The dimerization site-2 of the bacterial DNA-binding protein H-NS is required for gene silencing and stiffened nucleoprotein filament formation

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ABSTRACT

The bacterial nucleoid-associated protein H-NS is a DNA-binding protein, playing a major role in gene regulation. To regulate transcription, H-NS silences genes, including horizontally-acquired foreign genes. Escherichia coli H-NS is 137 residues long and consists of two discrete and independent structural domains: an N-terminal oligomerization domain and a C-terminal DNA-binding domain, joined by a flexible linker. The N-terminal oligomerization domain is composed of two dimerization sites, dimerization site-1 and site-2, which are both required for H-NS oligomerization, but the exact role of dimerization site-2 in gene silencing is unclear. To this end, we constructed an whole set of single amino acid substitution variants spanning residues 2 to 137. Using a well-characterized H-NS target, the slp promoter of the glutamic acid-dependent acid resistance (GAD) cluster promoters, we screened for any variants defective in gene silencing. Focusing on the function of dimerization site-2, we analyzed four variants, 170C/170A and L75C/L75A, which all could actively bind DNA, but are defective in gene silencing. Atomic force microscopy analysis of DNA-H-NS complexes revealed that all these four variants formed condensed complexes on DNA, whereas wild-type H-NS formed rigid and extended nucleoprotein filaments, a conformation required for gene silencing. Single-molecule stretching experiments confirmed that the four variants had lost the ability to form stiffened filaments. We conclude that the dimerization site-2 of H-NS plays a key role in the formation of rigid H-NS nucleoprotein filament structures required for gene silencing.

A group of nucleoid-associated proteins (NAPs) are involved in the regulation of transcription (1-5). H-NS, originally referred to as histone-like protein H1, is one of the major core NAPs. In Escherichia coli, more than 10,000 molecules of H-NS exist, mostly associated with the genome (5, 6). Recent super-resolution imaging and single particle tracking experiments determined that 95% of H-NS protein was bound to DNA (7). Genomic SELEX, ChIP-seq, and transcriptome analyses have identified around one thousand sites associated by H-NS, which regulates 5% of the genome (8, 9), including horizontally transferred foreign genes (10, 11).

H-NS recognizes and binds to intrinsically curved, AT-rich DNA, often located near promoters (12, 13). Promoter-associated DNA curvature is believed to provide H-NS with an initial contact site for transcriptional silencing (14). After initial binding, H-NS binds cooperatively to form a rigid nucleoprotein filament along the target genes (15, 16). A filamentous structure of DNA-H-NS complexes is required for gene silencing (4, 17). In E. coli and Salmonella, H-NS-mediated silencing can be relieved by a set of positive regulators such as Fis (18), CRP (18, 19), LeuO (8, 20, 21), Lcr (22), Lrp (21), SsrB (17, 23), and SlyA (24).

Molecular analysis of truncated H-NS proteins led to predictions that E. coli H-NS (137 amino acid residues), consisted of an N-terminal oligomerization domain (residues 1-79) (7) and a C-terminal DNA-binding domain (residues 95-137) (7, 25). The two
domains are joined by a flexible linker (residues 80-94), which has no secondary structure between the two domains (Fig. 1A). Recent analysis of the linker identified a role for the five charged residues in initial engagement with DNA, indicating it is much more than a passive tether (7). Both oligomerization and DNA-binding domains and the linker are highly conserved among H-NS family members (7, 26). Numerous single amino acid substitutions have been identified that were unable to repress promoter activities in vivo (27-30), based on the repression patterns of some model promoters (bglG, fimB, and proV). Ten substitutions were located within the oligomerization domain and twenty-five mapped within the DNA-binding domain (Fig. 1A, B and C). Most substitutions in the DNA-binding domain mapped within the core DNA-binding motif (TWTG-GR-P), between residues 108-116 (Fig. 1A and C).

Recent findings with Salmonella H-NS have identified two dimerization sites, dimerization site-1 (amino acids 1-42) and dimerization site-2 (residues 57-79), which serve as oligomerization interfaces in head-to-head and tail-to-tail oligomers (25). Dimerization site 1 is required for gene silencing. At least three substitutions in site 1: R15E, L26P, or L30P, fail to form a rigid nucleoprotein filament (14, 31, 32), resulting in the loss of gene silencing in vivo. Dimerization site-1 in Salmonella H-NS also interacts with Hha (33). Hha is also a NAP, and is partially required for H-NS silencing. In E. coli, Ueda et al. identified some genes that are co-silenced by H-NS, Hha, and/or YdgT (Cnu) (34). In contrast, dimerization site-2 has not been characterized, although amino acids in dimerization site-2 are also conserved among H-NS homologues (Fig. 1D). L65P, a mutant in site 2, is defective in silencing in vivo, but further analysis was not performed (30). In a recent paper, a double mutant (D68V and D71V) in this region showed enhanced H-NS oligomerization and transcriptional repression in vitro (35), although the underlying mechanistic basis of this behavior was not examined. Unfortunately, this study referred to this region as the linker (35), which generated confusion in the field. In H-NS family members, formation of a rigid nucleoprotein filament is essential for gene silencing (14, 31, 32), and mutants in the oligomerization or DNA binding domain of H-NS family proteins that are incapable of gene silencing are also incapable of forming rigid nucleoprotein filaments (16, 20). Thus, we were inspired to re-examine the role of dimerization site-2 in H-NS function.

