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Conserved amphiphilic feature is essential for periplasmic chaperone HdeA to support acid resistance in enteric bacteria

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The extremely acidic environment of the mammalian stomach (pH 1–3) represents a stressful challenge for enteric pathogenic bacteria, including *Escherichia coli*, *Shigella* and *Brucella*. The *hdeA* (*hns*-dependent expression A) gene was found to be crucial for the survival of these enteric bacteria under extremely low pH conditions. We recently demonstrated that HdeA is able to exhibit chaperone-like activity exclusively within the stomach pH range by transforming from a well-folded conformation at higher pH values (above pH 3) into an unfolded conformation at extremely low pH values (below pH 3). This study was performed to characterize the action mechanisms and underlying specific structural features for HdeA to function in this unfolded conformation. In the present study, we demonstrate that the conserved 'amphiphilic' feature of HdeA, i.e. the exposure of the conserved hydrophobic region and highly charged terminal regions, is essential for exhibiting chaperone-like activity under extremely low pH conditions. Mutations that disrupt this amphiphilic feature markedly reduced the chaperone-like activity of HdeA. The results also strongly suggest that this acid-induced chaperone-like activity of HdeA is crucial for acid resistance of the enteric bacteria. Moreover, our new understanding of this amphiphilic structural feature of HdeA helps to better interpret how this unfolded (disordered) conformation could be functionally active.

Key words: acid stress, chaperone, enteric bacterium, folding, *hns*-dependent expression A (HdeA), hydrophobic interaction.

INTRODUCTION

The extremely acidic environment of the mammalian stomach (pH 1–3) serves as a crucial natural barrier to prevent the entrance of micro-organisms [1]. Enteric pathogenic bacteria (e.g. *Shigella flexneri* and *Escherichia coli*) have evolved a mechanism to survive for several hours under this stress condition [2,3]. In *E. coli*, this acid resistance has been attributed to several cytoplasmic proteins that maintain the intracellular pH under extremely low extracellular pH conditions, e.g. the glutamate-dependent and arginine-dependent acid-resistance systems [4,5]. Nevertheless, the underlying molecular mechanism for protecting biomolecules in the periplasmic space is considerably less well understood. The periplasmic space is located between the inner membrane and the outer membrane of enteric bacteria. Since molecules smaller than 600 Da are allowed to diffuse freely across the outer membrane as a result of the presence of non-specific transporters such as porins [6,7], proteins in the periplasm are more exposed to stressful conditions. Thus periplasmic proteins may need more protection than cytoplasmic proteins when enteric bacteria are exposed to harsh environmental conditions, such as the low pH conditions in the mammalian stomach.

The *hdeA* (*hns*-dependent expression A) gene in *E. coli*, *S. flexneri* and *Brucella abortus* was revealed to be important for the acid-resistance phenotype: loss of function of *hdeA* made the organism highly acid-sensitive [8–10]. The HdeA protein was found to be acid-inducible [11] and is transported into the periplasmic space. We previously found that HdeA exhibits chaperone-like activity exclusively within the pH range of the stomach [12]. It was further demonstrated that HdeA employs an interesting strategy to modulate its chaperone activity: it possesses a well-folded conformation that is unable to bind denatured substrate proteins under neutral or mildly acidic stress conditions (pH 3–7) and transforms into a globally unfolded (disordered) conformation that is able to bind substrate proteins under extremely acidic conditions (pH 1–3) [12]. However, the underlying specific structural features which allow HdeA to function in this unfolded conformation are not well defined.

Molecular chaperones are a large family of proteins that can assist in the unfolding and refolding of native proteins, but primarily serve to suppress protein misfolding or aggregation under stressful cellular conditions, including non-optimal temperature, pH, osmotic pressure and the presence of toxic chemicals [13]. The molecular chaperones induced by heat-shock conditions (heat-shock proteins) have been well characterized. It is generally believed that hydrophobic interactions are involved in mediating the association between chaperones and denatured substrate proteins under neutral conditions [13]. By contrast, the molecular mechanism for chaperones functioning under acidic conditions has not been well elucidated [12,14,15].

We have previously observed the exposure of the internal hydrophobic region of HdeA under acidic conditions [12], which is suggested to be involved in binding substrate proteins. On the other hand, the N- and C-terminal regions of HdeA are highly enriched in positively charged lysine residues [12]. Given that mature periplasmic HdeA functions as a chaperone at extremely

Abbreviations used: ADH, alcohol dehydrogenase; ANS, 8-anilinonaphthalene-1-sulfonic acid; ΔC, C-terminal truncation; HdeA, *hns*-dependent expression A; IPTG, isopropyl β-D-thiogalactoside; LB, Luria–Bertani; ΔN, N-terminal truncation; RbsB, D-ribose transporter subunit B.

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Table 1 Oligonucleotide primers used in the present study

The restriction sites are underlined and the corresponding restriction enzymes are indicated in parentheses. ΔS, signal sequence deleted.

