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Periplasmic Protein HdeA Exhibits Chaperone-like Activity Exclusively within Stomach pH Range by Transforming into Disordered Conformation*

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The extremely acidic environment of the mammalian stomach, with a pH range usually between 1 and 3, represents a stressful challenge for enteric pathogenic bacteria such as *Escherichia coli* before they enter into the intestine. The *hdeA* gene of *E. coli* was found to be acid inducible and was revealed by genetic studies to be important for the acid survival of the strain. This study was performed in an attempt to characterize the mechanism of the activity of the HdeA protein. Our data provided in this report strongly suggest that HdeA employs a novel strategy to modulate its chaperone activity: it possesses an ordered conformation that is unable to bind denatured substrate proteins under normal physiological conditions (*i.e.* at neutral pH) and transforms into a globally disordered conformation that is able to bind substrate proteins under stress conditions (*i.e.* at a pH below 3). Furthermore, our data indicate that HdeA exposes hydrophobic surfaces that appear to be involved in the binding of denatured substrate proteins at extremely low pH values. In light of our observations, models are proposed to explain the action of HdeA in both a physiological and a molecular context.

The extremely acidic environment of the mammalian stomach, with pH values usually between 1 and 3, serves as an important natural barrier to defend against the entrance of microorganisms (1). The enteric pathogenic bacteria (*e.g. Shigella flexneri* and *Escherichia coli*), although normally living in a neutral pH environment, have evolved a mechanism for survival of several hours in such harsh conditions (2, 3). Several systems, including the glutamate-dependent and arginine-dependent systems in *E. coli*, have been identified as contributing to cytoplasmic acid resistance (4, 5). Nevertheless, the acid resistance mechanism for periplasmic space, though having greater survival value, is considerably less understood. The periplasmic space is enveloped by an outer membrane across which molecules smaller than 600 Da are allowed to diffuse freely due to the presence of nonspecific transporters like porins (6, 7). In view of the porous nature of the outer membrane, proteins present in the periplasm are more vulnerable, thus requiring greater protection than cytoplasmic proteins, when the enteric bacteria are exposed to the harsh environmental conditions encountered in the low pH conditions of the mammalian stomach.

Using random mutagenesis caused by transposon insertion, acid-sensitive mutants of *S. flexneri* (whose wild type is acid-resistant) were isolated (8). The *hdeA* (where *hde* stands for hns-dependent expression) genetic locus was revealed to be important for the acid resistance phenotype: insertional mutation in the *hdeA* gene made the organism highly acid sensitive, whereas a reintroduction of the functional *hdeA* gene restored the acid resistance phenotype (8). The *hdeA* gene in *E. coli* was found to be acid inducible (9) and essential for acid resistance (10). The HdeA protein, localized in the periplasm, was proposed to exhibit chaperone-like activity based on the observation that it prevented the aggregation of rhodanese proteins at low pH during renaturation after being denatured with guanidine hydrochloride (10). However, the detailed nature of this activity remains uncertain. Although the crystal structure of the HdeA homodimer was determined, it revealed limited information as to how the protein fulfils its physiological function (10, 11).

Molecular chaperones are a large family of proteins that mainly act to prevent aggregation of nonnative proteins under such stressful cellular conditions as non-optimal temperatures, pH, osmotic pressure, or presence of toxic chemicals (12). The most fully investigated types of molecular chaperones include those working under heat shock conditions. By contrast, molecular chaperones acting under acidic conditions represent a type that has rarely been investigated (13, 14).

This study was performed in an attempt to characterize such activity in detail, especially the response of this chaperone-like activity to changes in pH values. Data have been provided to demonstrate that HdeA exhibits chaperone-like activity only at a pH below 3 (corresponding to the mammalian stomach pH range) by transforming into a globally disordered conformation. In light of these observations, a working model for HdeA action in physiological contexts is proposed.

EXPERIMENTAL PROCEDURES

Materials—Glyceraldehyde 3-phosphate dehydrogenase and alcohol dehydrogenase (ADH)[1] were obtained from Sigma. Hydrochloric acid,

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[1] The abbreviations used are: ADH, alcohol dehydrogenase; ANS, 1-anilino-8-naphthalenesulfonate; HSQC, heteronuclear single quantum correlation.

sodium chloride, sodium sulfate, and trichloroacetic acid were of analytical grade.

Plasmid Construction, Expression, and Purification of Recombinant HdeA—The DNA fragment containing the *hdeA* gene, amplified by PCR from the genomic DNA of *E. coli* JM109(DE3), was inserted into the pET21b expression vector after both were digested with EcoRI and BamHI. The ligated plasmids were then transformed into *E. coli* BL21(DE3) cells for amplification and screening. The constructed plasmid was verified by DNA sequencing (Bioasia Inc., Shanghai, China).

The HdeA protein, overexpressed in pET21b-HdeA-transformed BL21(DE3) cells (present in the periplasmic space), was purified using methods previously described (being the 10-kDa protein in Ref. 15). The protein concentration was determined by the BCA method (Pierce) and was expressed as the molar concentration of the protomer.

