Removal of disulfide from acid stress chaperone HdeA does not wholly eliminate structure or function at low pH

M. Imex Aguirre-Cardenas\textsuperscript{a,b}, Dane H. Geddes-Buehre\textsuperscript{a}, Karin A. Crowhurst\textsuperscript{a,*}

\textsuperscript{a} Department of Chemistry and Biochemistry, California State University Northridge, 18111 Nordhoff St., Northridge, CA, 91330-8262, USA
\textsuperscript{b} Present address: Department of Chemistry, University of California Riverside, 900 University Ave, Riverside, CA, 92521, USA

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\textbf{ABSTRACT}

HdeA is an acid-stress chaperone that operates in the periplasm of various strains of pathogenic gram-negative bacteria. Its primary function is to prevent irreversible aggregation of other periplasmic proteins when the bacteria enter the acidic environment of the stomach after contaminated food is ingested; its role is therefore to help the bacteria survive long enough to enter and colonize the intestines. The mechanism of operation of HdeA is unusual in that this helical homodimer is inactive when folded at neutral pH but becomes activated at low pH after the dimer dissociates and partially unfolds. Studies with chemical reducing agents previously suggested that the intramolecular disulfide bond is important for maintaining residual structure in HdeA at low pH and may be responsible for positioning exposed hydrophobic residues together for the purpose of binding unfolded client proteins. In order to explore its role in HdeA structure and chaperone function we performed a conservative cysteine to serine mutation of the disulfide. We found that, although residual structure is greatly diminished at pH 2 without the disulfide, it is not completely lost; conversely, the mutant is almost completely random coil at pH 6. Aggregation assays showed that mutated HdeA, although less successful as a chaperone than wild type, still maintains a surprising level of function. These studies highlight that we still have much to learn about the factors that stabilize residual structure at low pH and the role of disulfide bonds.

1. Introduction

HdeA is an ATP-independent chaperone protein [1] found in the periplasm of several pathogenic bacteria including Shigella flexneri, Escherichia coli and Brucella abortus [2–4]. It, along with sister protein HdeB, helps to prevent the irreversible aggregation of other periplasmic proteins when the organism encounters a low pH environment (found in the stomach). In this way, HdeA aids in the survival of these bacteria so that they can enter the intestines of the host and cause dysentery, a disease which affects at least 120 million people each year [5,6]. Folded HdeA is a helical homodimer (Fig. 1). One of its particularly unusual characteristics is that its folded state (at near-neutral pH) is its inactive state; once the bacteria enter a low pH environment (below approximately pH 3) HdeA unfolds and assumes its active role as a chaperone [2,7].

HdeA contains one intramolecular disulfide bond between residues 18 and 66. It has been conjectured that it is instrumental in bringing two hydrophobic sites into close contact at low pH, thereby facilitating chaperone activity by enabling the formation of an extended client binding site [8]. Although the importance of the disulfide bond is mentioned in several publications on HdeA [7,9–11], relatively little investigation has been done. Hong et al. [7] originally reported that the chaperone activity of HdeA was unaffected by a reduction of the disulfide bond at low pH, but because they utilized dithiothreitol (DTT), which is functional only in the pH range 6.5–9.0, these observations were not valid. Tapley et al. [8] apparently demonstrated via light-scattering studies at 320 nm that, at low pH, reduced HdeA cannot act as a chaperone, although there are questions about those studies, given that DTT was once again used as the reducing agent. Finally, Zhai et al. [12] used TCEP (tris(2-carboxyethyl)phosphine), which is active over a wide pH range (1.5–8.5), to evaluate the chaperone activity of HdeA with a reduced disulfide at low pH, using SDS-PAGE to detect client protein aggregates. The authors suggest that the chaperone activity of reduced HdeA is heavily compromised, although their gel seems to show almost equal quantities of soluble and precipitated client protein in the presence of TCEP-treated HdeA [12]. In addition, it is unclear whether HdeA remains reduced during the assay, since the authors describe a ten-fold dilution of TCEP at one stage.
Considering that researchers assert the presence of the disulfide bond is crucial for HdeA chaperone activity but have not demonstrated this definitively, we decided to prepare a double mutant of HdeA in which the cysteines were conservatively mutated to serines. These mutations eliminate the possibility of disulfide bond formation without eliminating side chain hydrogen bond capabilities and allow for studies in the absence of chemical reductants. Our results show that the loss of the disulfide bond prevents HdeA-C18S–C66S (also called C18S–C66S) from folding at neutral pH. The mutant has near-random coil structure at pH 6.0, but surprisingly gains some secondary structure content at low pH, although it is still less structured than that of wild type HdeA at pH 2.0. Additionally, a comparison of chaperone activity of C18S–C66S versus TCEP-reduced wild type on client protein malate dehydrogenase (MDH) indicates that both have notable (although not complete) success in keeping the client protein soluble.

