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pH Effect on the Structure of Reduced NifU–like Protein from *Helicobacter pylori*

Ki–Young Lee[1], Ji–Hun Kim[2], Ye–Ji Bae[1], and Bong–Jin Lee[1*]

[1]Research Institute of Pharmaceutical Sciences, College of Pharmacy, Seoul National University, Seoul 151–742, Korea
[2]Department of Biotechnology, College of Biomedical and Health Science, Konkuk University, Chungju, Chungbuk 380–701, Korea

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Abstract *Helicobacter pylori* (*H. pylori*) survives in acidic and fluctuating pH conditions of the stomach. The pH effect on *H. pylori* proteins is important for the advanced understanding of its evolution and viability, although this bacterium has the molecular machinery that neutralizes the acidic condition. HP1492 is known as a conserved NifU–like protein from *H. pylori.* NifU is a nitrogen fixation protein that mediates the transfer of iron–sulfur (Fe–S) cluster to iron–sulfur proteins like ferredoxin. Commonly, the monomeric reduced state of NifU can be converted to the dimeric oxidized state by intermolecular disulfide bond formation. Because it remains unclear that HP1492 actually behaves as known NifU protein, we first found that this protein can adopt both oxidized and reduced forms using size exclusion chromatography. Circular dichroism experiment showed that HP1492 is relatively well–structured at pH 6.5, compared to other pH conditions. On the basis of the backbone resonance assignment of HP1492, we further characterized the residues that are sensitive to pH using NMR spectroscopy. These residues showing large chemical shift changes could be mapped onto the secondary structure of the protein. Our results could provide the foundation for structural and biophysical studies on a wide spectrum of NifU proteins.

Keywords *Helicobacter pylori,* NifU, redox state, pH sensitivity, chemical shift perturbation

Introduction

Helicobacter pylori (*H. pylori*) is a human pathogenic bacterium that can survive in the extremely acidic condition (pH 1–2) of the stomach, which might be intermittently changed during food ingestion. This bacterium have received an increasing attention for its strong correlation with human gastric diseases such as gastritis, peptic ulcer, gastric carcinoma, etc [1–3]. A combination of three or four broad–range antibiotics has been used to treat the *H. pylori* infection, also eliminating other beneficial bacteria in gut. In addition, the antibiotics–resistant *H. pylori* has increased over the past few decades [4–5]. Therefore, much effort has recently been paid to the discovery of new drug targets that are *H. pylori*–specific and essential for cell maintenance and survival. In particular, the knowledge of three–dimensional (3D) structure of target proteins is valuable for the development of antibiotic drugs. In line with this, many essential proteins of *H. pylori* have structurally and functionally studied to examine their potential as antibiotic drug targets.

HP1492 is one of *H. pylori* proteins, which is composed of 89 amino acid residues. This protein is annotated as a conserved hypothetical NifU–like protein. NifU is known as a nitrogen fixation protein to provide a scaffold to transfer iron–sulfur (Fe–S) cluster to redox proteins such as ferredoxin and thioredoxin [6–7]. These proteins are involved in TCA

* Address correspondence to: **Bong–Jin Lee,** Research Institute of Pharmaceutical Sciences, College of Pharmacy, Seoul National University, Seoul 151–742, Korea