Preparation of Periplasmic Protein Extracts and RbsB—Periplasmic extracts of JM109(DE3) and BL21(DE3) cells were obtained by osmotic shock according to methods previously described (15–17). The procedure for RbsB purification from periplasmic extracts has been described elsewhere (15).

Preparation of Polyclonal Antiserum against HdeA and Western Blotting Analysis—The polyclonal antiserum against HdeA from rabbit was prepared as described (18). Western blotting was performed using the anti-HdeA serum (at 1/1000 dilution) and the goat anti-rabbit anti-IgG (at 1/1000 dilution) conjugated to alkaline phosphatase.

Assay of Chaperone-like Activity—The chaperone-like activity was assayed by either qualitatively following the appearance of substrate proteins in the supernatant instead of in the pellet (Fig. 1, *A*, *C–E*) or quantitatively monitoring the decrease of the fluorescence light-scattering intensity of the aggregates (Figs. 1*E* and 4*C*). Sulfate, trichloroacetate, or chloride was added as the anion to achieve effective aggregation of the substrate proteins at low pH values (19, 20). For assaying the different substrate proteins (ADH, glyceraldehyde 3-phosphate dehydrogenase, RbsB), the pH values were adjusted with different acids (HCl or trichloroacetic acid). The presence of substrate proteins in the supernatants or pellets, after being incubated at 25 °C for 30 min and centrifuged at 10,000 × *g* for 10 min, was examined by SDS-PAGE (Fig. 1). Each sampling supernatant was neutralized to pH 7 by adding Tris base solution with the corresponding pellet resuspended in water to a volume equal to that of the supernatant.

The light-scattering intensity was monitored with a Hitachi F4500 fluorescence spectrophotometer equipped with a temperature-controlling system. The excitation and emission wavelengths were both set at 400 nm (Fig. 1*E*). The relative chaperone activities (as presented in Fig. 4*C*) were then calculated as $(F_{\text{ADH alone}} - F_{\text{ADH+HdeA+ANS}})/F_{\text{ADH alone}}$ (*F* represents fluorescence intensity at 400 nm). The maximal ADH aggregation in the presence of HdeA (with no ANS added) was defined as 100% relative chaperone activity.

Circular Dichroism (CD) Spectroscopy—This was performed using a J-715–150L spectrometer (JASCO, Tokyo, Japan). The far-UV CD spectra were recorded in the wavelength range of 200–250 nm, with the path length of the cuvette being 2 mm. Protein samples (at 20 μM) in 0.1 M Tris were each adjusted to a designated pH value using HCl and incubated at 25 °C for 1 h before the recording (Fig. 3*A*).

NMR Spectrometry—The ^{15}N-labeled HdeA was overexpressed and purified (using methods described above) from *E. coli* strain BL21(DE3) (transformed with the pET21b-HdeA plasmid) grown on a minimal medium containing 1 g/liter ^{15}N ammonium chloride as the sole nitrogen source. The NMR samples containing 0.5 mM ^{15}N-labeled HdeA were dissolved in buffers of three pH values: 50 mM phosphate, 0.03% (w/v) NaN_3 in 90% H_2O/10% D_2O at pH 5.0 and 50 mM sodium acetate, 0.03% (w/v) NaN_3 in 90% H_2O/10% D_2O at pH 3.5 or pH 2.5. Two-dimensional ^{1}H-^{15}N heteronuclear single quantum correlation experiments were performed at 25 °C using Bruker DMX 500- and 600-MHz NMR spectrometers (Fig. 3, *B* and *C*).

Detecting the Association and Dissociation between HdeA and the Denatured Substrate Proteins—Soluble complexes resulting from the association between HdeA and denatured substrate protein ADH were isolated by using size-exclusion chromatography and then examined using SDS-PAGE (Fig. 2). Samples (120 μl, 0.15 M NaCl, adjusted to pH 2 with HCl) containing both HdeA and ADH were incubated for 1 h and centrifuged at 10,000 × *g* for 10 min before 100 μl of the supernatant was loaded into a 10 mm × 30 cm Superdex HR-200 size-exclusion column (Amersham Biosciences). The sample was then eluted at a flow rate of 0.5 ml/min and collected at 0.5 ml/fraction. Proteins in the fractions collected were separated by SDS-PAGE before being visualized by silver staining.

For the dissociation of HdeA-ADH complexes, the sample mixture was neutralized to pH 7 at 25 °C for another hour after being incubated in 0.15 M NaCl at pH 2 for 1 h. The sample was centrifuged (10,000 × *g*, for 10 min) with the supernatant removed and the pellet resuspended to a volume equal to that of the supernatant before both were subjected to SDS-PAGE analysis.