We created an entire set of H-NS mutants, each carrying a single cysteine substitution from residues 2 to 137. We then examined the ability of these mutants to silence a well-characterized target, the slp promoter of the glutamic acid-dependent acid resistance (GAD) cluster promoters (36, 37). Two substitutions in dimerization site-2, I70C and L75C, resulted in a loss of gene silencing. We then examined the mode of DNA binding using atomic force microscopy (AFM) and transverse magnetic tweezers. Our results indicated that the mutants were capable of binding to DNA and forming compact DNA structures, but were unable to form higher-ordered nucleoprotein filaments. Thus, the second dimerization site coordinates N-terminal oligomerization and C-terminal DNA-binding. Amino acid substitutions can disrupt this site-dependent domain-domain communication, abrogating gene silencing. The Cys-scanned H-NS series constructed in this study will provide a useful tool to analyze H-NS mutants by cysteine modification.

RESULTS

Construction of an entire set of single Cys-substituted H-NS mutants—The GAD system is a major acid resistance system of E. coli, in which the transcription factor GadE plays a key role in regulating genes involved in acid resistance, including: slp, dctR, yhiD, hdeB, hdeA, hdeD, gadE, mdtE, mdtF, gadW, gadX, and gadA (37). These genes are organized into eight transcription units, slp-dctR, hdeAB-yhiD, hdeD, gadE, mdtEF, gadW, gadX, and gadAX (38). Since most of the GAD cluster genes are the targets of silencing by H-NS (34), we used the GAD cluster promoters as representative of H-NS-mediated gene silencing, and examined the effect of H-NS cysteine substitution on its silencing ability.

For detection of the promoter activity, we employed the lux reporter system. For this purpose, the target promoters, pgadA, pgadE, pgadW, phdeA, phdeD, and pslp, were inserted into the vector pLUX (38). The lux fusion plasmids were introduced into both the parent and the hns-deficient strains. Transformants were harvested at logarithmic phase (OD600 = 0.3) and then luciferase activity was measured. The activity of three promoters, pgadA, pgadW, and pslp, increased more than ten-fold in the hns-deficient strain as compared to the parent strain (data not shown). Among these, the slp promoter was selected for systematic analysis of H-NS in E. coli, because its activity was the highest in the hns-deficient mutant.

In order to examine the structural and functional roles of H-NS, we constructed an entire set of single Cys-substituted mutants from residue 2 to 136 of H-NS. For this purpose, lone Cys residue at position 21 in wild-type H-NS was first converted to Ser, and the resulting C21S mutant hns gene was cloned into pQE80Lhns-C21S (see EXPERIMENTAL PROCEDURES). Transformants harboring pLUXslp and pQE80Lhns or pQE80Lhns-C21S, were grown in 96-well plates at 37°C and luciferase activity was measured. In E. coli cells, Cys substitutions did not affect cell growth (data not shown). The activity of pslp increased in the hns-deficient strain, compared to the parent strain. This enhancement was suppressed by complementation with pQE80Lhns and pQE80Lhns-C21S (Fig. 2A). Using pQE80Lhns-C21S as a template, we next tried to
construct 136 single Cys-substituted H-NS mutants at all positions. Except for the A18C mutant, the entire set of single Cys-substituted mutants was obtained. These Cys mutants will be useful for future site-specific modification experiments.

**Identification of hns mutants that were unable to silence genes**—To determine the influence of Cys substitution on the silencing activity of H-NS, the pslp reporter plasmid was introduced into an entire array of hns mutants (Table S2). The level of lux reporter expression increased for a subset of H-NS mutants, indicating an inability to silence, as shown in Fig. 2B. The disruption in silencing activity of the H-NS mutants was essentially the same for all three promoters tested. Cys substitution resulted in a marked loss of silencing activity at seven positions in H-NS: four within the DNA-binding domain (T97C, W109C, G111C, and G113C); and three within dimerization site-2 (A67C, I70C, and L75C) (Figs. 1 and 2). Mutants in dimerization site-1 rarely affected H-NS promoters, although others reported that R12C and R54C derepressed proV (28).