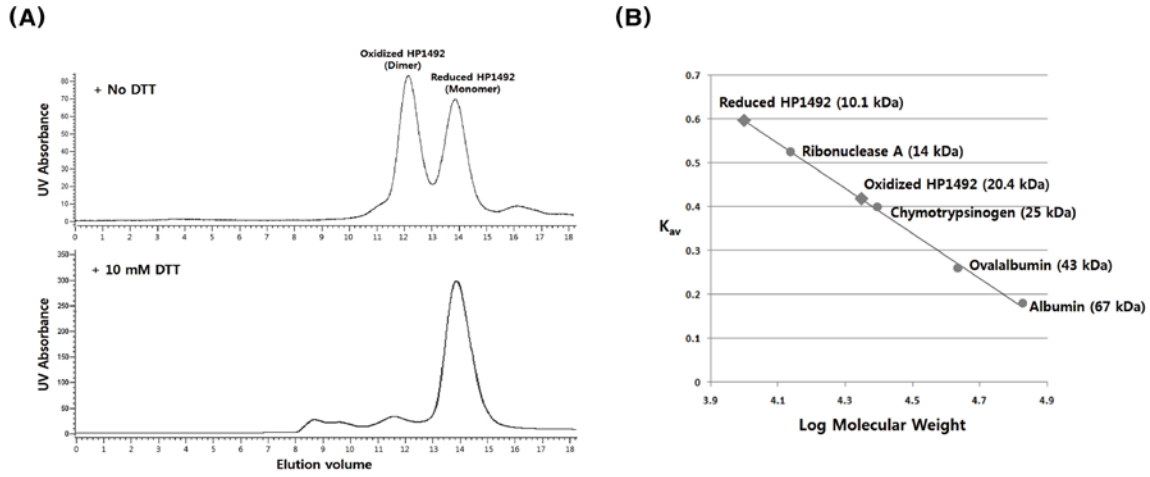

Figure 1. Identification of oxidized and reduced states of HP1492 using size exclusion column. (A) Upper and lower profiles were obtained in the absence and presence of 10mM DTT, respectively. (B) Size exclusion column was calibrated by using molecular standards, and the molecular weight of the two forms of HP1492 was calculated.

cycle, heme biosynthesis, iron homeostasis, etc [8–9].
Incorporation of iron cofactors into redox proteins is a finely tuned process to avoid a cellular toxicity from free iron. It is possible that cell death is induced by the inhibition of Fe–S cluster transfer. One of new antibiotic approaches might be the suppression of the function of HP1492.
In this paper, we present that HP1492 can adopt both oxidized and reduced conformations using size exclusion chromatography. Circular dichroism experiments were carried out to examine the pH effect on the structure of HP1492. Based on the previously reported backbone resonance assignment of HP1492 [10], we characterize the residues that are sensitive to pH using NMR experiments.

Experimental Methods

Cloning and sample preparation- The *hp1492* gene was acquired from *H. pylori* 26695 genomic DNA and subcloned into the plasmid pCOLD–1 (Takara, Inc.). The recombinant plasmid was transformed into *Escherichia coli* (*E. coli*) strain BL21 host cells. This construct contains N–terminal $(His)_6$–tag which is easily removed by factor Xa cleavage and used for protein purification. Overexpression was induced with 0.5 mM isopropyl ß–Dthiogalactopyranoside (IPTG) in *E. coli* BL21 for 24 h at 15°C after the cells had grown to an optical density of 0.5–0.6. These cells were harvested and sonicated in lysis buffer (pH 8.0) containing 50mM Tris–HCl and 500mM NaCl. The lysed cells were centrifuged, and the supernatant was loaded onto a nickel–chelating column (Chelating Sepharose Fast Flow resin; GE Healthcare, Inc.). The protein was eluted with an imidazole concentration of 100 mM. After factor Xa cleavage of the protein, the final protein purification was performed using size exclusion chromatography (SuperdexTM 75 10/300; GE Healthcare, Inc.) with the AKTA primeTM system (GE Healthcare, Inc.). Size exclusion chromatography was also used to determine oligomeric and redox states of HP1492 in the absence and presence of 10 mM DTT. The buffer includes 20 mM MES (pH 5.5), 150 mM NaCl, and 1 mM EDTA.

Circular dichroism- Circular dichroism (CD) spectra for 20 μM HP1492 were recorded at 298 K and at a scanning speed of 20 nm/min in the far–UV region of 200–250 nm using a J–715 spectropolarimeter (JASCO Inc.). All buffers for CD measurements contained 20 mM MES (pH 5.5), 20 mM Bis–Tris (pH 6.5), 20 mM NaH_2PO_4/Na_2HPO_4 (pH 7.5), and 20 mM Tris (pH 8.5), respectively. The buffers commonly

included 150 mM NaCl, 10 mM DTT, and 1 mM EDTA. The raw CD data were processed by blank subtraction and line smoothing before analysis.