Gene product	Strand	Primer sequence
hdeA (wild-type)	+	5′-CGCTCATGAAAAAAGTATTAGGCGTTATTC-3′ (FatI/BspHI)
	−	5′-GCGTCTAGATTACATATCTTTCTTAATTTTGTCCC-3′ (XbaI)
ΔS	+	5′-CGCCATATGGCGGATGCGCAAAAAGCAGCT-3′ (NdeI)
	−	5′-GCGGAATTCTTACATATCTTTCTTAATTTTGT-3′ (EcoRI)
V52K/V58K 5′ fragment	+	5′-CGCTCATGAAAAAAGTATTAGGCGTTATTC-3′ (FatI/BspHI)
	−	5′-GGTCTTGGTTGCAATACCCTGCTTATCTAA-3′
V52K/V58K 3′ fragment	+	5′-TTAGATAAGCAGGGTATTGCAACCAAGACC-3′
	−	5′-GCGTCTAGATTACATATCTTTCTTAATTTTGTCCC-3′ (XbaI)
V52K/V58K	+	5′-CGCTCATGAAAAAAGTATTAGGCGTTATTC-3′ (FatI/BspHI)
	−	5′-GCGTCTAGATTACATATCTTTCTTAATTTTGTCCC-3′ (XbaI)
V52K/V58K/ΔS5′ fragment	+	5′-CGCCATATGGCGGATGCGCAAAAGCAGCT-3′ (NdeI)
	−	5′-GGTCTTGGTTGCAATACCCTGCTTATCTAA-3′
V52K/V58K/ΔS3′ fragment	+	5′-TTAGATAAGCAGGGTATTGCAACCAAGACC-3′
	−	5′-GCGGAATTCTTACATATCTTTCTTAATTTTGT-3′ (EcoRI)
V52K/V58K/ΔS	+	5′-CGCCATATGGCGGATGCGCAAAAGCAGCT-3′ (NdeI)
	−	5′-GCGGAATTCTTACATATCTTTCTTAATTTTGT-3′ (EcoRI)
ΔN 5′ fragment	+	5′-CGCTCATGAAAAAAGTATTAGGCGTTATTC-3′ (FatI/BspHI)
	−	5′-CCAGG AGTTG ACCGG TGCAT TGCTC ACAAC-3′
ΔN 3′ fragment	+	5′-GTTGT GAGCA ATGCA CCGGT CAACT CCTGG-3′
	−	5′-GCGTCTAGATTACATATCTTTCTTAATTTTGTCCC-3′ (XbaI)
ΔN	+	5′-CGCTCATGAAAAAAGTATTAGGCGTTATTC-3′ (FatI/BspHI)
	−	5′-GCGTCTAGATTACATATCTTTCTTAATTTTGTCCC-3′ (XbaI)
ΔN/ΔS	+	5′-CGCCATATGCCGGTCAACTCCTGGACCTG-3′ (NdeI)
	−	5′-GCGGAATTCTTACATATCTTTCTTAATTTTGT-3′ (EcoRI)
ΔC	+	5′-CGCCATATGA AAAAAGTATTAGGCGTTATTC-3′ (NdeI)
	−	5′-GCGGAATTCTTAAAAGTTGGCTTGTTTATCC-3′ (EcoRI)
ΔC/ΔS	+	5′-CGCCATATGGCGGATGCGCAAAAAGCAGCT-3′ (NdeI)
	−	5′-GCGGAATTCTTAAAAGTTGGCTTGTTTATCC-3′ (EcoRI)
*K42A/K44A/*ΔS5′ fragment	+	5′-CGCCATATGGCGGATGCGCAAAAGCAGCT-3′ (NdeI)
	−	5′-CGCATCTTCTGGCGCATCCGCGTTGTTCAG-3′
*K42A/K44A/*ΔS3′ fragment	+	5′-CTGAACAACGCGGATGCGCCAGAAGATGCG-3′
	−	5′-GCGGAATTCTTACATATCTTTCTTAATTTTGT-3′ (EcoRI)
K42A/K44A/ΔS	+	5′-CGCCATATGGCGGATGCGCAAAAGCAGCT-3′ (NdeI)
	−	5′-GCGGAATTCTTACATATCTTTCTTAATTTTGT-3′ (EcoRI)

low pH values (pH < 3) [12], protonation allows the lysine residues to remain positively charged, but results in the neutralization of the negatively charged aspartate and glutamate residues. Previous NMR spectrometry analysis showed that the clustered positively charged lysine residues in the N- and C-terminal regions are exposed to the solvent exclusively at pH values below pH 3 [12]. These highly charged N- and C-terminal regions are suggested to be crucial for increasing the solubility of the HdeA–substrate complex at extremely low pH values because of increased hydration. In the present paper, the term 'amphiphilic' is used to refer to the distinct pattern of charge and hydrophobicity distribution of HdeA, i.e. N- and C-terminal regions being markedly positively charged and the internal region being markedly hydrophobic [12].

The present study was performed in an attempt to mechanistically understand the role of the amphiphilic feature, especially the importance of the hydrophobic region and charged terminal regions, for the function of HdeA. Our results showed that truncation of the highly charged N- or C-terminal regions decreases the solubility of the HdeA–substrate complex, whereas replacement of two hydrophobic valine residues in the conserved hydrophobic region greatly reduces the substrate-binding activity of HdeA, supporting the hypothesis that the amphiphilic structural feature is required for HdeA to exhibit chaperone-like activity. Furthermore, this amphiphilic structural feature also helps to better interpret how unfolded (disordered) conformations could be functionally active.

EXPERIMENTAL

Bacterial strains and growth conditions

E. coli strain BL21(DE3) was used for the expression and purification of wild-type and mutant HdeA; *E. coli* Δ*hdeA* mutant strain JWK347 (the *hdeA* single-gene-knockout strain [16]), derived from the parent strain BW25113 {*rrnB3* Δ*lacZ4787 hsdR514* Δ*(araBAD)567* Δ*(rhaBAD)568 rph-1* [17]}, was used for the acid-resistance assay. Bacterial cells were grown in LB (Luria–Bertani) broth or on LB agar medium (Sigma). Antibiotics were used at final concentrations of 60 μg/ml for ampicillin and 50 μg/ml for kanamycin (Sigma). *E. coli* strains BW25113 and JWK3478 were obtained from the Nara Institute of Science and Technology (Ikoma, Nara, Japan).

Construction of plasmids for gene expression

For purification of the wild-type and mutant HdeA proteins, pET21b plasmids (Novagen) with inserted wild-type or ΔS (signal sequence deleted) *hdeA* genes were generated to express the proteins in the cytoplasm of *E. coli* BL21(DE3) cells. The genomic DNA of *E. coli* JM109(DE3) was used as a template for PCR. The primers used for the amplification of the DNA fragments in the present study are listed in Table 1. Point mutations were generated via overlap PCR [18]. The products were digested with NdeI and EcoRI and subcloned into pET21b before being transformed into *E. coli* BL21(DE3).

For acid-resistance assays, the plasmids pTrc99A-HdeA and pTrc99A-HdeA V52K/V58K were generated to express the wild-type and mutant HdeA proteins respectively in the periplasmic space of *E. coli* JWK3478 cells. The DNA fragment containing the *hdeA* gene with the signal sequence was amplified by PCR from *E. coli* JM109(DE3) genomic DNA using primers incorporating BspHI and XbaI restriction sites (see Table 1). Point mutations were generated via overlap PCR. The products were digested and inserted between the NcoI and XbaI restriction sites of the pTrc99A expression vector (BspHI and NcoI generate the same sticky ends). The ligated plasmids were then transformed into *E. coli* JWK3478 cells for amplification and expression. The constructed plasmids were verified by DNA sequencing (Bioasia, Shanghai, China).