We further examined effects of site-1 on the GAD promoter. A truncated H-NS, lacking N-terminal residues 1-46 (containing dimerization site-1), could not silence the slp promoter (Fig. 2C). We suspected that additional factors were involved in H-NS function for slp silencing because Hha and YdgT (Cnu) are modulators that interact with oligomerization site-1 of H-NS (33). The slp promoter activity increased in an hha- and ydgT-double mutant strain as well as an hns null strain (Fig. 2C), indicating that silencing of the slp promoter requires H-NS, Hha, and YdgT. Therefore, site-1 mutants might prevent slp silencing by Hha and/or YdgT activity (see DISCUSSION).

As Ile70 and Leu75 are completely conserved among orthologues and paralogues of *E. coli* H-NS (Fig. 1D), these mutants displayed the largest defect in silencing pslp. We focused on them for a detailed analysis of H-NS structure and function. As an aside, the expression levels of I70C and L75C H-NS were similar to or higher than wild-type (Fig. S1), indicating that the loss of silencing was attributable to a loss of function, and not due to a reduced level of protein.

**H-NS Cys-substitutions in dimerization site-2 bind DNA**—The loss of the silencing phenotype observed above could be due to an inability to bind slp promoter DNA. To examine this possibility, the DNA binding activity of I70C, I70A, L75C and L75A mutants was analyzed using an electrophoretic mobility assay (EMSA). Wild-type and the substituted proteins were purified from *E. coli*. To confirm the secondary structure of the mutant proteins, circular dichroism (CD) spectrometry analysis was performed, resulting that purified H-NS-C21S-I70C displayed two negative maxima at ~208 and 222 nm and a positive maximum at ~192 nm and was similar to the wild-type H-NS and H-NS-C21S (Fig. S2). Estimation of secondary structure indicated that all of the H-NS wild-type and mutant proteins had similar alpha-helix content (92%) (Table S1).

We next examined the DNA binding of the H-NS mutants in detail by EMSA. In the presence of wild-type H-NS, shifted complexes were first apparent at 120 nM (lane 4, Fig. 3A). The pattern of complex formation of the H-NS-C21S protein was similar to the wild-type, but required >2.5-fold higher protein, indicating the binding property of H-NS-C21S differs from wild-type H-NS. The two single Cys-substituted mutants, H-NS-C21S-I70C and H-NS-C21S-L75C, also formed protein-DNA complexes (Fig. 3C and D), as did the Ala-substituted mutants (Fig. 3E and F) although the binding pattern was different between Cys- and Ala-substituted H-NS (see DISCUSSION). These results indicate that the loss of silencing activity of these two H-NS mutants was not due to an inability to bind DNA.

**Dimerization site-2 mutants fail to form stiffened filaments**—In order to understand the molecular basis of the silencing defect of the mutants in dimerization site-2, we examined DNA binding using AFM. In low Mg²⁺ buffers, H-NS forms a rigid filament on DNA and this filament is the basis for gene silencing (4, 15, 16). In the absence of protein, the naked DNA formed random coils (Fig. 4A). At 600 nM of H-NS or H-NS-C21S (2 monomers/base pairs), the DNA-protein complexes formed extended, thick filaments (Fig. 4B, C and H), indicating that both H-NS and H-NS-C21S were capable of forming stiffened H-NS nucleoprotein filaments. In contrast, H-NS-C21S-I70C (Fig. 4D and H), H-NS-C21S-L75C (Fig. 4E and H), H-NS-I70A (Fig. 4F and H), and H-NS-L75A (Fig. 4G and H) formed condensed DNA-protein complexes that were distinct from the nucleoprotein filaments formed by wild-type H-NS and H-NS-C21S (Fig. 4H). The quantification of AFM images by the radius of gyration illustrated that Cys- or Ala-substituted H-NS were similar pattern to naked DNA, while H-NS and H-NS-C21S formed nucleoprotein filaments (Fig. 4H). In addition, formation of condensed structures did not differ between Cys- and Ala-substituted H-NS (Fig. 4H).

The size of the nucleoid in cells expressing H-NS, H-NS-C21S, H-NS-C21S-I70C, H-NS-C21S-L75C, H-NS-I70A or H-NS-L75A were quantified in cells stained with DAPI, using structured-illumination microscopy (SIM) as described (7). The average nucleoid area of cells expressing H-NS or H-NS-C21S was 0.32 ± 0.12 µm² or 0.38 ± 0.14 µm² (n = 108), respectively. In contrast, that of H-NS-C21S-I70C, H-NS-C21S-L75C, H-NS-I70A or H-NS-L75A was 0.36 ± 0.13 µm² (n = 101), 0.36 ± 0.13 µm² (n = 101), 0.35 ± 0.13 µm² (n = 105), or 0.35 ± 0.11 µm² (n = 107) (Fig. S4). Thus, the site-2 substitution between Cys- and Ala-substituted H-NS does not change DNA compaction *in vitro*.