NMR experiments- Uniformly ^{15}N–labeled HP1492 was prepared by growing the cells in M9 medium, which includes 99% $^{15}NH_4Cl$ (Cambridge Isotope Laboratories, Inc.). NMR samples were finally acquired at a concentration of approximately 0.4 mM in 90% H2O/10% D2O. For pH titration and NMR experiments, we selected pH–optimal buffers that include 20 mM NaAc (pH 4.5), 20 mM MES (pH 5.5), 20 mM Bis–Tris (pH 6.5), and 20 mM NaH_2PO_4/Na_2HPO_4 (pH 7.5), respectively. All of the buffers commonly contain 150 mM NaCl, 10 mM DTT, and 1 mM EDTA. The NMR experiments were carried out at 298 K on a Bruker AVANCE DRX 600 spectrometer that was equipped with a cryogenic probe. Two–dimensional (2D) NMR data were processed with the program NMRPipe [11] and analyzed with the program NMRView [12]. The average chemical shift perturbation values for ^{15}N and ^{1}H nuclei were derived as follows from Equation 1:

$$\Delta\delta_{avg} = [(0.2 \times \Delta\delta^2_N + \Delta\delta^2_H)/2]^{1/2}$$

where $\Delta\delta_N$ and $\Delta\delta_H$ represent the chemical shift perturbation values for the amide nitrogen and proton, respectively..

Results and Discussion

Oxidized and reduced conformations of HP1492- HP1492 is annotated as a conserved hypothetical NifU–like protein from *H. pylori*. But it has not been confirmed that this protein actually behave as a well–known NifU protein. Therefore, we first examined whether HP1492 can adopt both oxidized and reduced conformations, which is found in NifU protein. Size exclusion experiments detected dimeric oxidized form and monomeric reduced form of the protein, as shown in Figure 1A. The result indicated that the oxidized form is a little more favorable than the reduced form in the absence of reducing agent. In the presence of the excess of reducing agent (10 mM DTT), the dimeric oxidized form was almost completely converted to the monomeric reduced form (Figure 1A), probably by the breakage of the intermolecular disulfide bond. For precise identification, elution volumes of the two forms were compared with those of several protein markers including Ribonuclease A (13.7 kDa), Chymotrypsinogen A (25.0 kDa), Ovalumin (43.0 kDa), and Albumin (67.0 kDa). Blue Dextran 200 and Trp amino acid were used to know void and geometric volumes of size exclusion column, respectively. The molecular weight of the oxidized and reduced forms was calculated to be approximately 20.4 and 10.1 kDa, respectively, by constructing calibration curve (Figure 1B). These values are highly similar to their theoretical molecular weights of 20.52 and 10.26 kDa, respectively.

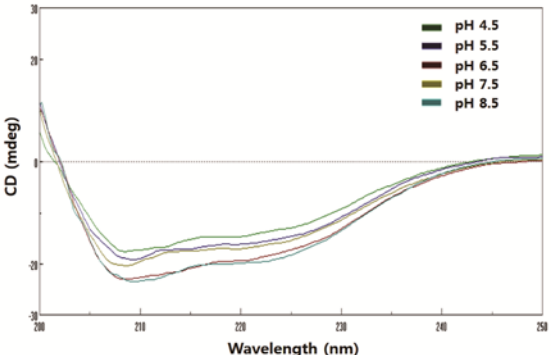

Figure 2. CD spectra of reduced HP1492. The spectra acquired at a variable pH were color–mapped.

Circular dichroism spectra in a variable pH- One of important factors to change the structure and biochemistry of protein is an amount of protons in solution, which is parameterized as pH. Especially, the pH effect on the structure of *H. pylori* proteins has a biological significance because *H. pylori* survives in acidic and fluctuating pH conditions of the stomach. We carried out far–UV circular dichroism (CD) experiments to examine the pH effect on the secondary structure of HP1492. The largest CD values were observed at pH 6.5 and 8.5 in the wavelength of 200–250 nm, and the lowest CD value was observed at pH 5.5 (Figure 2). The result

indicated that HP1492 is well–structured at pH 6.5 and 8.5.