2. Materials and methods

Isotopes were obtained from Cambridge Isotope Laboratories, and chromatography columns were from GE Lifesciences.

2.1. Preparation of HdeA

Site-directed mutagenesis was executed using the QuikChange Lightning kit from Agilent. HdeA-C18S–C66S was expressed and purified as outlined previously for wild type HdeA [10,13,14], except that a Superdex 75 HiLoad 26/600 column was required rather than the usual HR 10/30 column. All samples were uniformly 15N-labeled.

2.2. NMR experiments

C18S–C66S samples were prepared for NMR through dialysis into 50 mM citrate buffer at the desired pH and had final concentrations in the range of 0.1 – 1.0 mM. The results were not concentration dependent. Reduced samples of wild type HdeA contained 5 mM tris(2-carboxyethyl)phosphine (TCEP); to obtain spectra at pH 6.0 the protein sample was first reduced in the unfolded state at low pH and then dialyzed to 6.0 in the presence of TCEP. NMR data were recorded at 25 °C on an Agilent DD2 600 MHz spectrometer equipped with a triple resonance probe. All raw data were processed using NMRPipe/ NMRDraw [15,16] and resulting spectra were viewed and analyzed using NMRViewJ [17,18].

Chemical shift assignment. Because C18S–C66S is unfolded at pH 6.0 and 2.0, backbone chemical shift assignments at both pHs required HNCaCb, CbCa(CO)NH and HN(Ca)CO experiments were required at pH 6.0. The assignment data have been deposited at the BioMagResBank (BMRB), acquisition number S0437. Wild type HdeA assignments at pH 2.0 (BMRB acquisition number S0421, [20]) and pH 6.0 (BMRB acquisition number 19165, [13]) were reported previously, and assignments of spectra from wild type HdeA in TCEP were made by overlaying with mutant spectra.

Backbone amide chemical shift differences (C3Ds, or Δδ) were calculated using the equation:

\[ Δδ = \sqrt{(ΔH)^2 + (ΔN)^2} \]

where ΔH and ΔN refer to the differences in backbone 1H and 15N chemical shifts for a given residue between different pHs or between wild type and mutant at a specific pH.

Secondary structure propensity. SSP analysis was performed on HdeA and HdeA-C18S–C66S using only Ca and Cβ chemical shifts, as recommended for an unfolded protein [21].

2.3. Aggregation assays

The chaperone activity of C18S–C66S to prevent or rescue aggregation of malate dehydrogenase (MDH) was tested at pH 2.0 and 6.0, using Aggregation Buffer (20 mM citrate, 100 mM sodium chloride and 150 mM ammonium sulfate) [12]. All samples contained 10 μM MDH and some contained 30 μM wild type HdeA or mutant, plus 5 mM TCEP when appropriate. At pH 2.0, the mixtures were incubated at 37 °C for 1 h, then centrifuged at 14,000 × g for 10 min to separate the supernatant and pellet. The pellets were washed once with Aggregation Buffer and centrifuged again (to remove surface supernatant); the supernatants were partially neutralized with 0.13 vol of 0.5 M sodium phosphate, pH 8 in advance of SDS-PAGE analysis. At pH 6.0, MDH was pre-treated with 2 M guanidinium hydrochloride in Aggregation Buffer and incubated at 100 °C for 20 min to ensure MDH was denatured before mixing with HdeA, and to test the ability of the chaperone to “rescue” aggregated MDH at pH 6.0. The mixtures were incubated at 37 °C for 1 h, then centrifuged to separate the supernatant and pellet. Pellets were washed once with Aggregation Buffer and centrifuged again. All samples were run on 15% SDS-PAGE gels.