The extended, stiffened DNA-protein filament formed by H-NS provides structural rigidity, which can be probed by single-molecule stretching experiments.
H-NS dimerization site 2 is required for polymerization

(15, 16). AFM imaging showed that dimerization site-2 mutants formed compact structures (Fig. 4). We next performed a force-jumping procedure to measure the force-extension curve of DNA (see EXPERIMENTAL PROCEDURES for details), which minimizes interference from DNA folding during the measurement (4, 15, 16). In the absence of H-NS, naked DNA gradually extended with increased pulling force (Fig. 5, solid line). The extension of DNA complexed with H-NS or H-NS-C21S was significantly increased compared to naked DNA at the same force (Fig. 5, squares and circles), indicating that their binding increased the apparent DNA bending rigidity. In contrast, the extension of DNA complexed with H-NS-C21S-I70C or H-NS-C21S-L75C was similar to naked DNA at the same force (Fig. 5, triangles). Similar observations were also found for H-NS-I70A or H-NS-75A. The effects of protein binding on the apparent DNA bending rigidity can be quantified from such single-DNA stretching experiments (Fig. 5). The elastic behavior of a DNA polymer under tension can be modeled using the worm-like chain model, where the DNA bending rigidity is represented by a quantity with the dimension of length referred to as the persistence length. For naked DNA, the value of the bending persistence length has been determined to be ~50 nm (39, 40). The persistence length of a DNA can be quantified in single-DNA stretching experiments by fitting the measured force-extension curve using the Marko-Siggia formula (39, 40). Using this method, DNA fully coated with H-NS and H-NS-C21S had persistence lengths of 1130.29±40.53 nm and 852.21±53.34 nm, respectively (Table 1), which indicated significant DNA stiffening as compared to the naked DNA with a persistence length of 54.58±1.25 nm. The persistence lengths of complexed H-NS-C21S-I70C or H-NS-C21S-L75C were 47.06±5.33 nm and 34.67±1.36 nm, respectively. The persistence lengths of complexed H-NS-I70A or H-NS-L75A were 40.61±4.82 nm and 46.63±3.06 nm, respectively. These values were similar to naked DNA, indicating the absence of DNA stiffening. These results confirm the formation of a rigid nucleoprotein filament when DNA is complexed with H-NS or H-NS-C21S and the absence of a filament in the dimerization site-2 mutants.

**DISCUSSION**

H-NS is composed of two discrete domains, an N-terminal oligomerization consisting of dimerization site-1 and -2 and a C-terminal DNA-binding domain, connected by a flexible linker. Because of the difficulty in crystallizing intact H-NS, structural analyses have been performed on the isolated N-terminal oligomerization and C-terminal DNA binding domains (25, 41, 42). Starting from the entire set of single Cys-substitutions of H-NS, we identified two mutants in dimerization site-2, I70C and L75C, which suppressed the silencing activity of H-NS. These amino acid residues in dimerization site-2, I70 and L75, were completely conserved among orthologues and paralogues of *E. coli* H-NS (Fig. 1D).

The crystal structure of the N-terminal oligomerization domain of truncated *Salmonella* H-NS (residues 1-83) suggests a role for dimerization sites-1 and site-2 in forming a higher-order oligomers (25). In this model, the N-terminus forms a higher-order structure in tandem by interactions between upper sites (for head-to-head), referred to as dimerization site-1, and between lower sites (for tail-to-tail), referred to as dimerization site-2 (Fig. 1B). Impairment of one of these two dimerization sites is expected to result in the disruption of high-order oligomers. Substitution at dimerization site-1 also abrogates gene silencing, as well as the ability to form nucleoprotein filaments (16). The phenotypes of site-1 mutants which fail to silence target genes are well known as the presence of DNA binding ability but not form nucleoprotein filaments. However, the role of site-2 has not been characterized. Recently, van der Valk et al. showed that *E. coli* H-NS mutant, Y61-L64D, does not form multimeric structure and stiffer nucleoprotein filament *in vitro* (44). Hence, the site-2 seems to have an important role for H-NS function. Therefore, we re-examined the site-2 function in detail.