Table 1. pH–sensitive residues of HP1492 with chemical shift perturbation (CSP) values

Residue Number	Amino Acid	CSP (pH7.5 and pH 6.5)	CSP (pH 6.5 and pH 5.5)
3	GLU	0.081566	0.069939[a]
5	SER	0.056565	0.084741
21	ARG	0.023372	0.035929
23	TYR	0.023555	0.035016
25	LEU	0.073667	N/A[b]
27	ASP	0.052609	0.039939
28	GLY	0.042643	0.042706
30	ASN	0.0932	0.07416
32	GLU	0.0984	0.17522
46	GLU	0.145156	N/A[b]
47	GLY	0.109816	N/A[b]
48	ALA	0.15113	0.14257
57	ILE	0.070311	0.018451
58	THR	0.086133	N/A[b]
66	GLN	0.035626	0.041651
74	ASN	0.028092	0.112583[a]
82	ASN	0.08114	0.0835
86	PHE	0.066212	0.082101

[a] Chemical shift values of two split peaks were averaged.
[b] N/A is abbreviated from 'not applicable'. The corresponding peaks disappeared.

pH–sensitive residues of HP1492- We further attempted to obtain the residue–specific information on pH–sensitive structural changes of HP1492. An excess of reducing agent (10 mM DTT) was included in NMR buffers to secure a homogeneity of reduced form of the protein [13]. Four NMR buffers included NaAc, MES, Bis–Tris, and NaH_2PO_4/Na_2HPO_4, respectively, each of which are optimal for chosen pH conditions (pH 4.5, 5.5, 6.5, and 7.5). Tris buffer (pH 8.5) was not used because higher pH is not suitable for NMR measurement. For robust interpretation of NMR spectra, acquisition and processing parameters for all samples were set to be same. We obtained 2D ^{1}H–^{15}N HSQC spectra of reduced HP1492 in a variable pH range of 4.5 – 7.5 (Figure 3A–D). We found that peaks on the spectrum acquired at pH 6.5 are relatively well–separated with homogeneous intensities (Figure 3B), in comparison with other spectra. The spectrum acquired at pH 5.5 showed newly generated peaks, possibly due to an initial unfolding of the protein (Figure 3C). These peaks could not be traced from the previously assigned peaks acquired at pH 6.5 [10]. In addition, the spectrum recorded at pH 4.5 showed densely clustered peaks with line broadenings at approximately 8.3 ppm, indicative of unfolded and aggregated forms of the protein (Figure 3D). This result is consistent with our CD analysis showing that the protein is the most unfolded at pH 4.5 among a pH range of 4.5 – 8.5. However, it should be noted that the protein concentration used for NMR experiments was approximately 20–fold higher than than for CD experiments, more facilitating the protein aggregation.

Overlay of three spectra acquired in pH 5.5, 6.5, and 7.5 showed the residues that are sensitive to pH changes (Figure 3E). For these residues, chemical shift perturbations are more predominant than resonance broadenings, and these values were quantitatively calculated from the standard chemical shift perturbation equation, as shown in table 1. It would be possible that these changes are caused by a combination of pH–induced structural changes and protonation effects. The structural changes were confirmed by the CD data showing different degrees of the secondary structure formation in a variable pH range, retaining the possibility of concomitant tertiary structure changes. The pH–sensitive residues are classified into three groups and mapped onto the secondary structure of HP1492 (Figure 4), which is previously determined by chemical shift index (CSI) and the TALOS program [10, 14, 15]. The residues showing largest chemical shift changes include Asn30, Glu32, Glu46, Gly47, Ala48, Thr58, and Asn82. Taken together, our results could contribute to the understanding of the pH effect on the NifU structure.