For the dissociation of the HdeA homodimer, the HdeA proteins (100 μM) were incubated in an HCl solution of pH 2 (with 0.15 M NaCl), an HAc solution of pH 4 (with 0.05 M NaAc and 0.15 M NaCl), or a 50 mM Na_3PO_4 solution of pH 7 (with 0.15 M NaCl) at 25 °C for 60 min. For the reassociation of the HdeA homodimer, the sample (100 μM HdeA) was neutralized to pH 7 at 25 °C for another hour after being incubated in an HCl solution of pH 2 (with 0.15 M NaCl) for 1 h. The samples were then analyzed by size-exclusion assay as described above.

ANS Binding Assay to HdeA—The binding of ANS to HdeA was monitored by measuring the increase of fluorescence intensity for ANS upon its binding to proteins, using a Hitachi F4500 fluorescence spectrophotometer equipped with a temperature control system. To obtain the fluorescence spectra, HdeA samples (at 15 μM) were excited at 395 nm, with emission being recorded between 400 and 600 nm (Fig. 4*A*). To estimate the number of ANS molecules that were bound to HdeA, the protein (15 μM) was titrated to saturation with increasing dye amounts (0–100 μM). The samples were excited at 395 nm, and maximal emission from ANS was measured. The molar ratios of the bound ANS to HdeA were calculated from fluorescence intensity values as $(F_{\text{HdeA+ANS}}/F_{10\ \mu\text{M ANS}}$ at saturation) × (10 μM/[HdeA]) (in which $F_{\text{HdeA+ANS}}$, $F_{10\ \mu\text{M ANS}}$ at saturation and [HdeA], respectively, represent maximal fluorescence intensity at indicated ANS concentrations, maximal fluorescence intensity for 10 μM ANS that was fully saturated by excessive amounts of HdeA, and the molar concentration of HdeA protein). All the data points were fitted to sigmoidal plot. To explore the effect of ANS binding on chaperone-like activity of HdeA, preincubation of HdeA with ANS was carried out for 3 min before being assayed for the relative chaperone activity as described above.

Primary Structure Analysis—The theoretical charge titration curve for HdeA was generated using the program provided at www.iut-arles.up.univ-mrs.fr/w3bb/d_abim/compo-p.html. The hydropathy index for HdeA was calculated using the program provided at cn.expasy.org/tools/protscale.html, which uses the method of Kyte and Doolittle (21). The window size of the index was set to all 89 residues of HdeA.

RESULTS

HdeA Exhibits Chaperone-like Activity Exclusively at pH Lower than 3—We have been focusing on understanding certain features of periplasmic proteins of *E. coli*, including their stability under various conditions (15). In an attempt to investigate whether such proteins are resistant to aggregation caused by acid treatment, the proteins in the whole periplasmic extracts from two different *E. coli* strains, JM109(DE3) and BL21(DE3), were seen to behave very differently under extremely low pH conditions. Although many proteins in the periplasmic extract of BL21 formed aggregates and thus appeared in the precipitate (Fig. 1*A*, *lanes 5* and *6*), almost none of JM109 formed such aggregates and thus remained in the supernatant (*lanes 3* and *4*) under treatment of 1 M HCl. This difference is apparently attributed to the existence of HdeA in the JM109 extract and the absence of it in the BL21 extract, as revealed by the Western blotting analysis (*lanes 1* and *2*). Supporting this claim is the effective prevention of the acid-induced aggregation of BL21 periplasmic extract when exogenous HdeA was added (*lanes 7* and *8*).

To further elucidate the nature of HdeA action, the aggregation-suppressing process was systematically examined using two typical substrates of chaperone-like activity assays (ADH and glyceraldehyde 3-phosphate dehydrogenase) and one protein of the periplasmic space (RbsB) at various pH values (adjusted with two acids, hydrochloric acid and trichloroacetic acid). The data clearly demonstrate that the aggregation of all three proteins at pH values below 2.5 was effectively suppressed by the addition of an excessive amount of HdeA (Fig. 1*B*, *lanes 1–4*; Fig. 1*C*, *lanes 1–6*; Fig. 1*D*, *lanes 1–4*). Nevertheless, the aggregation of these substrate proteins induced at pH higher than 3 was not suppressed by addition of HdeA (Fig. 1*B*, *lanes 5* and *6*; Fig. 1*C*, *lanes 7* and