Our findings with Ile70C/A and Leu75C/A shed light on the role of the H-NS dimerization site-2 in gene silencing. EMSA and AFM indicated the ability of DNA binding in both mutants but changed the binding patterns between Cys and Ala mutants (Fig. 3). This was evident at low H-NS concentrations (120–240 nM, Fig. 3 lane 4–6), suggesting initial binding to DNA differs between Cys- and Ala-substituted H-NS. In addition, the difference between binding patterns might be affected by polar or structural changes of Cys-substituted H-NS compared to Ala-substitution. On the other hand, formation of condensed structure was similar between Cys- and Ala- mutants (Fig. 4H). As same as DNA complex *in vitro*, nucleoid formation *in vivo* did not differ between Cys- and Ala- substitutions (Fig.S4 and Table S2). Thus, only H-NS does not play a significant role in overall DNA compaction in the cell. Furthermore, the effects of protein binding on the apparent DNA bending rigidity can be quantified from such single-DNA stretching experiments (Fig. 5 and Table 1). DNA fully coated with H-NS and H-NS-C21S indicated significant DNA stiffening as compared to the naked DNA. On the other hand, H-NS-C21S-I70C, H-NS-C21S-L75C, H-NS-I70A or H-NS-L75A indicates the absence of DNA stiffening. These results demonstrate that silencing-defective mutants were unable to stiffen DNA upon binding. Recently the high-resolution structure based on solid-state NMR spectra from full-length H-NS in *E. coli* has been determined (45), in which R54 and K57 were identified to contact E74 and D68, respectively, strongly supporting the involvement of dimerization site-2 in the molecular...
interplay of H-NS dimers forming long filaments needed for gene silencing. As this region is linked to the C-terminal DNA binding domain, this interaction is potentially important for tail-to-tail linkage of H-NS molecules, as well as the position and direction of the C-terminal DNA-binding domain in an H-NS binding unit. Thus, we conclude that the phenotype of dimerization site-2 mutants is similar to that of site-1 \textit{in vitro}; the presence of DNA binding ability but nucleoprotein filaments are absent. However, the role between site-1 and -2 seems different \textit{in vivo}. Our tested promoter, \textit{slp}, was suppressed by site-2 mutants while the site-1 mutants showed minor effects on this promoter (Fig. 2). We suspect these effects due to other NAPs \textit{in vivo} because the Hha binding site is located in site-1 (33). Hha is a NAP, but it does not have the DNA binding domain. Therefore, Hha cannot bind to DNA alone. Previously Ueda et al. indicated GAD region including \textit{slp} was highly distributed by H-NS and H-NS distribution was decreased in \textit{huo}-deleted strain. Resulting, \textit{slp} promoter is under the control of both H-NS and Hha (34). Our result enhances that H-NS silences \textit{slp} promoter with Hha and YdgT (Fig. 2C). On the other hand, a typical promoter such as \textit{bglG} and \textit{proV} in previous studies had minor effects on Hha \textit{in vivo} (34). Site-1 mutants of \textit{Salmonella} H-NS, I11A and R12H, disrupt H-NS/Hha and H-NS/YdgT interaction without affecting DNA binding \textit{in vitro}, resulting in the decrease of \textit{hilA} and \textit{ssrB} silencing \textit{in vivo} (33). In addition, the NMR-based structural model of the complex between Hha and the truncated \textit{Salmonella} H-NS (residues 1-46) reveals that the formation of a three-protein charge zipper with interdigitated complementary charged residues from Hha and the two units of the H-NS dimer (43). Our comprehensive mutants assay showed that I11C slightly suppressed the silencing activity of H-NS (Fig. 2B) since a cysteine substituted for isoleucine at 11st residue in the site-1 might partially affect electric interactions between H-NS and Hha.

In summary, our findings implicate the conserved dimerization site-2 of H-NS as playing an important role in gene silencing and nucleoprotein filament formation in bacteria.

**EXPERIMENTAL PROCEDURES**

\textit{E. coli} strains, plasmids, and growth media—\textit{E. coli} strains and plasmids used in this study are listed in Table S2. Luria-Bertani (LB) or LB Agar (LB supplemented with 1.5% w/v bacto agar; pH 7) was used for standard cloning procedures.