3. Results and discussion

3.1. Chemical shift assignment

Upon overlaying the 15N-HSQC of C18S–C66S with wild type HdeA at pH 2.0, it was clear that the mutations significantly alter the residual low-pH structure of HdeA (Figure S1a). After comparing C18S–C66S spectra at pH 2.0 and 6.0 (Figure S1b) it was also clear that the loss of the disulfide bond results in unfolded protein at both pHs, but the ensembles of structures are not the same. To aid in chemical shift assignment of unfolded protein at each pH, we employed the 3D HNN experiment [19] in addition to the HNCaCb/CbCa(CO)NH and, at pH 6.0, the HN(Ca)CO/HNCO suites of experiments. We achieved near-complete backbone assignment: 99% of 1H–15N atoms and Ca/Cβ atoms at pH 2.0, as well as 95% of 1H–15N, 99% of Ca/Cβ, and 100% of Cα(CO) atoms at pH 6.0 were successfully assigned. Missing assignments at pH 2.0 were located at P12 (Cβ), G34 (Ca) and K87 (1H–15N), while at pH 6.0 missing assignments were at D2 (1H–15N), K42 (1H–15N), K44 (Cβ), D83 (1H–15N) and I85 (1H–15N). The data have been deposited at the BioMagResBank (BMRB),
3.2. Chemical shift differences indicate some long-range impacts of disulfide removal

The sites of the largest differences (Δδ, or CSD) in backbone amide chemical shift were evaluated. When comparing wild type HdeA to C18S–C66S at pH 2.0, it is noteworthy that the amide shifts of the first and last 15 residues in the protein are essentially indistinguishable (Fig. 2a). As one might expect, the largest CSDs can be found near (but not always at) the mutated cysteines (Fig. 2b). In a trend that is consistent with previously reported experiments [10, 20], the largest changes are on the C-terminal side of position 18 (residues 20–25) and the N-terminal side of position 66 (residues 57–65). Within the folded structure, portions of these two regions (residues 20–25 and 62–65) are adjacent to each other; this proximity seems to be maintained in the unfolded state of the wild type, primarily due to the disulfide tether [10]. However, residues 57–61 extend along the entire C-terminal half of helix C, far from residues 20–25 and far from the site of the removed disulfide in the mutant (Fig. 2b). When assessing secondary structure propensities (based on chemical shift assignments), the loss of the disulfide not only eliminates the helical propensity of helix C that is seen in the wild type, its secondary structure flips to weak β-sheet content (Fig. 3 and Table S1); this, or an allostERIC effect, could explain the large chemical shift changes.

Unlike the segments described above, residues 14–16 at the N-terminal side of position 18 and 70–74 at the C-terminal side of position 66, which are also adjacent to each other in the folded protein, do not have significant CSDs at pH 2.0 when comparing mutant to wild type. In folded wild type HdeA, this region acts like a clasp that is opened at low pH to expose hydrophobic client binding sites as part of its chaperone activation [20]. Fig. 2c shows the positions of each hydrophobic residue in HdeA. The chemical shift similarities imply structural similarities between wild type and C18S–C66S in this segment of the protein at pH 2.0; these results support the notion that this region is indeed open and less structured in the wild type when the clasp is released at low pH.

Upon initial inspection, the CSDs between C18S–C66S at pH 6.0 and 2.0 (Fig. 2d), seem to have no pattern to the magnitude of Δδ value. However, upon closer examination, it becomes clear that 14/19 residues with Δδ > 1 standard deviation above the mean are aspartate or glutamate, all of which undergo neutralization in transitioning from pH 6.0 to 2.0. The remaining 5/19 residues have large CSDs due to their proximity to these ionizable groups.

3.3. Secondary structure propensity analysis shows that the mutant maintains some structure at low pH but is virtually random coil in near-neutral conditions