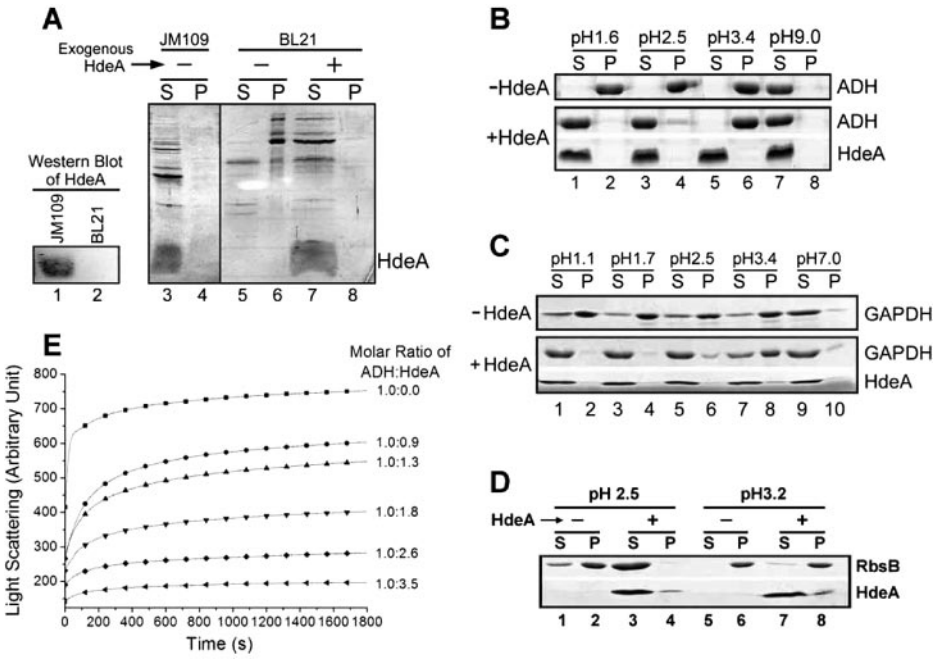

FIG. 1. **HdeA exhibits chaperone-like activity only at pH values lower than 3.** *A*, results of SDS-PAGE analysis of the supernatant (*S*) and pellet (*P*) fractions of the whole periplasmic extracts of *E. coli* JM109 (DE3) or BL21(DE3) cells that were subjected to acid treatment without (*lanes 3–6*) or with the addition of exogenous HdeA (*lanes 7* and *8*). Shown on the left is the immunoblotting result of the whole periplasmic extracts using anti-HdeA antibodies (*lanes 1* and *2*). *B–D*, results of SDS-PAGE analysis of the supernatant (*S*) and pellet (*P*) fractions of ADH (10 μM, containing 70 μM HdeA and 0.1 M trichloroacetate, *B*), glyceraldehyde 3-phosphate dehydrogenase (11 μM, containing 40 μM HdeA and 0.2 M sulfate, *C*), or RbsB (12 μM, containing 50 μM HdeA and 0.1 M trichloroacetate, *D*) that were subjected to acid-induced aggregation at the indicated pH values in the absence or presence of the above indicated excessive amount of HdeA. The pH values were adjusted as described under "Experimental Procedures." *E*, recorded light-scattering intensity curves of ADH (10 μM) aggregation induced at pH 1.5 in the presence of increasing concentrations of HdeA (as indicated on the *curves*).

8; Fig. 1*D*, *lanes 5–8*). The degree of prevention of protein aggregation for ADH in acid (pH 1.6) was found to highly correspond to the increasing molar ratio of HdeA to ADH (Fig. 1*E*). A small amount of HdeA was found in the precipitate together with ADH, most likely in the form of insoluble HdeA-ADH complexes, when ADH was present in an excessive amount (data not shown).

These results strongly indicate that HdeA is able to effectively and nonspecifically suppress the aggregation of periplasmic proteins occurring at extremely low pH values. We have also observed that HdeA appears to be completely incapable of suppressing the thermal aggregation of ADH and the aggregation of insulin induced by dithiothreitol (data not shown), both of which are typically effectively suppressed by other molecular chaperones (22, 23).

HdeA Binds to the Denatured Substrate Proteins at pH Lower than 3 but Releases Them at Neutral pH—Given that HdeA is able to effectively suppress protein aggregation at extremely low pH values (Fig. 1), it must then be determined whether HdeA forms soluble complexes with the denatured substrate proteins, as molecular chaperones usually do. The formation of such soluble complexes at pH 2 is clearly demonstrated by the appearance of an absorption peak on the elution curve of the size-exclusion chromatography (Fig. 2*A*) and the coexistence of HdeA and ADH in the fractions at the peak (fractions from 11 to 15 ml) as detected by SDS-PAGE analysis (Fig. 2*A*, *inset*). The broad feature of the peak indicates a certain extent of heterogeneity for the HdeA-ADH complexes. The reason that the integrated absorbance values of curve 2 (ADH) and curve 3 (HdeA) do no add up to curve 1 (ADH+HdeA) is that curve 2 (for free ADH) was performed after the aggregated ADH was partially removed from the sample by centrifugation. Similar soluble complexes between HdeA and the periplasmic proteins RbsB or DegP were also detected at pH 2 but were almost completely undetectable when the assay was performed at pH values higher than 3 (data not shown).