Construction of H-NS expression plasmids—pQE80Lhns for expression of His\textsubscript{6}-H-NS was kindly provided by Dr. Taku Oshima. To construct pQE80LhnsN1, the truncated \textit{hns} gene was amplified by PCR using a pair of primers H-NS-F-47 and H-NS-R and the template DNA as W3110 type genome. The resulting PCR fragment was digested by \textit{SalI} and \textit{SphI} and ligated into pQE80L at corresponding sites. To construct a whole set of single Cys-substituted \textit{hns} mutants, the original cysteine residue at position 21 was substituted with serine by site-directed mutagenesis. In a round of PCR cycles, a pair of primers hnsC21S-F and hnsC21S-R2 (Table S2) annealed to the template DNA pQE80Lhns, replicating the plasmid DNA with the mutation. The resulting DNA pool (mutant and parental) was treated with DpnI to destroy the parental methylated DNA, leaving the newly synthesized unmethylated mutant DNA intact to transform \textit{E. coli} cells. Thus, the plasmid for the original C21-substituted H-NS, named pQE80Lhns-C21S, was prepared. Similar to H-NS-C21S, a set of cysteine-scanned \textit{hns} mutants were constructed using pQE80Lhns-C21S as the template DNA and a pair of complementary primers with a mutation (Table S2). In addition, a set of alanine scanning \textit{hns} mutants for Ile70 to Leu75 was constructed using pQE80Lhns and pQE80Lhns-C21S as the template DNA and a pair of complementary primers with a mutation (Table S2). All of the plasmids were confirmed by DNA sequencing with pQE-forward and pQE-reverse primers for pQE80L derivatives.

\textit{Measurement of luciferase activity in \textit{E. coli}—} LB medium supplemented with 50 µg/ml kanamycin and 100 µg/ml ampicillin was used for the luciferase assay. A single colony of a strain carrying a \textit{lux} reporter plasmid (Km\textsuperscript{r}) and an \textit{hns}-expressing plasmid (Ap\textsuperscript{r}) was grown overnight at 37°C with reciprocal shaking. The overnight culture was diluted 100-fold into the medium containing 10 µM IPTG to express \textit{lac}-inducible H-NS. The culture was grown overnight again and luciferase activity was measured as described in Yamanaka et al. (46). For identification of H-NS mutants affecting silencing, 96-well plates were used for incubation. Assays were performed in triplicate with three independent colonies for each strain, including that carrying the vector pQE80L in place of an \textit{hns}-expressing plasmid, to obtain the mean with the standard deviation of luciferase activity relative to that of the wild-type strain.

\textit{Purification of H-NS proteins—} To purify H-NS proteins, each pQE80L-derivative plasmid was introduced into the \textit{E. coli} BL21 (DE3). In a typical procedure (47), a single colony of transformant was grown to OD\textsubscript{600} = 0.6 at 30°C with shaking in LB medium supplemented with 100 µg/ml ampicillin. His\textsubscript{6}-H-NS was induced with 0.5 mM IPTG at 30°C for 3 hour with shaking. Cells were isolated by centrifugation and resuspended in lysis buffer (1 M NaCl, 50 mM Tris-HCl pH 8.0, 1 mM DTT) containing 2% Triton X-100. Cells were treated with lysozyme and then subjected to sonication. The lysate was centrifuged, and the supernatant was mixed with 0.5 ml of Ni-nitrilotriacetic acid (NTA) agarose resin (Qiagen) and loaded onto a column. The column was washed with lysis/ 2% Triton X-100 buffer and then washed with lysis/ 2% Triton X-100 buffer containing 25 mM imidazole. Proteins were
eluted with each elution buffer (lysis/ 2% Triton X-100 buffer with 0.1 M, 0.2 M, 0.3 M, 0.4 M, or 0.5 M imidazole), and peak fractions of H-NS were pooled and dialyzed against a storage buffer (1 M NaCl, 50 mM Tris-HCl pH 8.0, 1 mM DTT, and 50% glycerol). The protein purity was then analyzed on SDS-PAGE.

Western blotting analysis — E. coli cells grown in LB medium were harvested by centrifugation and suspended in lysis buffer containing 8 M urea and sonicated. After centrifugation, the same volume of supernatant was subjected to 18% SDS-PAGE and blotted on to PVDF membranes using an iBlot semi-dry transfer apparatus (Invitrogen). Membranes were first immuno-detected with anti-H-NS serum (46) and HRP-conjugated anti-rabbit IgG (Nacalai Tesque) antibodies and then developed with a chemiluminescence kit (Nacalai Tesque). The image was analyzed with a LAS-4000 IR multi colour imager (Fuji Film).

Circular dichroism (CD) spectroscopy — CD spectra of H-NS were measured using a J-820 spectropolarimeter (Jasco). The CD measurements were carried out in a wavelength range between 190 and 250 nm in a cell with a path length of 0.2 cm (volume 400 µl) at 25°C in the binding buffer (10 mM Tris-HCl pH 7.4 and 50 mM KCl). The spectra are the average of two or three independent measurements of each five scans recorded in 0.5 nm increments at a scan speed of 20 nm/min. The estimation of secondary structure content was performed using a Spectra Manager (Jasco).