As mentioned above, the chemical shift data were also used to calculate and compare secondary structure propensities (SSP). As recommended by Marsh et al. [21], only Cα and Cβ shifts were used to calculate SSP values for the unfolded proteins. Fig. 3 shows an overlay of SSP values as a function of residue number for wild type and C18S–C66S at pH 2.0, as well as C18S–C66S at pH 6.0. See Table S1 for the numerical values and Figure S2 for a plot showing the change in mutant SSP (ΔSSP) between pH 2.0 and 6.0 as a function of residue number. As has been observed in previous publications [11, 20], even at pH 2.0 the “unfolded” wild type maintains notable residual helical secondary
structure, albeit in slightly shifted positions compared to the folded structure [20]. However, when evaluating the SSP values for C18S–C66S, it is truly remarkable that the mutant has more structural content at low pH compared to near-neutral conditions (Fig. 3). At pH 6.0 C18S–C66S has extremely low SSP values, almost exclusively in the -0.1 to 0.1 range, suggesting that the protein is very close to random coil. However, at pH 2.0, the SSP values in the mutant are significantly strengthened in almost every region of the protein compared to pH 6.0; its helical secondary structure propensity even outperforms wild type at several residues on the N-terminal side of the C18S mutation. Additionally, at pH 2.0 the C18S–C66S mutant displays notable β structure propensity at the C-terminus, while wild type shows no persistent secondary structure in that region (Fig. 3 and Table S1). C18S–C66S also displays greater β propensity at the N-terminus at both pHs compared to unfolded wild type, suggesting that the N- and C-termini of the mutant may have an increased tendency to form semi-stable β-sheet structures with each other; this is even higher than the tendency we observed when modeling the unfolded wild type with MD simulations [10]. In trying to explain these results we wondered whether hydrogen bonds are stronger at low pHs compared to neutral pH. If this were so, we could argue that even weak hydrogen bonds between S18 and S66 (or other residues) in the mutant could help maintain a portion of the secondary structure that is observed in the wild type at pH 2.0. Alternatively, there may be other features, in addition to the disulfide in wild type HdeA, that help the protein maintain a partially folded conformation at low pH and that are strengthened in the mutant. Unfortunately, we have yet to find published evidence of either hypothesis; the phenomenon may be worth pursuing computationally.

3.4. C18S–C66S has similar (but not identical) chemical shifts to wild type HdeA in TCEP

We were curious whether our double mutant behaved similarly to wild type HdeA in the presence of the reducing agent TCEP. When comparing the CSDs at pH 2.0 and 6.0 (Figures S3 and S4), the presence of TCEP clearly results in a similar ensemble of unfolded structures for wild type HdeA as those observed for the double mutant. The chemical shift perturbations are, overall, an order of magnitude smaller than comparisons between unfolded, oxidized wild type HdeA and C18S–C66S at pH 2.0 (compare Fig. 2a and S4). Even so, the CSDs at each pH follow distinctly different trends. At pH 6.0, CSDs between reduced wild type and C18S–C66S are very low, except in the region immediately surrounding the mutation sites. However, at pH 2.0, the average CSD is more than double what is observed at pH 6.0, and although the largest values are also near the mutation sites, there are other notably high values near the N-terminus and between residues 47–51 (Figure S4a). Neither of these regions are close to the disulfide in the folded state, but the N-terminus of one protomer is in contact with residues 47–51 in the other protomer of the folded dimer. Five out of six residues with the highest CSDs among those residues are aspartates suggesting that TCEP may specifically interact with aspartates in solution, altering their backbone amide chemical shifts; it is unclear, however, why the other aspartates (or glutamates) in the protein are not similarly affected.

3.5. Loss of the disulfide does not wholly eliminate chaperone activity in HdeA

We were interested to evaluate the chaperone activity of C18S–C66S, and to compare it to the activity of wild type HdeA in TCEP. It was previously suggested that the disulfide may have a crucial role in chaperone activation by clustering hydrophobic patches in HdeA and creating a larger client binding site in the partially unfolded state [8]. It was therefore expected that neither the mutant nor chemically reduced wild type HdeA could protect a client protein from aggregation. We performed aggregation assays at pH 2.0 and 6.0, employing methods similar to those reported by other groups, using malate dehydrogenase (MDH) as the client protein [8,12,22,23]. Figures S5 and S6 show the gel images and plots of quantified band density, and Table 1 summarizes the density values. The results at pH 6.0 are as one might expect – even wild type HdeA is not expected to rescue aggregated MDH since it is folded and inactive as a chaperone at that pH (Figure S5). At pH 2.0, MDH without chaperone is found almost exclusively in the pellet and MDH in the presence of wild type HdeA is found almost exclusively in the supernatant, as expected (Figure S6). Interestingly, at least one-third of the

Table 1

|                | pH 6.0 | pH 2.0 |
|----------------|--------|--------|
| P - MDH only   | 96.2a  | 89.4a  |
| S - MDH only   | 3.8    | 10.6   |
| P - WT HdeA    | 94.6   | 1.5    |
| S - WT HdeA    | 5.4    | 98.5   |
| P - WT HdeA + TCEP | 91.4 | 39.8   |
| P - WT HdeA + TCEP | 8.6   | 40.2   |
| P - HdeA-C18S-C66S | 95.5 | 63.5   |
| P - HdeA-C18S-C66S + TCEP | 4.5  | 36.5   |
| S - HdeA-C18S-C66S + TCEP | 95.5 | 64.9   |
| S - HdeA-C18S-C66S + TCEP | 4.5  | 35.1   |

Notes: a) Numbers estimated using volume integration data from Bio-Rad Image Lab software. Values correspond to % relative volume (each pellet-supernatant pair adds up to 100%).