The fate of such soluble HdeA-ADH complexes (formed at pH 2) when returned to neutral pH was then examined. Their dissociation at pH 7 was indicated by the disappearance of ADH from the supernatant and its appearance in the precipitate (Fig. 2*B*, *lanes 1* and *2*), most likely because of the release

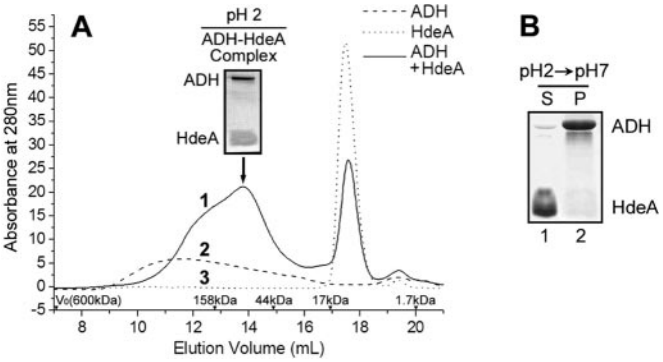

FIG. 2. **HdeA binds ADH at pH 2 and releases it at pH 7.** *A*, the size-exclusion chromatography elution curve of the sample that contained 100 μM HdeA and 30 μM ADH and was preincubated at pH 2 (*curve 1*) and the elution curves of samples containing 30 μM ADH (*curve 2*) or 100 μM HdeA alone (*curve 3*). The samples were subjected to size-exclusion chromatography at pH 2 (containing 0.15 M NaCl, adjusted by HCl). The elution positions of the standard molecular mass markers as performed at pH 7 are indicated *above the abscissa*. The SDS-PAGE analysis result of the elution fraction having the coexistence of HdeA and ADH is shown in the *inset*. *B*, results of SDS-PAGE analysis of the supernatant (*S*) and pellet (*P*) of the sample that contained the soluble HdeA-ADH complexes formed at pH 2 but was subjected to a pH readjustment to 7.

and subsequent aggregation of ADH from the soluble HdeA-ADH complexes.

HdeA Is Transformed into a Globally Disordered Conformation upon a Decrease of pH to Lower than 3—The nature of HdeA conformation, which allows it to exhibit chaperone-like activity at pH values lower than 3 but not at pH higher than 3, was then examined using CD spectrometry. The far-UV CD spectra recorded at various pH values, shown in Fig. 3*A*, revealed a sharp change in its secondary structure at a pH around 3, from a highly ordered form at pH above 3.1 (as indicated by a high content of α-helices, revealed by the appearance of the maximum ellipticity values at 208 and 222 nm; also see Ref. 10) to a highly disordered form at pH below 2.5 (as indicated by the appearance of the maximum ellipticity values at 203 nm and the simultaneous disappearance of those at 208 and 222 nm). Similar changes in tertiary structure were also

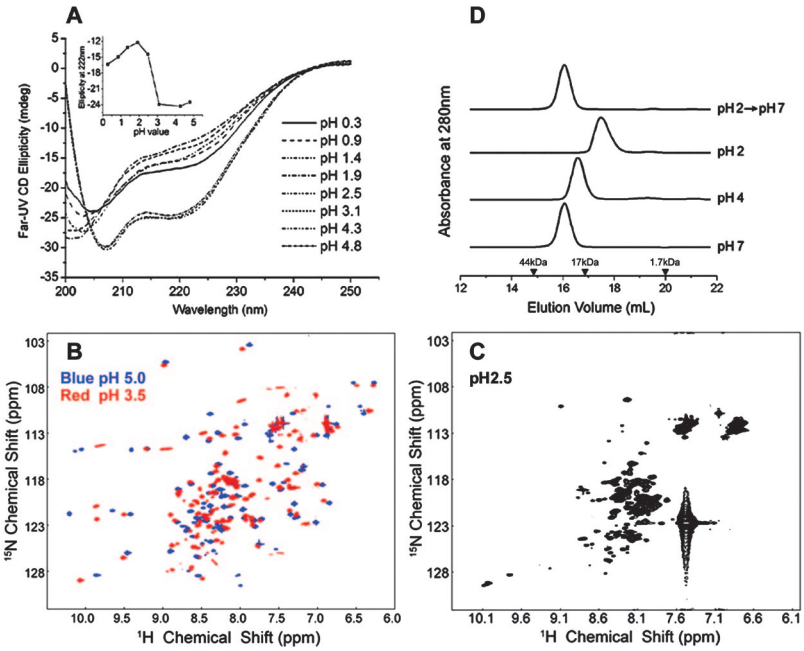

FIG. 3. **HdeA is transformed into a disordered conformation upon the decrease of pH to values lower than 3.** *A*, the far-UV spectra (reflecting the composition of their secondary structures) of HdeA recorded at the indicated pH values. The HdeA samples (all at 20 μM) were first incubated at the indicated pH values for 30 min before being recorded. The ellipticity values at 222 nm for the far-UV spectra were plotted against the corresponding pH values (*inset*) to better illustrate the pH-induced transition of the secondary structure for HdeA. *B*, superimposed ^{1}H-^{15}N HSQC spectra of HdeA at pH 5.0 (*blue spots*) and pH 3.5 (*red spots*). *C*, the ^{1}H-^{15}N HSQC spectrum of HdeA recorded at pH 2.5. All the ^{1}H-^{15}N HSQC spectra were recorded at the HdeA protein concentration of 0.5 mM. *D*, size-exclusion chromatography elution curves, monitored at 280 nm, of HdeA recorded at the indicated pH conditions (*bottom three curves*). Also shown is the elution curve (*upper curve*) of the HdeA sample that was pretreated at pH 2 for 1 h before being returned to pH 7 and subjected to analysis. The elution positions of the standard molecular mass markers as performed at pH 7 are indicated *above the abscissa*.