Electrophoretic mobility shift assay (EMSA) — Probes were amplified by PCR using the pLUXsipp as a template, with a pair of primers: a specific primer and a FITC-labeled primer (Table S2). PCR products with FITC at their termini were purified using the QIAquick PCR purification kit (Qiagen). For EMSA, mixtures of the FITC-labeled probes (~700bp) and purified each H-NS protein were incubated at room temperature for 15 min in the binding buffer. After addition of a DNA dye solution, the mixture was directly subjected to 5% PAGE. Fluorescent-labeled DNA in gels was detected using LAS-4000 (Fuji Film).

Atomic force microscopy (AFM) — The AFM imaging experiments were performed on glutaraldehyde-coated mica surfaces following Winardhi et al. (22). The sI promoter DNA (~700bp) was amplified by PCR using W3110 genome as templates, with a pair of primers (Table S2). PCR product was purified using the QIAquick PCR purification kit (Qiagen). The promoter DNA fragment was incubated with 600 nM of each H-NS protein for 15 min in a test tube in binding buffer at room temperature. Following this, the DNA or protein-DNA complexes were deposited for surface fixation on the glutaraldehyde-coated mica for 15 min. The sample is then gently washed with deionized water, dried with N2 gas, and imaged. AFM imaging was performed using a Dimension FastScan AFM (Bruker Corporation) using tapping mode with silicon nitride probe (FastScan-A, Bruker Corporation). Images were acquired with a resolution of 1024 x 1024 pixels and processed with Gwyddion software (gwyddion.net). Radius of gyration distributions were obtained by application of threshold for image segmentation between the DNA or protein-DNA complexes and the background, followed by calculation of the radius of gyration for each of the distinct object.

Transverse Magnetic Tweezers — The magnetic tweezers used in this study were similar to previous studies (15, 16, 22). λ-DNA labeled with biotin on both end was tethered between a streptavidin-coated glass coverslip edge and streptavidin-coated paramagnetic bead. A pair of permanent magnet was used to stretch the DNA along the focal plane. The pulling force was controlled by adjusting the distance between the magnet and the magnetic bead. The force-extension data was obtained by using force-jumping procedure, which was carried out as follows: A single DNA was initially held at a high force (~10 pN) and then jumped to a series of lower forces for around 2 s for extension measurement. Following each force jump to lower forces, the force was jumped back to ~10 pN to ensure that protein-induced DNA folding was minimal (approximately <300 nm below that of naked DNA at force ~10 pN) and to unfold the nucleoprotein complex, if any, before the measurement resumed. The force-extension curve is thus obtained with minimal contribution from DNA folding.

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Author contributions: YY conducted most of the experiments, analyzed the results, RSW, YY and JY conducted the single-molecule experiments and analyzed the results. EY conducted the promoter assays and analyzed the results.
SN, YS and IK constructed the Cys-substitution *hns* mutant plasmids and analyzed the results. KY, AI and LJK conceived the project, and YY, IK, AI, RSW, JY, KY and LJK wrote the manuscript.
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**FOOTNOTES**

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The abbreviations used are: NAP, nucleoid associated protein; SELEX, systematic evolution of ligands by exponential enrichment; ChIP-chip, chromatin immunoprecipitation with DNA chip; GAD, glutamic acid dependent acid resistance; AFM, atomic force microscopy; IPTG, isopropyl β-D-1-thiogalactopyranoside; OD600, optical density of 600 nm; NTA, nitrilotriacetic acid.
Table 1. The value of bending rigidity of the DNA-H-NS complex

|                          | Contour length (nm) | Persistence length (nm) |
|--------------------------|---------------------|-------------------------|
| Naked DNA                | 16365±53            | 54.58±1.25              |
| H-NS                     | 16151±25            | 1130.29±40.53           |
| H-NS-C21S                | 15956±33            | 852.21±53.34            |
| H-NS-C21S -I70C          | 16265±75            | 47.06±5.33              |
| H-NS-C21S -L75C          | 16552±41            | 34.67±1.36              |
| H-NS-I70A                | 16542±11            | 40.61±4.82              |
| H-NS L75A                | 16533±24            | 46.63±3.06              |

The values were indicated using the average of three independent DNAs.
FIGURE LEGENDS