Fig. 3. Plot of secondary structure propensities (SSP) as a function of residue number for wild type HdeA at pH 2.0 (red), and HdeA-C18S-C66S at pH 2.0 (blue) and 6.0 (yellow, shown 50% transparent to reveal values from the other samples underneath). Positive SSP values indicate helical secondary structure (value of 1.0 equals 100% helical propensity) and negative SSP values indicate β-sheet secondary structure (value of -1.0 indicates 100% β structure propensity). Approximate positions of residual helix structure in wild type HdeA at low pH are indicated at the top [20]. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
MDH can be found in the supernatant when either C18S–C66S or HdeA plus TCEP are present (Table 1 and Figure S6). This implies that, while the disulfide bond is important to the success of HdeA as a chaperone, the lack of disulfide does not fully eliminate its capabilities.

Although wild type HdeA plus TCEP has similar chaperone properties as the C18S–C66S mutant, the NMR data indicate there are still differences in the conformational ensembles (Figures S3a and S4a). Given these results, we argue it is advantageous to use the mutant in any future studies, since it eliminates the variability and complication of employing a chemical additive which may also have unintended effects on other solution components, such as a client protein.

3.6. Results provide evidence for the maintenance of some hydrophobic clustering in mutant HdeA

Considering that HdeA maintains some chaperone activity at low pH in the absence of the disulfide, it is worth taking a deeper look at the data. We have suggested that hydrogen bonding between the serines in C18S–C66S can help maintain some residual structure (and therefore chaperone activity) at pH 2.0, but this alone is unlikely to fully explain our assay results. Helix C in wild type HdeA contains numerous hydrophobic groups and constitutes a portion of client binding site I proposed by Yu et al. [11]. However, at pH 2.0 most of this region shows significant chemical shift perturbation in C18S–C66S compared to wild type (Fig. 2a), and most of its secondary structure propensity is lost (Fig. 3). These data therefore suggest that there is little residual hydrophobic clustering in this segment and therefore does not contribute to the protein’s chaperone activity. On the other hand, for residues 28–39, which include proposed client binding site II from Yu et al. [11], the mutant has very similar amide chemical shifts to wild type at pH 2.0 (Fig. 2), and it maintains some residual helical structure (Fig. 3). It is also the same segment that is believed to become accessible (due to the opening of the clasp region) when the wild type unfolds and becomes chaperone-active [20]. In addition, the SSP data indicate increased β structure propensity at both the N- and C-termini in C18S–C66S (Fig. 3) compared to wild type; previous studies provided evidence that the termini transiently form a β-sheet as part of HdeA’s chaperone activation [10]. Taken together, it is possible that this strengthened β-sheet formation (compared to wild type) helps C18S–C66S maintain a more compact shape, subsequently preserving the hydrophobic cluster near client binding site II and possibly explaining the mutant’s partial chaperone activity at pH 2.0. Future studies will investigate the importance to chaperone activity of this β-sheet formation as well as the specific location of the disulfide.

4. Conclusions

Replacement of the cysteines that form the disulfide bond in HdeA with serines results in a near-random coil structure at pH 6.0, but notably higher structural content at pH 2.0. HdeA-C18S–C66S also retains greater chaperone activity than previously suggested. Both low-pH results for the mutant are unexpected, highlighting the need for more investigation of positions 18 and 66, located at a “pinch point” of the HdeA structure [20], as well as other residues in the vicinity and at the N- and C-termini that may collectively maintain partial chaperone activity when the cysteines are removed or reduced. These results underscore the complexities of the structure-function relationship in this acid stress chaperone and may open new areas of inquiry into the role of long-range disulfide bonds in small proteins.

CRediT authorship contribution statement

M. Imex Aguirre-Cardenas: Investigation, Visualization, Formal analysis, Writing – review & editing. Dane H. Geddes-Buehre: Investigation, Formal analysis, Writing – review & editing. Karin A. Crowhurst: Conceptualization, Funding acquisition, Supervision, Project administration, Investigation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2021.101064.

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