Purification of HdeA proteins and preparation of periplasmic protein extracts

The expression of wild-type and mutant HdeA in pET21b-transformed BL21(DE3) cells was induced at an attenuance (D_{600}) of 0.6 by adding 0.5 mM IPTG (isopropyl β-D-thiogalactoside) (Sigma) and the bacteria were grown for a further 4 h at 37 °C. The overexpressed proteins were purified using methods described previously [12]. HdeA V52K/V58K, HdeAΔC (where ΔC is C-terminal truncation), HdeAΔN (where ΔN is N-terminal truncation) and HdeA K42A/K44A proteins were verified by measuring their molecular masses via MS. Samples were analysed in an Ultraflex MALDI TOF/TOF (matrix-assisted laser-desorption ionization–tandem time-of-flight) mass spectrometer (Bruker Daltonics) under the control of FlexControl™ 2.2 software (Bruker Daltonics). The protein concentrations were determined by the BCA (bicinchoninic acid) method (Pierce), with BSA used as a standard. The molar concentrations for HdeA, as well as the substrate proteins throughout the present paper, all refer to the monomeric form. The purified HdeA proteins used in the *in vitro* assays have no signal peptide.

The expression of wild-type and V52K/V58K mutant HdeA in pTrc99A-transformed JWK3478 cells was induced as described above. Periplasmic protein extracts of *E. coli* BW25113, JWK3478 and JWK3478 strains transformed with genes encoding wild-type HdeA or HdeA V52K/V58K were obtained by osmotic shock following methods described previously [19,20].

Primary structure analysis

Sequences of three HdeA homologues from *E. coli*, *S. flexneri* and *B. abortus* were aligned by using T-coffee [21]. The hydropathy index of HdeA was calculated using the program provided at http://cn.expasy.org/tools/protscale.html, which applied the method of Kyte and Doolittle [22]. The window size of the index was set to all residues of HdeA.

ANS (8-anilinonaphthalene-1-sulfonic acid)-binding assay

The binding of ANS to wild-type or V52K/V58K mutant HdeA was monitored by measuring the increase of fluorescence intensity of ANS on its binding to the proteins, using a Hitachi F4500 fluorescence spectrophotometer equipped with a temperature-control system. To obtain the fluorescence spectra, samples containing 15 μM protein were excited at 395 nm, with the emission recorded at 420–600 nm.

Chemical cross-linking

Chemical cross-linking by glutaraldehyde was performed mainly following methods described previously [23]. Glutaraldehyde (0.05 %) was added to react with wild-type or V52K/V58K mutant HdeA (10 μM) for 3 min at room temperature (20 °C). The reactions were quenched with 1 M Tris/HCl (pH 7.0) and then SDS/PAGE loading buffer was added. The cross-linked samples were then analysed by urea/SDS/PAGE (12 % gels) and visualized by silver staining.

CD spectroscopy

CD spectroscopy was performed using a J-715-150L spectrometer (Jasco). The far-UV CD spectra were recorded in the wavelength range 200–250 nm, with the pathlength of the cuvette being 2 mm. Samples containing 20 μM protein were each adjusted to a designated pH value using ethanoic (acetic) acid/HCl (for pH values < 2.9), glycine/HCl (for pH 2.9) or sodium acetate/ethanoic acid (for pH values > 2.9) and incubated at 25 °C for 1 h before recording.

Assay of chaperone-like activity

Chaperone-like activity was assayed by following the appearance of substrate proteins in the supernatant instead of the pellet, and this was mainly performed according to methods described previously [12]. Trichloroacetate was added as an anion to achieve effective aggregation of the substrate proteins at low pH values [12]. The presence of substrate proteins in the supernatant or pellet, after being incubated at 25 °C for 60 min and centrifuged at 13 000 ***g*** for 10 min at 25 °C. Each sample supernatant was neutralized to pH 7 by adding 2 M Tris (pH ~11.5), with the corresponding pellet resuspended in water to a volume equal to that of the supernatant before being examined by urea/SDS/PAGE (12 % gels) and visualized by Coomassie Brilliant Blue staining. Western blotting was used to detect HdeAΔC (as this mutant protein stained poorly with Coomassie Brilliant Blue). Blocking of membranes was performed using blocking buffer [5 % (w/v) non-fat dried skimmed milk powder in 20 mM Tris/HCl (pH 7.5), 150 mM NaCl and 0.05 % Tween 20] for 12 h at 4°C. The rabbit polyclonal anti-HdeA antibody (1:1000 dilution) used to detect wild-type HdeA and HdeAΔC has been described previously [12]. Alkaline phosphatase-conjugated goat anti-rabbit IgG (1:1000 dilution; Sigma) was used as the secondary antibody. All antibody incubations were performed for 2 h at room temperature. Gel bands and Western blots were scanned and quantified using the Quantiscan program (Biosoft). ADH (alcohol dehydrogenase; monomeric molecular mass of 35.3 kDa) was obtained from Sigma and RbsB (D-ribose transporter subunit B) (monomeric molecular mass of 28.5 kDa) was purified according to a procedure described previously [24].

Size-exclusion chromatography

Size-exclusion chromatography was performed mainly according to procedures described previously [12]. For the dissociation of the HdeA homodimer, HdeA (100 μM) was incubated in an HCl buffer [0.015 M HCl and 0.15 M NaCl (pH 2.0)] or a 50 mM sodium phosphate buffer [50 mM sodium phosphate and 0.15 M NaCl (pH 7.0)] at 25 °C for 60 min. For the association between wild-type or mutant HdeA and denatured ADH (see Figure 5), 120 μl samples [in 0.15 M NaCl/HCl (pH 2.0)] containing both HdeA (100 μM) and ADH (30 μM) were incubated for 1 h at 20 °C and centrifuged at 13 000 ***g*** for 10 min at 20 °C, before 100 μl of the supernatant was loaded on to a Superdex HR-200 size-exclusion column (10 mm × 30 cm, Amersham Biosciences).

Acid-resistance assay

E. coli cells were grown overnight in LB broth with aeration at 37 °C. An aliquot (50 μl) of each overnight culture was then inoculated into LB broth (20 ml). Expression of wild-type or mutant HdeA was induced by adding IPTG to a final concentration of 0.5 mM when the culture reached an attenuance (D_{600}) of 0.6. The cultures were grown into stationary phase with aeration at 37 °C and diluted 1:1000 into 1 ml of LB (either neutral pH or pH 2.0 (adjusted with HCl)] pre-warmed at 37 °C. The pH value of the acidic medium did not vary at this dilution. Viable cell counts in neutral LB broth were determined by plating serial dilutions on to LB agar, which were used as initial cell populations. The acidic LB broth (pH 2.0) inoculated with *E. coli* was incubated at 37 °C for 2 h, and viable cell counts in acidic LB broth were determined and used as final cell populations. The percentage survival was determined as the percentage of the viable cell counts after the acid stress compared with the initial viable cell counts (neutral pH) at the zero time point.