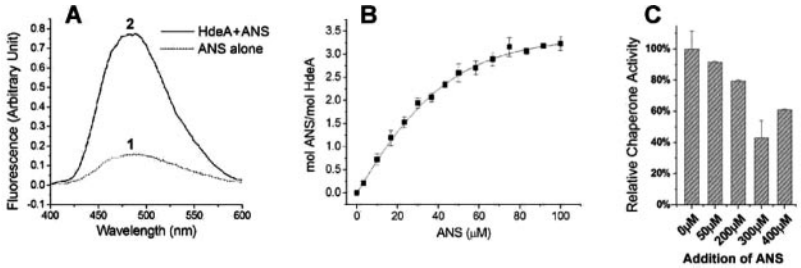

FIG. 4. **HdeA prevents the acid-induced aggregation of ADH via hydrophobic interactions.** *A*, fluorescence emission spectra of ANS (100 μM, in HCl solution of pH 1.5 with 150 mM NaCl) in the presence of HdeA. Fluorescence spectra for ANS were recorded for the dye alone (*curve 1*) and in the absence (*curve 1*) or presence of 15 μM HdeA (*curve 2*) after excitation at 395 nm. *B*, titration of HdeA with ANS. HdeA (15 μM) was titrated with an increasing concentration of ANS. The samples were excited at 395 nm with the maximal emission recorded. The fluorescence values were utilized to calculate the molar ratio of ANS to HdeA, which was then plotted against the concentration of ANS. *C*, the relative chaperone activities of HdeA (15 μM), assayed using ADH (4.3 μM) as the substrate protein and measured at pH 1.5 (as in Fig. 1*E*), in the presence of different concentrations of ANS. The light-scattering intensity was converted into relative chaperone activity as described under "Experimental Procedures." *Error bars* represent S.E. of the mean.

detected using both near-UV CD spectrometry and intrinsic fluorescence spectroscopy (data not shown).

To add further support for the occurrence of such conformational changes for HdeA accompanying the pH changes, two-dimensional ^{1}H-^{15}N heteronuclear single quantum correlation (HSQC) experiments were performed. The ^{1}H-^{15}N HSQC spectra of HdeA recorded at pH 5.0 and 3.5 showed well dispersed signals, indicating the presence of well folded structures (Fig. 3*B*). The chemical shifts of the NH cross peaks of HdeA at pH 7.0 (not shown) was almost completely identical to that at pH 5.0. However, the NH signals of HdeA significantly changed when the pH was lowered from 5.0 (Fig. 3*B*, *blue spots*) to 3.5 (*red spots*), indicating a conformational change that was marginally detected by far-UV CD spectroscopy (Fig. 3*A*). The ^{1}H-^{15}N HSQC spectrum of HdeA recorded at pH 2.5 markedly differs from those recorded at pH 5.0 or 3.5. These chemical shift dispersions clearly suggest that HdeA adopts a fully unfolded structure at pH 2.5 (Fig. 3*C*), being highly consistent with the far-UV CD spectroscopy observations (Fig. 3*A*). In addition, the large cross peak at chemical shifts of ~7.5 ppm for ^{1}H and ~123 ppm for ^{15}N is a peak aliased four times in the ^{15}N dimension. Its actual ^{15}N chemical shift is about 33 ppm. This peak should be the overlap of all 12 lysine side chain NH_3 signals. Normally, at neutral pH, lysine side chain NH_3 has fast exchanges with the solvent and is invisible for NMR. Apparently, it shows up at pH 2.5, probably because of a change in the exchange rate.

The fact that HdeA exhibits chaperone-like activity only at pH values lower than 3, where the protein possess a globally disordered conformation, strongly suggests that a structural transformation from ordered to disordered at ~pH 3 is needed for HdeA to gain the capacity to bind the denatured substrate proteins.

Because previous studies have demonstrated that HdeA ex-

ists as homodimers at neutral pH and starts to dissociate significantly into monomers at ~pH 4, it was proposed that the dissociation of the dimeric form of HdeA allows it to bind denatured substrate proteins at pH 2 (10). In view of the observations reported here, one issue requiring clarification is the importance of dimeric dissociation and conformation disordering for HdeA to work. Results from both chemical cross-linking studies (data not shown) and size-exclusion chromatography analysis performed at pH 7 demonstrated that HdeA exists as a homodimer. Although our size-exclusion chromatography analysis also revealed a similar decrease of the apparent size of the HdeA protein at pH 4 (Fig. 3*D*), the data reported here demonstrate the unambiguous inability of HdeA to prevent the aggregation of substrate proteins at pH values higher than 3 (Fig. 1*B*, *lanes 5* and *6*; Fig. 1*C*, *lanes 7* and *8*; and Fig. 1*D*, *lanes 5–8*). These data all together suggest that it is the disordered conformation, instead of dissociation *per se*, that is essential for HdeA to work.

