temperature was shifted to 20 $^\circ\text{C}$ for 10 min before the samples were neutralized to pH 7 by the addition of 0.5 M Na_2HPO_4 . Aliquots were taken after 2 hr of incubation at 20 $^\circ\text{C}$ and assayed for MDH activity. MDH activity upon neutralization in the absence (white bars) or presence of HdeB (black bars) is shown. Standard deviation derived from at least 3 independent measurements is shown. Figure is modified from Dahl *et al.*¹⁰. This research was originally published in the Journal of Biological Chemistry. Dahl JU, Koldewey P, Salmon L, Horowitz S, Bardwell JC, Jakob U. HdeB functions as an acid-protective chaperone in bacteria. J Biol Chem. 2015, 290(1):65-75. doi: 10.1074/jbc.M114.612986. Copyright the American Society for Biochemistry and Molecular Biology. [Please click here to view a larger version of this figure.](#)

Our *in vitro* data revealed that HdeB binds different model client proteins at pH 4, prevents their aggregation and facilitates client refolding once neutral pH conditions are restored. To investigate the effects of HdeB *in vivo*, pH-dependent survival assays were conducted using the temperature-sensitive *rpoH* deletion strain. This strain lacks most chaperones and is therefore more susceptible to elevated temperature, low pH or oxidative stress¹⁵. Overexpression of HdeB under neutral pH conditions showed no effect on the growth rate of the strain, which grew comparably well to the control strain that harbors the empty vector pBAD18 (**Figure 4**, untreated). In contrast, we found clear differences in their ability to resume growth upon pH 3 or pH 4 treatment with the HdeB overexpressing strain showing reproducibly improved recovery from low pH treatment than the control strain. In contrast to our *in vitro* data, however, presence of HdeB had also a significantly protective effect at pH 3. This might be due to the high concentration of HdeB in the cell, which might shift the oligomerization state of HdeB towards dimers even at pH 3. Note that shifting cells to pH 2 or pH 3 resulted in very fast and highly toxic effects, while no significant killing was observed when cells were incubated at pH 4^{9,10,22}.

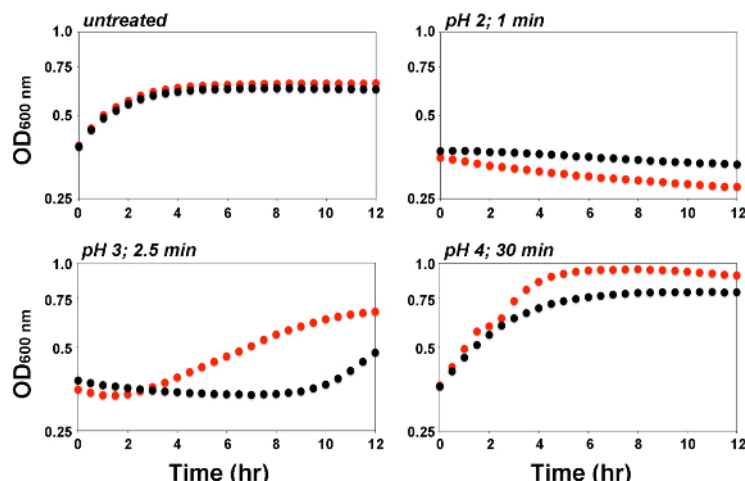


Figure 4: HdeB protects *E. coli* against acidic pH. HdeB (red circles) was overexpressed in BB7224 ($\Delta rpoH$) in the presence of 0.5% arabinose at 30 °C. BB7224 cells harboring the empty vector pBAD18 were used as control (black circles). Upper left panel shows growth of both strains at 30 °C, pH 7. Cells were shifted to the indicated pH by adding 5 M HCl, and incubated for 1 min at pH 2 (upper right panel), 2.5 min at pH 3 (lower left panel) or 30 min at pH 4 (lower right panel). Subsequently, cultures were neutralized by adding appropriate volumes of 5 M NaOH and growth was monitored in liquid media at 30 °C. Figure is modified from Dahl *et al.*¹⁰. This research was originally published in the Journal of Biological Chemistry. Dahl JU, Koldewey P, Salmon L, Horowitz S, Bardwell JC, Jakob U. HdeB functions as an acid-protective chaperone in bacteria. J Biol Chem. 2015, 290(1):65-75. doi: 10.1074/jbc.M114.612986. Copyright the American Society for Biochemistry and Molecular Biology. [Please click here to view a larger version of this figure.](#)

Discussion

In order to study the mechanism of activation and chaperone function of HdeB, large quantities of HdeB have to be expressed and purified. A number of expression vector systems are available for the production of high levels of a target protein, including pTrc or pBAD vectors, both of which were used in this study. The promoters are readily accessible for *E. coli* RNA polymerase and thus allow strongly upregulated expression of HdeB in any *E. coli* strain. This aspect is especially relevant for *in vivo* overexpression study of HdeB under acid stress conditions, where the *rpoH*-deficient strain was utilized. This strain lacks most of the chaperones and thus is more sensitive towards various stressors, including elevated temperature, low pH and oxidative stress¹⁵. As alternative, survival studies similar to the ones presented here can be performed in mutant strains that lack the gene of interest.