RESULTS

The amphiphilic feature of HdeA is highly conserved

We suggested previously an amphiphilic feature to explain how the mature HdeA protein of *E. coli* exhibits chaperone-like activity under extremely low pH conditions. The major feature is that HdeA is transformed into an unfolded structure on the decrease of the pH to values lower than 3, which is characterized by the positively charged lysine residues in the N- and C-terminal regions (suggested to enhance the solubility of the HdeA–substrate complex) and the exposure of an internal hydrophobic region (suggested to be involved in substrate binding) [12]. To demonstrate the essential role of these sequence features in the function of HdeA, we began by analysing how these features are conserved among the HdeA homologues so far reported to confer acid resistance to the host species [8–10]. For this purpose, the amino acid sequences of the HdeA homologues from *E. coli*, *S. flexneri* and *B. abortus* were compared (Figure 1A). It was revealed that HdeA from *E. coli* shares 100 % amino acid identity with that from *Shigella* and 46 % amino acid identity (84 % similarity) with that from *Brucella*. A detailed examination of the distribution of the positively charged residues (Figure 1A) as well as the hydropathy plots (Figure 1B) indicate that the HdeA homologues from the three different species exhibit a similar amphiphilic sequence pattern, i.e. highly positively charged at the two terminal regions and highly hydrophobic in the internal region. Furthermore, the internal hydrophobic region (amino acid residues 17–66) apparently exhibits a higher level of residue conservation, sharing 58 % amino acid identity and 92 % similarity, than the terminal regions (residues 1–16 and 67–89), which share 30 % amino acid identity and 75 % similarity. In addition, two positively charged lysine residues, Lys^{42} and Lys^{44}, are conserved in all three HdeA homologues, generating a valley on the hydropathy curve in the middle of the internal hydrophobic region.

HdeAΔN co-precipitates with denatured substrate proteins

To test the importance of the amphiphilic feature of HdeA for its chaperone-like activity, we generated different mutant HdeA proteins in which the conserved amphiphilic feature is disrupted. The chaperone-like activity of these mutant proteins under extremely acidic conditions was then tested using RbsB (a periplasmic protein) and ADH (a commonly used substrate for chaperone-like activity assays) as substrate proteins at

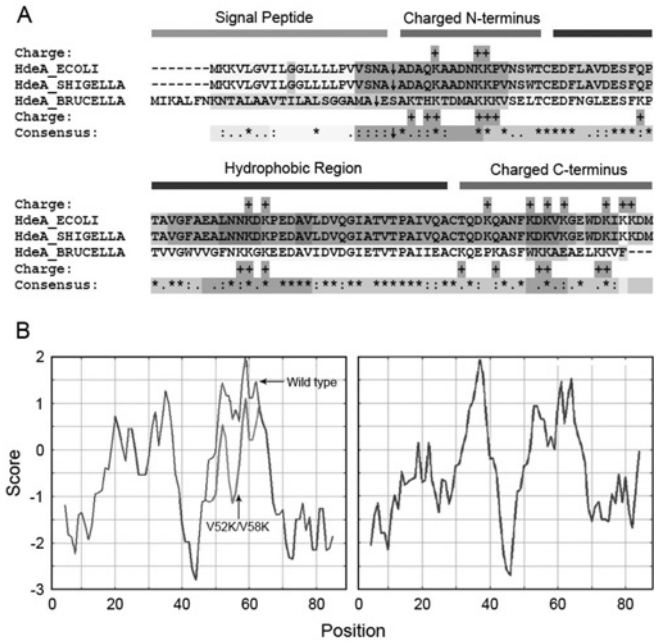

Figure 1 Analysis of the primary structure of HdeA

(**A**) Alignment of amino acid sequences of the HdeA homologues from *E. coli* (HdeA_ECOLI), *Shigella* (HdeA_SHIGELLA) and *Brucella* (HdeA_BRUCELLA). The signal peptide, the positively charged N- and C-termini, and the hydrophobic region of HdeA are indicated. Residues positively charged under acidic conditions ('+'), identical residues ('*') and similar residues ('.' or ':') are shown. Shading of the sequences indicates the degree of conservation (with no shading indicating lowest conservation, and dark grey indicating the highest conservation). (**B**) Hydropathy plots of the HdeA homologues from *E. coli*/*Shigella* (left panel) and *Brucella* (right panel), and the HdeA V52K/V58K mutant (left panel).

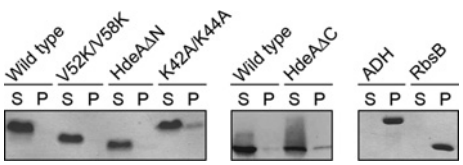

Figure 2 Analysis of the solubility of the various mutant HdeA proteins and substrate proteins under extremely acidic condition

Urea/SDS/PAGE analysis of the supernatant (S) and pellet (P) fractions of the indicated proteins after treatment at pH 1 for 60 min. Proteins were used at the following concentrations: wild-type and mutant HdeA at 24 μM, ADH at 10 μM and RbsB at 5 μM. Trichloroacetate (0.1 M) was added as the anion to achieve effective aggregation of the substrate proteins. The proteins were all visualized by Coomassie Brilliant Blue staining, except for HdeAΔC, which was visualized by Western blotting using a polyclonal antibody against wild-type HdeA because it stained poorly with Coomassie Brilliant Blue for unknown reasons. Wild-type HdeA was also visualized by Western blotting as a control.

three monomeric molar ratios of chaperone/substrate. We first examined the role of the positively charged N-terminal region of HdeA in increasing the solubility of the HdeA–substrate complex under extremely low pH conditions by truncating 11 amino acid residues, including three positively charged lysine residues, from the N-terminal region of the mature HdeA protein (see Figure 1). As shown in Figure 2, HdeAΔN itself remained soluble under extremely low pH conditions in the absence of denatured substrate proteins. However, HdeAΔN exhibited much lower chaperone-like activity on both RbsB and ADH compared with the wild-type protein at the three molar ratios of chaperone/substrate tested, and showed more co-precipitation with the substrate proteins than the wild-type protein did (Figures 3 and 4), suggesting that truncation of the N-terminal positively charged residues