Data presented in Fig. 3*D* also demonstrate that HdeA monomers are able to reassociate to form dimers when the pH value is raised from pH 2 to 7, suggesting a reversible nature for the dissociation process. The ordered conformation of HdeA is apparently also resumed upon such a switch of pH from 2 to 7, as shown by CD spectroscopy and NMR spectrometry studies (not shown).

HdeA Exposes Hydrophobic Surfaces That Appear to Be Involved in Binding Denatured Substrate Proteins at pH Lower than 3—It is generally believed that molecular chaperones interact with their denatured substrate proteins via hydrophobic interactions (12). To explore whether HdeA employs a similar strategy in preventing the aggregation of substrate proteins at extremely low pH values, the extent of hydrophobic surfaces exposed at neutral and extremely low pH values was examined. Data shown in Fig. 4*A* demonstrate the hydrophobic exposure at pH 1.5 (compare *curves 1* and *2*) and its absence at pH 7.0 (data not shown), as reflected by the increase of the intensity of fluorescence emission of a commonly used hydrophobic probe, ANS (19, 24). Furthermore, titration of HdeA with ANS indicates a binding curve apparently with a sigmoidal feature (Fig. 4*B*). The number of ANS molecules that bind to HdeA was estimated to be 3.2. A decrease of the relative chaperone-like activity of HdeA was observed when it was preincubated with excessive ANS (Fig. 4*C*), whereas the same amounts of ANS exhibited little influence on the aggregation of ADH alone (data not shown). This decrease is likely a result of the binding of ANS to the hydrophobic surfaces of HdeA, which would block the access of the substrate proteins, also suggesting the involvement of exposed hydrophobic surfaces in binding denatured substrate proteins.

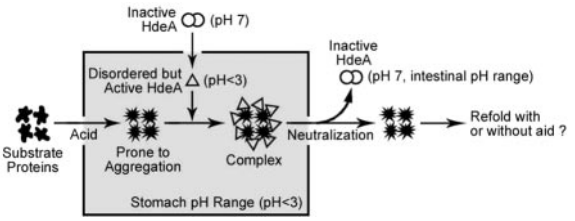

FIG. 5. **A scheme illustrating the possible mode of action for HdeA under a physiological context.** The *shadowed area* represents the mammalian stomach environment.

DISCUSSION

This study represents a major effort to understand how HdeA, a periplasmic protein found to be required for *E. coli* cells to survive in acidic environments, works to prevent the aggregation of proteins at extremely low pH values. Experimental observations include the following: (*a*) HdeA exhibits chaperone-like activity, *i.e.* being able to bind to the denatured substrate proteins, exclusively at extremely low pH values and releases them at neutral pH; (*b*) HdeA possesses a globally disordered conformation at extremely low pH values but an ordered conformation at neutral pH; and (*c*) HdeA exposes hydrophobic surfaces that appear to be involved in binding denatured substrate proteins at extremely low pH values. What is most noteworthy is the parallel appearance of a globally disordered conformation and the chaperone-like activity, both exclusively below pH 3, that coincides closely to the pH range found in the mammalian stomach.

In light of the properties of HdeA presented here, together with the previous observation that HdeA is necessary for enterobacteria to survive at extremely low pH values (10), it is proposed that HdeA might have evolved to protect *E. coli* cells for survival in the extremely low pH environment that bacteria typically encounter in mammalian stomachs. In particular, HdeA appears to be able to act as an acid-responsive molecular chaperone that prevents protein aggregation in the periplasmic space (that is exposed to the outside environment because of the porous nature of the outer membrane) at such extremely low pH values. The physiological role of HdeA during the pas-