FIGURE 1. The domain organization and structure of H-NS with mutated positions previously identified by random mutagenesis. (A) Arrows indicate the amino acid residue positions of H-NS substitutions previously identified with secondary structures (27-29). On the bottom, the substituted positions of H-NS, which did not silence slp promoter (identified in this study) were represented. Substituted amino acids are colored as the follows: light-green- a previously identified residue; red- a residue isolated from this study; yellow- a residue identified by both previous studies and the present work. (B) The identified amino acid residues shown in A are colored with light-green in the three-dimensional dimer structure of the E. coli N-terminal truncated H-NS (PDB ID code 3NR7) (24). (C) The substituted amino acid residues, shown in A, are colored in the three-dimensional structure of the E. coli C-terminal truncated H-NS (PDB ID code 1HNR) (25). (D) Amino acid sequences of dimerization site-2 (residues 57-79) were aligned using ClustalW2 site (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The completely conserved amino acid residues are bold. The H-NS homologues are as follows: H-NSs from Escherichia coli, Shigella flexneri, Salmonella enterica serovar Typhimurium, Serratia marcescens, Yersinia enterocolitica, Erwinia chrysanthemi, Proteus vulgaris, Haemophilus influenzae; StpA from E. coli; VicH from Vibrio cholerae. Amino acid residues highlighted in gray are mutants isolated in this study and in red are studied in detail.

FIGURE 2. Single Cys-substitutions in H-NS that cannot silence slp promoter activity. (A) hns plasmids (pQE80Lhns and pQE80Lhns-C21S) or an empty plasmid (a vector pQE80L) were introduced into wild-type or a Δhns strain harbouring the pLUXslpp. Transformants were grown in 96-well plate containing LB medium with 10 µM IPTG at 37°C overnight, and then the promoter activity was calculated as described in EXPERIMENTAL PROCEDURES. (B) An entire set of single Cys-substituted hns-expressing plasmids (Table S2) was used for each promoter activity in Δhns strains as described above. The horizontal axis in the right panel indicates the position of H-NS amino acid residues. The A18C mutant was not measured (asterisk). (C) hns plasmids (pQE80Lhns and pQE80LhnsN1) or an empty plasmid (a vector pQE80L) were introduced into wild-type, a Δhns strain, a Δshha strain, a ΔydgT strain, or a ΔydgTΔydgT strain harbouring the pLUXslpp. Transformants were grown in 96-well plate containing LB medium with 10 µM IPTG at 37°C overnight, and then the promoter activity was calculated as described in EXPERIMENTAL PROCEDURES.

FIGURE 3. EMSA of H-NS binding to slp promoters. The DNA probe was amplified by PCR using the reporter plasmids as templates and the following primer pairs; pLUXslpp and a pair of SLP- F-2 and Lux-R-FITC. FITC-labeled probe (0.033 µM) was incubated with H-NS (A), H-NS-C21S (B), H-NS-C21S-I70C (C), H-NS-C21S-L75C (D), H-NS-I70A (E), or H-NS-L75A (F) protein with 0 (lane 1), 0.006 (lane 2), 0.06 (lane 3), 0.12 (lane 4), 0.18 (lane 5), 0.24 (lane 6), 0.3 (lane 7), 0.45 (lane 8), 0.6 (lane 9), 0.9 (lane 10), 1.2 (lane 11), 1.5 (lane 12), 3 (lane 13) µM. The DNA-H-NS complex was analyzed by native PAGE. In the gel, non-specific amplifying DNA was also found in both DNA probes. The arrow indicates the free DNA.

FIGURE 4. AFM imaging of DNA-H-NS complexes. (A–G) The AFM imaging experiments were performed on glutaraldehyde-coated mica surfaces. slp promoter DNA (0.2 ng/µL) was incubated with 600 nM purified H-NS protein for 15 min in a test tube in 10 mM Tris-HCl pH 7.4 and 50 mM KCl at room temperature. All AFM images were 0.7 µm x 0.7 µm. The color gauge represents the height of molecules in the AFM image. (H) The quantification of AFM imaging by measuring radius of gyration.

FIGURE 5. DNA stretching assay of DNA-H-NS complexes. Force extension curves of λ-DNA incubated with (dotted line) or without (solid line) 600 nM H-NS proteins using force-jumping (described in EXPERIMENTAL PROCEDURES). H-NS wild-type (opened-circle), H-NS-C21S (opened-square), H-NS-C21S-I70C (filled-triangle), H-NS-C21S-L75C (filled-diamond), H-NS-I70A (opened-triangle), and H-NS-L75A (opened-diamond).
Yamanaka et al. (Fig. 1)
A

B

Oligomerization domain

Dimerization site-1

Dimerization site-2

DNA-binding domain

C

[Graphs and bar charts showing promoter activity for different conditions, including vectors with H-NS, Hha, and YdgT deletions, and the amino acid residues affected by these modifications.]

Yamanaka et al. (Fig. 2)
Yamanaka et al. (Fig. 3)
Yamanaka et al. (Fig. 4)
Yamanaka et al. (Fig. 5)
The dimerization site-2 of the bacterial DNA-binding protein H-NS is required for gene silencing and stiffened nucleoprotein filament formation

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