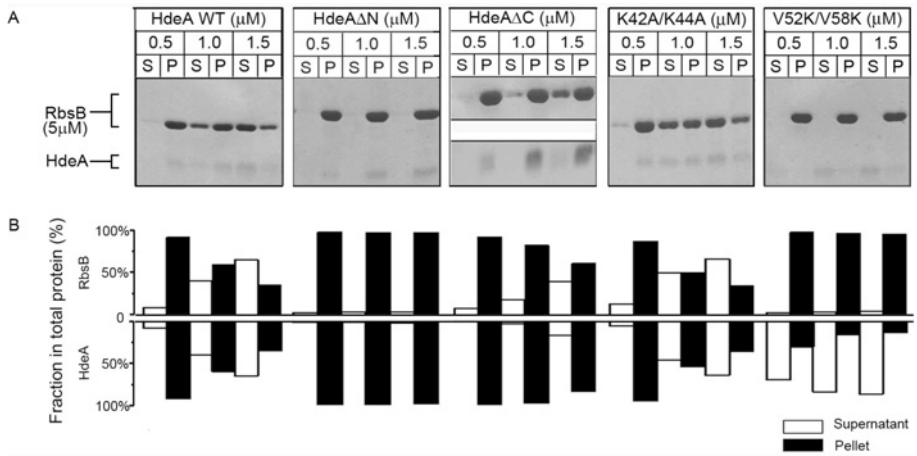

Figure 3 Analysis of chaperone-like activity of the various mutant HdeA proteins under extremely acidic conditions using RbsB as a substrate protein

(**A**) Representative results of urea/SDS/PAGE analysis of the supernatant (S) and pellet (P) fractions of the RbsB substrate protein (5 μM, containing 0.1 M trichloroacetate) subjected to acid treatment in the presence of 0.5, 1.0 or 1.5 μM of either wild-type or mutant HdeA. Proteins were visualized by Coomassie Brilliant Blue staining. For HdeAΔC, the HdeA proteins were visualized by Western blotting using polyclonal antibodies against wild-type HdeA because HdeAΔC stained poorly with Coomassie Brilliant Blue for unknown reasons. Protein concentrations were determined by using the BCA (bicinchoninic acid) method, using BSA as a protein standard. (**B**) Quantitative analysis of the gels and Western blots in (**A**) demonstrating the percentage of RbsB and HdeA in the supernatant and pellet fractions as a fraction of total protein present as determined by densitometry (see the Experimental section for details).

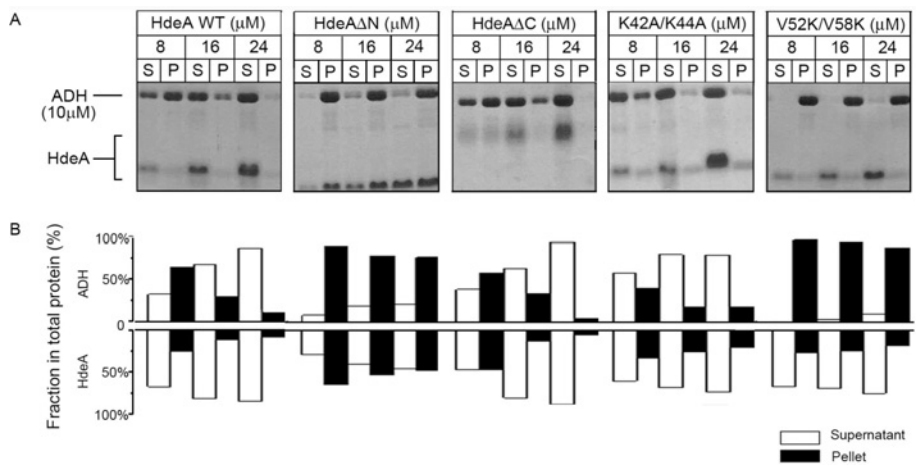

Figure 4 Analysis of chaperone-like activity for the various mutant HdeA proteins under extremely acidic conditions using ADH as a substrate protein

(**A**) Representative results of urea/SDS/PAGE analysis of the supernatant (S) and pellet (P) fractions of the ADH substrate protein (10 μM, containing 0.1 M trichloroacetate) subjected to acid treatment in the presence of 8, 12 or 24 μM of either wild-type or mutant HdeA. Proteins were visualized by Coomassie Brilliant Blue staining. Protein concentrations were determined by using the BCA (bicinchoninic acid) method, using BSA as a protein standard. (**B**) Quantitative analysis of the gels shown in (**A**) demonstrating the percentage of ADH and HdeA found in the supernatant and pellet fractions as a fraction of the total protein present as determined by densitometry (see the Experimental section for details).

decreases the solubility of the HdeA–substrate complex. The co-precipitation of HdeAΔN and denatured substrate proteins also suggested that the formation of the HdeA–substrate complex was not affected by the truncation. These results demonstrate that the positively charged N-terminal region is crucial to increasing the solubility of the HdeA–substrate complex under extremely low pH conditions.

HdeAΔC co-precipitates with denatured substrate protein

The role of the positive charges at the C-terminal region of HdeA was examined by truncating 15 amino acid residues, including six lysine residues, from the C-terminus (see Figure 1). Similarly to HdeAΔN, HdeAΔC also remained largely soluble under extremely low pH conditions in the absence of denatured substrate proteins (Figure 2), but its activity in preventing the aggregation of RbsB under extremely acidic conditions was greatly decreased in comparison with the wild-type protein at the three molar ratios of chaperone/substrate examined. Furthermore, HdeAΔC showed stronger co-precipitation with RbsB (Figure 3) than the wild-type protein did, suggesting that truncation of the C-terminal positively charged residues decreases the solubility of the HdeA–RbsB complex. In the case of ADH, however, the chaperone-like activity of the mutant protein was equivalent to that of the wild-type protein (Figure 4). These observations demonstrated that the positive charges at the C-terminal region of HdeA do not play a critical role in binding the substrate proteins, but they are crucial for increasing the solubility of the complex between HdeA and substrate proteins such as RbsB under acidic conditions. Our effort to express exogenous HdeA with double deletions of both the N- and C-terminal regions was not successful in *E. coli* (results not shown), possibly because the

simultaneous truncation of both regions greatly destabilized the protein.