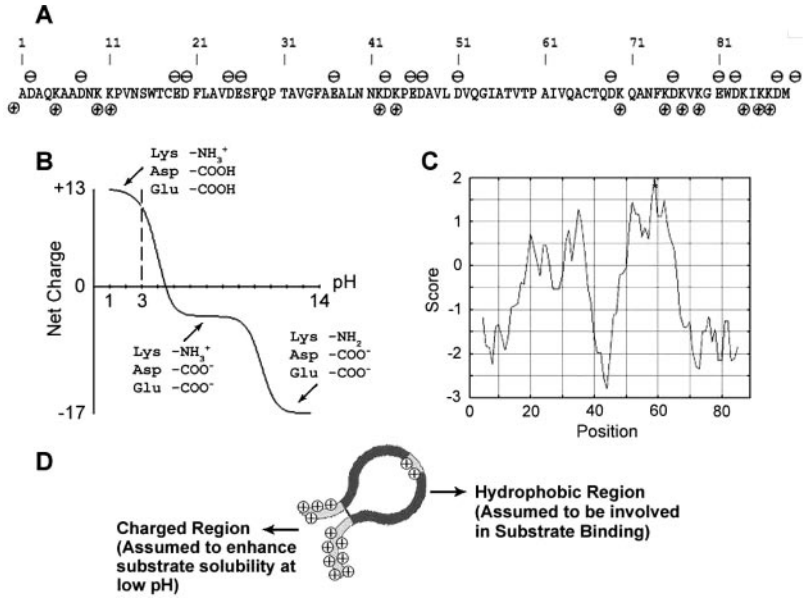

FIG. 6. **Analysis of the primary structure of HdeA.** *A*, the amino acid sequence of HdeA with the positively and negatively charged residues marked. *B*, the theoretical charge titration curve of HdeA, marked with the protonation states of charged residues (*Lys*, *Asp*, and *Glu*). *C*, the hydropathy index of HdeA was calculated based on the method of Kyte and Doolittle (21), with the higher plus value corresponding to a higher level of hydrophobicity and a higher minus value to a higher level of hydrophilicity. *D*, a proposed model of HdeA in its disordered conformation, illustrating an "amphipathic" feature.

sage of the enterobacteria through the stomach may be visualized as follows (as illustrated in Fig. 5). Upon the entrance of the enterobacteria from a neutral environment into an acidic stomach, whose pH is typically maintained in the range of 1–3 by the secreted HCl, the structure of the HdeA protein is immediately transformed from an ordered conformation into a disordered one, which allows it to effectively bind to the denatured periplasmic proteins and thereby prevent their aggregation. When the enterobacteria reach the small intestine, where the pH is typically around 7, the proteins bound to HdeA are released and may eventually refold into their native conformation with or without aid (15, 25) or they may be degraded (25).

An analysis of the primary structure of HdeA suggests a possible explanation for such acid-induced conformational disordering as well as the structural basis for chaperone-like activity in a disordered conformation. Two significant features are apparent. First, as shown in Fig. 6*A*, the positively charged residues, all being Lys, are concentrated at the N- (residues 1–17) and C-terminal (residues 67–89) regions of the protein chain, whereas the negatively charged residues (Asp and Glu) are dispersed throughout the entire sequence. These two Lys-rich terminals may be brought close to each other in space by a disulfide bond between Cys-18 and Cys-66, the existence of which was revealed before by structural determination (10, 11). Upon a decrease of pH, protonation allows the Lys residues of HdeA to remain positively charged but results in the neutralization of the negatively charged Asp and Glu residues (as shown in Fig. 6*B*). Such pH-dependent charge alterations might play a role in the conformational transformations from the ordered to the disordered. This feature of charge distribution for HdeA was also noticed previously (26). In addition, the sole usage of Lys residues as positively charged residues might allow the protonation process to be synchronized, leading to an effective, marked change in the conformation from ordered to disordered. Secondly, hydropathy plotting (Fig. 6*C*) reveals the existence of two relatively hydrophobic fragments (residues 19–41 and 45–65). Such fragments most likely correspond to the exposed hydrophobic surfaces as detected by ANS binding assays (Fig. 4, *A* and *B*) and are thus involved in binding substrate proteins (Fig. 4*C*). The exposure of these fragments in HdeA might be a result of the combined effects of the protonation, the dimer dissociation, and the conformation disordering.

All of these experimental observations and primary structure analyses appear to support the following. Upon the decrease of pH to values lower than 3, conformational disordering somehow transforms HdeA into an "amphipathic molecule" having a hydrophobic tail (consisting of the two hydrophobic fragments) that is involved in substrate binding and a positively charged hydrophilic head (consisting of the two Lys-rich terminals) that enhances the solubility of HdeA-substrate complexes via the hydration of the charges (as schematically shown in Fig. 6*D*). Such a postulated "amphipathic molecule" would act as an effective molecular chaperone in preventing the aggregation of substrate proteins.

A certain degree of structural disordering has been increasingly recognized as an important feature for molecular chaperones to bind their denatured substrate proteins (27–29). HdeA apparently represents an extreme case of this in that it binds the denatured substrate proteins only in its globally disordered conformation. It is conceivable that the conformational disordering might allow the optimal exposure of the hydrophobic fragments, which would play a similar role to the hydrophobic surfaces present in a relatively ordered conformation of other molecular chaperones.

Our preliminary studies demonstrate that the chaperone-like activity of HdeA changed little when the disulfide bond was reduced by dithiothreitol (data not shown), indicating that the breakage of the disulfide bond does not interfere with the hydration of the charged residues. The detail role of the disulfide bond is being investigated.

We have provided evidence in a series of studies that molecular chaperones, at least of the small heat shock family, are able to modulate their chaperone activity via oligomeric dissociation (22, 23). HdeA appears to employ a markedly different strategy for modulating its chaperone activity: it possesses an ordered conformation that is unable to bind denatured substrate proteins under normal physiological conditions (*i.e.* at neutral pH) and transforms into a globally disordered structure that is able to bind the substrate proteins under stress conditions (*i.e.* at extremely low pH).

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