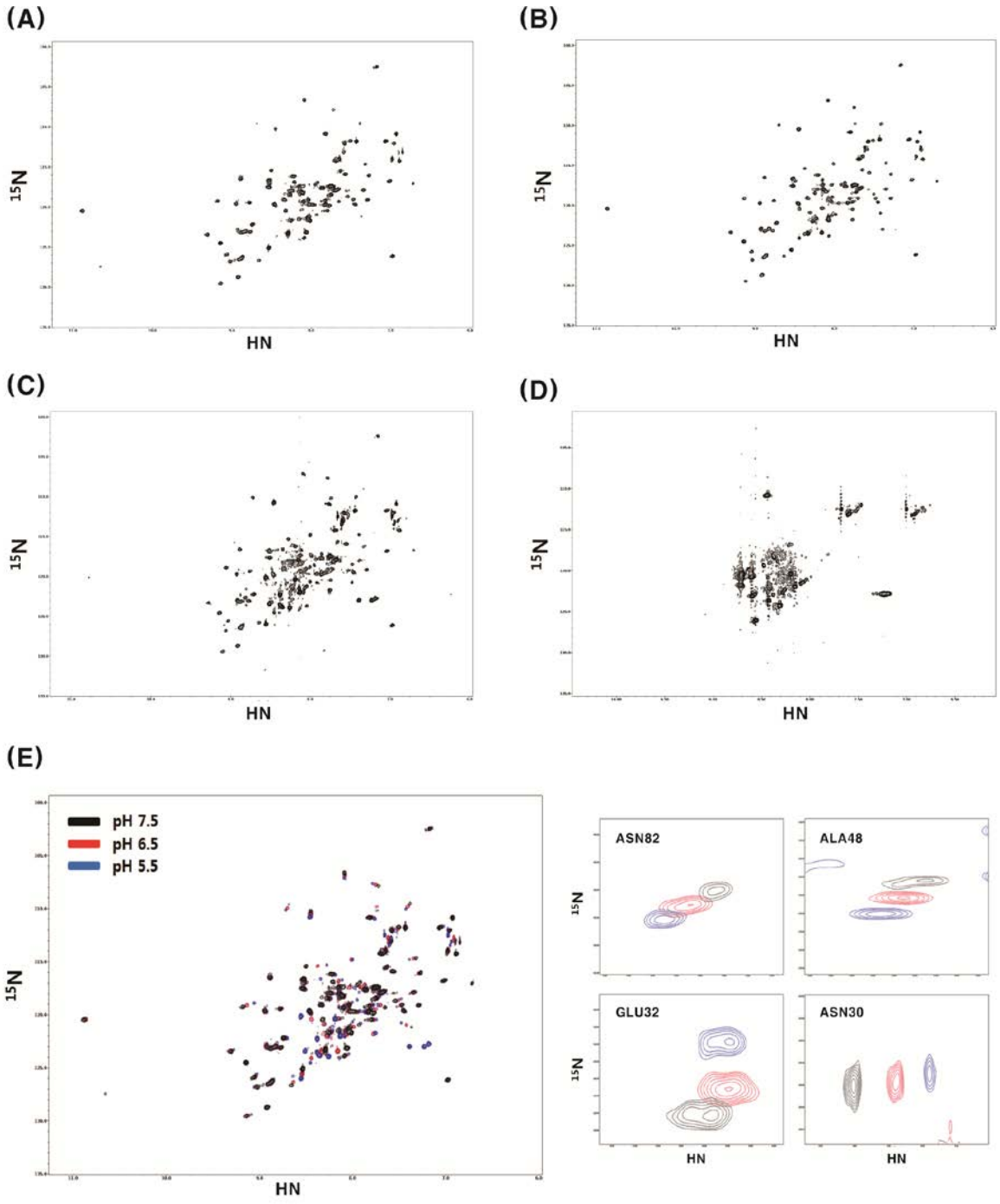

Figure 3. 2D $^{1}H–^{15}N$ HSQC spectra of reduced HP1492 were recorded in a variable pH of (a) 7.5, (b) 6.5, (c) 5.5 and (d) 4.5. (e) The overlay of the spectra were acquired at pH 5.5, 6.5, and 7.5. These spectra are color–mapped, and peaks showing significant chemical shift changes are shown in an expanded view.

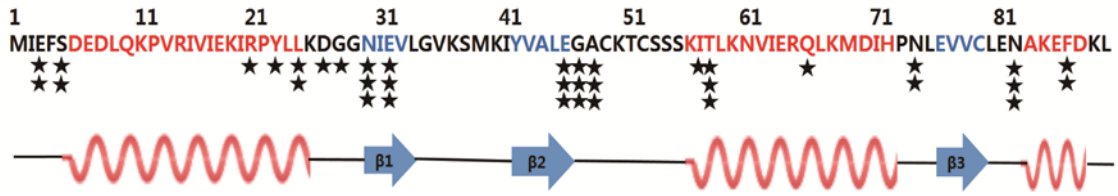

Figure 4. Mapping of the chemical shift perturbation (CSP) onto the secondary structure of HP1492. The number of asterisks is indicated according to the chemical shift perturbation value for each residue. One, two, and three asterisks indicate a CSP value of 0.2–0.6, 0.6–0.8, and above 0.8, respectively, each of which is averaged between the two values as shown in table 1. Red and blue colors indicate alpha–helix and beta–strand, respectively.

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