Replacement of conserved positively charged residues in the hydrophobic region has little effect on the chaperone-like activity of HdeA

Two positively charged lysine residues (Lys[42] and Lys[44]) in the middle of the hydrophobic region are conserved among HdeA homologues, resulting in a valley in the hydropathy plot of HdeA. To test whether they play any critical role in the chaperone-like activity of HdeA, we replaced these two residues in *E. coli* HdeA simultaneously with two alanine residues (HdeA K42A/K44A). This replacement resulted in a slight increase in the hydrophobicity of the internal region of HdeA, as indicated by hydropathy plotting (results not shown). The HdeA K42A/K44A mutant exhibited partial aggregation under extremely acidic conditions (Figure 2), possibly as a result of enhanced hydrophobic interactions between the mutant HdeA protein molecules as a result of the replacement of the two positively charged lysine residues. The chaperone-like activity of the mutant HdeA on RbsB was found to be equivalent to that of the wild-type protein (Figure 3). Similar results were obtained using ADH as the substrate, except that the mutant protein showed slightly higher activity than the wild-type protein at 8 μM HdeA, possibly due to an increased hydrophobic interaction with the substrate protein.

Replacement of conserved hydrophobic residues in the hydrophobic region greatly reduces chaperone-like activity of HdeA

To test the role of the internal hydrophobic region in the chaperone-like activity of HdeA, we replaced two conserved hydrophobic residues in the hydrophobic region, Val[52] and Val[58], with hydrophilic lysine residues. The hydropathy plot of the HdeA V52K/V58K mutant indicates a partial loss of hydrophobicity in the conserved hydrophobic region (Figure 1B, left panel). The mutant protein remained soluble in the absence of denatured substrate proteins under extremely acidic conditions (Figure 2). However, its ability to suppress the aggregation of both RbsB (Figure 3) and ADH (Figure 4) under extremely acidic conditions was much lower than that of wild-type HdeA at the three molar ratios of chaperone/substrate tested. Nevertheless, unlike HdeAΔN and HdeAΔC, the majority of the HdeA V52K/V58K mutant protein remained in the supernatant fraction, whereas most of the substrate proteins were found in the pellet fraction. These observations indicate that the failure of the HdeA V52K/V58K mutant to suppress the aggregation of ADH and RbsB is probably the result of a lack of capacity to bind denatured substrate proteins under extremely low pH conditions. Indeed, as shown by size-exclusion chromatography analysis (Figure 5), wild-type HdeA was able to form a soluble complex with ADH, demonstrated by the appearance of an absorption peak on the elution curve [12]; however, no complex between ADH and HdeA V52K/V58K was detected. These results strongly suggest that the hydrophobic feature of the conserved internal region of HdeA is essential for mediating the association between HdeA and denatured substrate proteins, and is therefore critical for chaperone-like activity.

Replacement of conserved hydrophobic residues in the hydrophobic region reduces exposure of the hydrophobic surface, but has no effect on acid-induced structural transformation of HdeA

Previous work has suggested that HdeA exists as homodimers at neutral pH and starts to dissociate significantly into monomers at approx. pH 4 [9]. It was further found that HdeA transforms from a well-folded conformation to a globally unfolded

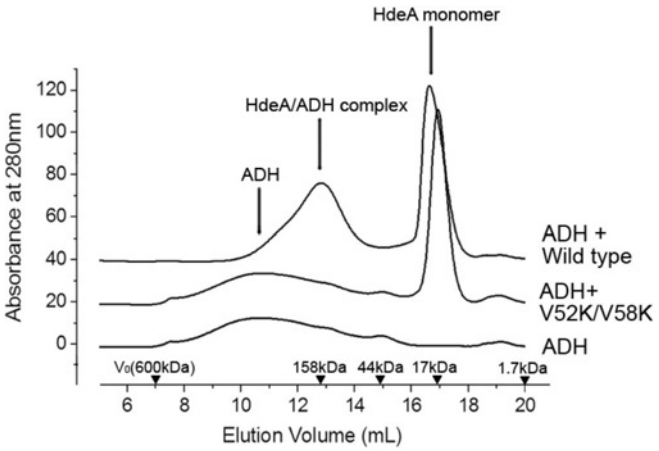

Figure 5 Replacement of Val[52] and Val[58] with lysine residues in the hydrophobic region markedly reduces the capacity of HdeA to bind to unfolded substrate protein at pH 2

Elution curves of size-exclusion chromatography of samples containing ADH (30 μM) alone or in the presence of wild-type HdeA or the HdeA V52K/V58K mutant (both at 100 μM). The samples were analysed after being pre-incubated at pH 2 for 60 min. Protein elution was detected by measuring the absorbance at 280 nm (A_{280}), with molecular-mass standards shown (in kDa) along the *x*-axis. V_0, void volume.

conformation on decreasing the pH to less than 3, and exposes hydrophobic surfaces that appear to be involved in binding denaturing substrate proteins [12]. Therefore the loss of substrate-binding activity of the HdeA V52K/V58K mutant protein could be due to either an inability to expose the hydrophobic surfaces or a failure in oligomeric status or secondary-structure transformation. To test these possibilities, we first investigated the effect of the V52K/V58K mutation on the exposure of the hydrophobic surface of HdeA at extremely low pH by examining the fluorescence emission spectra of the fluorescence probe ANS [12,23]. ANS binds preferentially to hydrophobic sites in proteins, and its binding is accompanied by an enhancement in ANS fluorescence intensity and a shift of the emission maximum to shorter wavelengths. The HdeA V52K/V58K mutant exhibited a markedly reduced fluorescence intensity of bound ANS at pH 1.5 in comparison with the wild-type protein (Figure 6), strongly suggesting a corresponding decrease in the exposure of the hydrophobic surface in the HdeA V52K/V58K mutant under extremely low pH conditions. The maximum fluorescence emission wavelength was shifted to shorter wavelengths to a lesser degree for the mutant protein in comparison with the wild-type protein (Figure 6, inset), consistently reflecting a markedly decreased exposure of the hydrophobic surface in the HdeA V52K/V58K mutant.