The experimental design for any chaperone activity or refolding assay has to be carefully considered, both in regards to the type of client, the concentration of the client protein and the buffer conditions. In typical chaperone assays, model client proteins, such as citrate synthase, luciferase, or malate dehydrogenase are denatured in high concentrations of urea or guanidine-HCl, and diluted into denaturant-free buffer to induce aggregation^{23,13}. Measurements in the presence of chaperones reveal the extent to which they prevent protein aggregation. Alternatively, the clients are thermally unfolded and protein aggregation is monitored. In both cases, light scattering measurements are used as readout for protein aggregation. Active chaperones prevent the aggregation of unfolding client proteins, thereby causing a decrease in the light scattering signal⁶. Both of these experimental setups can be combined with low pH incubation. In addition to assessing the molecular chaperone activity by monitoring their ability to prevent aggregation of particular client proteins, the influence of chaperones on client refolding upon return to non-stress conditions can be tested²⁴. This is especially straightforward when the client proteins possess enzymatic activity, which can be used as quantitative readout for their inactivation and reactivation. While chaperone-mediated refolding is naturally an ATP-dependent process, periplasmic chaperones such as HdeA, HdeB and Spy have been shown to facilitate client refolding in an ATP-independent fashion, consistent with the lack of energy in the periplasm^{9,25}.

Studying the pH optimum of an acid-protective chaperone such as HdeB is challenging due to various reasons: (i) aggregation behaviors of even well-established chaperone-client proteins such as citrate synthase differ at acidic pH; and (ii) only few buffer systems work in the pH range between 2-5 and are suitable for both the chaperone of interest and the client protein. We decided to use phosphate buffer, although we are aware that this is a non-ideal buffer system under acidic pH conditions. However, phosphate buffer was found to be well-suited to characterize HdeA as acid activated chaperone^{9,22}. Aggregation measurements are very sensitive towards changes in temperature or buffer content. To eliminate false-positive results, we therefore recommend to always test the influence of chaperone storage buffer on client aggregation (Figure 1, buffer control). Sometimes aggregation of the client protein occurs so fast that even the best chaperone might not be capable of competing with the aggregation process. It is therefore essential to conduct preliminary tests to find the optimal assay conditions. A good example for such a situation is given in our ultracentrifugation experiments where incubation of LDH at temperatures of >42 °C is so fast that even the presence of an excess of HdeB does not prevent LDH aggregation. In addition, the chaperone/co-chaperone ratio or chaperone/client ratio has to be determined carefully²³. We started using a fairly high HdeB:MDH ratio of 50:1 in preliminary experiments and that helped us in identifying pH 4 as the optimal pH for the chaperone activity of HdeB. We then continued analyzing HdeB:MDH ratios between 1:1 and 50:1 at pH 4, identifying 25:1 to be the most effective ratio. In contrast, HdeA suppressed MDH aggregation as 10:1 chaperone:client ratios^{6,9,10,22}. Thus, we conclude that HdeA, in comparison to HdeB, is more effective in suppressing MDH aggregation as lower chaperone:client ratios were sufficient to completely suppress MDH aggregation. Another approach to investigate chaperone-mediated suppression of protein aggregation involve spin-down assays, in which client aggregates are removed by centrifugation and quantified by SDS PAGE. This approach is also suited for monitoring the influence of chaperones on protein aggregation *in vivo*. Mutant strains that either overexpress or lack the chaperone of interest are exposed

to protein unfolding stress conditions. Subsequently, the cells are lysed and soluble and aggregated fractions are separated and quantified^{15,16,26}.

For detection of the client-chaperone complex, we applied analytical ultracentrifugation. It shall be noted here that based on the experimental setup it is not possible to directly quantify the amount of HdeB and LDH monomers bound in this complex, as both proteins absorb at 280 nm. If desired, the stoichiometry of the chaperone-client complex can be determined by separately labeling chaperone and client protein with a chromophore, whose excitation maximum lies within the visible range. Alternatively, the stoichiometry of clients to chaperones within complexes can be determined by using native PAGE coupled with quantitative western blot.

By following the protocols presented here, we were able to characterize two molecular chaperones, HdeA, and HdeB^{9,10,22}. In general, these assays can be also used to investigate the role of potential inhibitors of molecular chaperones in protein refolding *in vitro* and *in vivo* or can be applied to test synthetic chaperones for their ability to prevent client aggregation under acid stress. In addition, the protocols presented here can be used for analyses of point-mutations and/or truncated variants of the acid-activated chaperones in order to shed light into the mechanism of their activation.

Disclosures

The authors have nothing to disclose

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