We then examined the effect of this residue substitution on the oligomerization and the pH-dependent conformational transformation of HdeA. Chemical cross-linking demonstrated that the HdeA V52K/V58K mutant protein is similar to the wild-type protein in forming homodimers at pH 7 (Figure 7A). Size-exclusion chromatography analysis demonstrated that V52K/V58K homodimers are able to dissociate into monomers at pH 2, also similar to the wild-type HdeA protein (Figure 7B). These results suggest that the replacement of Val[52] and Val[58] in HdeA has little effect on subunit association at neutral pH or dissociation at acidic pH. Moreover, the HdeA V52K/V58K mutant protein also resembled the wild-type protein with a well-folded α-helix-rich conformation at a pH value higher than 3.9, which became almost totally unfolded at a pH lower than

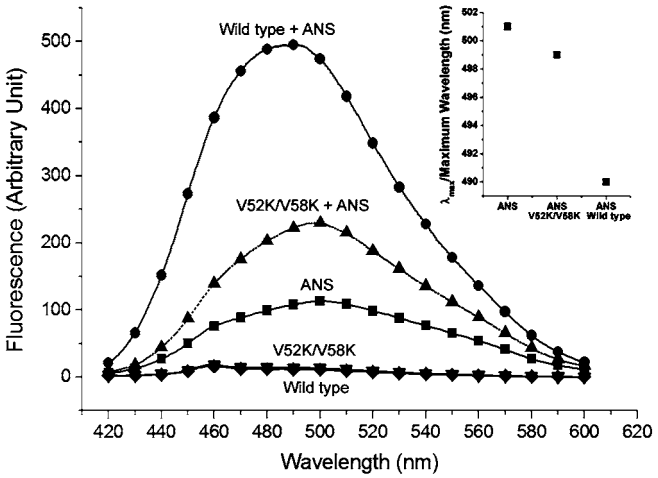

Figure 6 Replacement of Val[52] and Val[58] with lysine residues in the hydrophobic region markedly reduces the exposure of hydrophobic surface of HdeA at acidic pH

Fluorescence emission spectra of ANS at pH 2 in the presence of either wild-type or V52K/V58K mutant HdeA. The fluorescence curves were recorded for ANS alone or in the presence of 15 μM wild-type or V52K/V58K mutant HdeA after being excited at 395 nm. Also shown are the fluorescence spectra of wild-type or mutant HdeA (both at 15 μM) in the absence of ANS.

2.9 (Figure 7C). All these results consistently suggest that the V52K/V58K mutation did not affect subunit interactions or acid-induced conformational transformation (from well-folded to unfolded) of HdeA, and that the hydrophobic feature of the conserved internal region, which primarily depends on the primary sequence rather than secondary and tertiary structures, is essential for HdeA to bind to denatured substrate proteins under extremely acidic conditions.

Mutated HdeA with reduced exposure of the hydrophobic surface cannot support acid resistance in *E. coli*

The capacity of the various mutant HdeA proteins described above to support acid resistance of *E. coli* cells was examined using an *hdeA* single-gene-knockout strain designated Δ*hdeA*. The Δ*hdeA* strain exhibited a survival ratio of 14 % after being exposed to acid stress at pH 2.0 for 2 h, whereas the wild-type *E. coli* strain exhibited a survival ratio of 38 % under the same conditions (Figure 8A), consistent with previous reports that the knockout of the *hdeA* gene resulted in an acid-sensitive phenotype [8–10]. Plasmids carrying genes encoding wild-type HdeA, HdeA V52K/V58K, HdeAΔN or HdeAΔC with the signal sequence were each transformed into the Δ*hdeA* strain. SDS/PAGE analysis indicated that proper amounts of only wild-type and V52K/V58K mutant HdeA were expressed and correctly localized into the periplasmic space of Δ*hdeA* cells (Figure 8B), whereas HdeAΔN and HdeAΔC were undetectable in the periplasmic space (results not shown). The Δ*hdeA* strain transformed with the wild-type *hdeA* gene exhibited an increased survival ratio of over 27 %, whereas the Δ*hdeA* strain transformed with the gene encoding HdeA V52K/V58K exhibited a survival ratio of less than 10 % after acid stress at pH 2.0 for 2 h (Figure 8A), indicating that the wild-type *hdeA* gene was able to restore acid resistance, whereas the gene encoding HdeA V52K/V58K was not. These results strongly suggest that the hydrophobic feature of the conserved internal hydrophobic region of HdeA is crucial for supporting acid resistance in *E. coli*, and that the chaperone-like activity of HdeA is required for acid resistance.

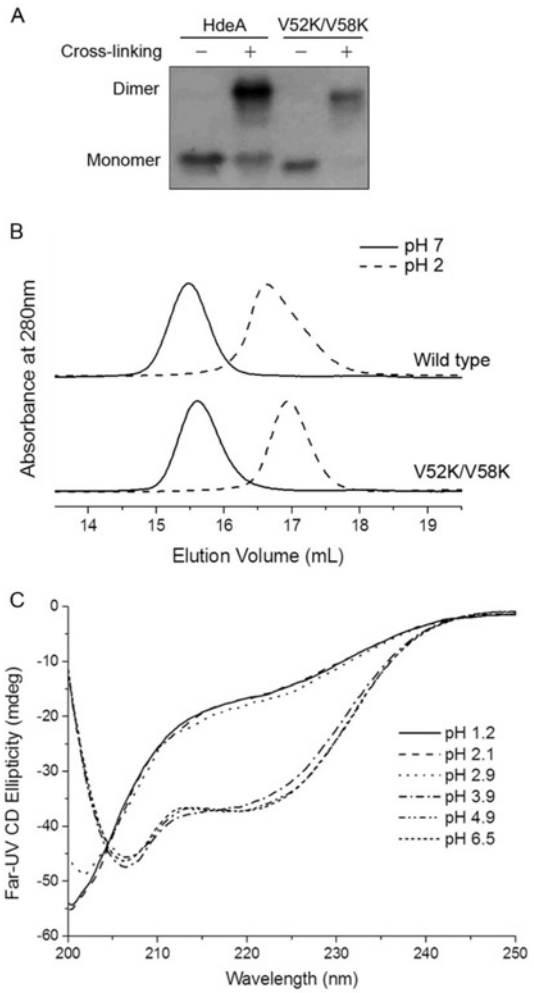

Figure 7 Replacement of Val[52] and Val[58] with lysine residues in the hydrophobic region has little effect on the dimerization/dissociation and acid-induced structural transformation of HdeA

(**A**) Urea/SDS/PAGE analysis of wild-type HdeA and HdeA V52K/V58K mutant (10 μM) alone (−) or after being subjected to cross-linking with 0.05 % (v/v) glutaraldehyde (+) at neutral pH. Proteins were visualized by silver staining. (**B**) Elution curves from size-exclusion chromatography analysis of wild-type HdeA and HdeA V52K/V58K mutant (both at 100 μM) after being pre-incubated at pH 2 (broken lines) or pH 7 (solid lines) for 60 min. (**C**) The far-UV CD spectra of the HdeA V52K/V58K mutant recorded at the indicated pH values. mdeg, [θ] (measured in degrees · cm^2 · $dmol^{-1}$).

DISCUSSION

This present study represents our effort to understand whether the conserved amphiphilic feature, which is largely dependent on the primary structure rather than the secondary and tertiary structures, is essential for HdeA to exhibit chaperone-like activity and to support acid resistance in *E. coli*. Our experimental observations include the following: (i) truncation of the positively charged N- and C-terminal regions reduced the chaperone-like activity of HdeA as a result of decreased solubility of the HdeA–substrate complex under extremely low pH conditions; (ii) the replacement of two conserved valine residues in the conserved hydrophobic region of HdeA with lysine residues markedly reduced the exposure of the hydrophobic surface and substrate-binding activity of HdeA under acidic conditions; and (iii) the HdeA V52K/V58K mutant protein could neither prevent the aggregation of unfolded substrate proteins nor support acid resistance in

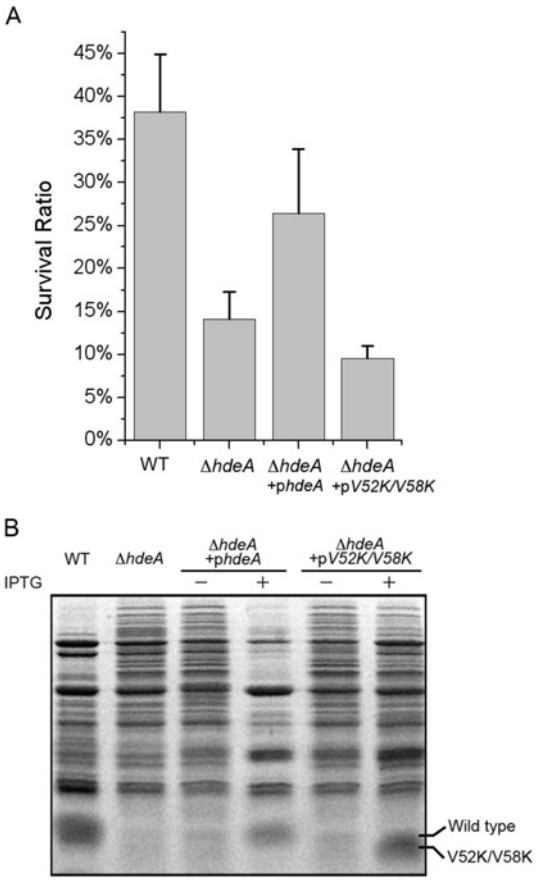

Figure 8 The HdeA V52K/V58K mutant protein fails to support acid resistance in *E. coli*

(**A**) Survival ratios of the *E. coli* BW25113 strain (WT; having the wild-type *hdeA* gene), the *hdeA* single-gene-knockout strain JWK3478 (Δ*hdeA*) and the JWK3478 strain transformed with either the pTrc99A-HdeA (Δ*hdeA*+p*hdeA*; plasmid expressing wild-type HdeA) or pTrc99A-HdeA-V52K/V58K (Δ*hdeA*+p*V52K/V58K*; plasmid expressing HdeA V52K/V58K) after being exposed to acid stress at pH 2.0 for 2 h. Results are means ± S.D. ($n = 2$). (**B**) Urea/SDS/PAGE analysis of proteins present in the whole periplasmic extracts of the strains indicated in (**A**) after acid stress. Expression of the exogenous HdeA proteins were induced by adding IPTG to a final concentration of 0.5 mM after the cells were grown to an attenuance (D_{600}) of 0.6 (+). Cells grown in the absence of IPTG (−) were also analysed as a control.

E. coli cells. Taken together, these observations strongly suggest that the conserved amphiphilic feature, i.e. the exposure of the internal hydrophobic region and the positively charged terminal regions under extremely low pH conditions, is essential for HdeA to accomplish its physiological function, in allowing the organism to survive under extremely acidic conditions.

The mechanism of action of molecular chaperones acting under acidic conditions has not been thoroughly investigated [12,14,15]. For those chaperones that function under neutral conditions, it is generally believed that they act by binding to the denatured substrate proteins via hydrophobic interactions. Proteins are often prone to unfolding and exposure of hydrophobic surfaces under acidic conditions, which usually results in aggregation [12,25–27]. We found that HdeA and its substrate proteins largely expose hydrophobic surfaces [12], and hydrophobic interactions play a crucial role in the association between HdeA and denatured substrate proteins under extremely low pH conditions. The reason that substrate proteins have a strong propensity to aggregate, but HdeA or HdeA–substrate complexes are resistant to aggregation, is probably the result of exposure of the highly charged terminal segments of HdeA, which increases the solubility of both HdeA itself and HdeA–substrate complexes under extremely low pH conditions.

Unlike all the other molecular chaperones studied so far, HdeA is unique in that it is transformed from a well-folded conformation at a pH higher than 3, an inactive state as a molecular chaperone, into an unfolded structure at a pH lower than 3, an active state as a molecular chaperone [12]. Results of the present study further support our proposal that the amphiphilic feature of the unfolded HdeA molecule is essential for the protein to exhibit chaperone-like activity. A certain degree of structural disordering has been increasingly recognized as an important feature for protein function in general [28,29], and for molecular chaperones to interact with their denatured substrate proteins in particular [30]. To our knowledge, examples of well-characterized structural disordering are still rare [31]. HdeA apparently represents an extreme case of this type in that it binds the denatured substrate proteins only in its conditionally induced disordered conformation, in which HdeA exhibits an amphiphilic structural feature that is dependent on the primary sequence rather than on higher levels of structure. Given the substrate non-specificity and the immediate response to acid stress of HdeA [12], this structural feature is suggested to facilitate the process of molecular recognition, including sampling over a large configurational space, contacting the partner over a large binding surface for a protein of a given size, structurally adapting to different partners and enabling an increased speed of interaction. These properties were previously proposed to be the functional benefits of structural disordering [32]. The immediate transformation of HdeA in structure and function in response to the stress conditions of extreme acidity was proposed to be essential for enteric bacteria to pass through the extremely acidic stomach environment and travel to the intestine, which has a neutral pH environment. HdeA might also be regarded as a primitive form of a chaperone molecule by utilizing the most basic structural feature to function.

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