Interaction between the bacterial nucleoid associated proteins Hha and H-NS involves a conformational change of Hha

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The H-NS family of proteins has been shown to participate in the regulation of a large number of genes in Gram-negative bacteria in response to environmental factors. In recent years, it has become apparent that proteins of the Hha family are essential elements for H-NS-regulated gene expression. Hha has been shown to bind H-NS, although the details for this interaction are still unknown. In the present paper, we report fluorescence anisotropy and NMR studies of the interaction between Hha and H-NS₆₄, a truncated form of H-NS containing only its N-terminal dimerization domain. We demonstrate the initial formation of a complex between one Hha and two H-NS₆₄ monomers in 150 mM NaCl. This

complex seems to act as a nucleation unit for higher-molecular-mass complexes. NMR studies suggest that Hha is in equilibrium between two different conformations, one of which is stabilized by binding to H-NS₆₄. A similar exchange is also observed for Hha in the absence of H-NS when temperature is increased to 37°C, suggesting a key role for intrinsic conformational changes of Hha in modulating its interaction with H-NS.

Key words: fluorescence anisotropy, Hha, H-NS, NMR, nucleoid-associated protein, protein–protein interaction.

INTRODUCTION

Bacterial nucleoid-associated proteins play both structural and modulatory roles. In Gram-negative bacteria, the H-NS family of proteins has deserved extensive research efforts [1]. Proteins belonging to this family are involved in organizing the nucleoid structurally and in the regulation of the expression of many operons (for recent reviews, see [2–4]). Among others, environmental factors such as osmolarity or temperature [5,6] switch regulatory responses requiring the presence of H-NS. In *Escherichia coli*, up to 5 % of the genes are subjected to H-NS modulation [7]. H-NS proteins do not exhibit a preference for specific DNA sequences, but rather for DNA structures: curved DNA sequences are usually targets for H-NS [8]. To repress transcription, binding to two distant sites located nearby the promoter, H-NS oligomerization and nucleation of the DNA region appear as key steps [9–11]. Nevertheless, the specific molecular details of the process remain to be elucidated.

In recent years, there has been evidence that at least some of the processes that require H-NS to modulate gene expression may require H-NS to interact directly with other proteins. Generation of heterodimers between H-NS and its paralogue StpA has been reported [12–14]. Interaction of H-NS with StpA protects StpA from Lon-mediated proteolysis [13]. It has also been shown that StpA can act as a molecular adapter for some species of truncated H-NS proteins to repress the *bgl* operon [15]. However, details of the modulatory role of such heterodimers remain to be elucidated.

Characterization of the Hha/YmoA family of nucleoid-associated proteins has also provided evidence for protein–protein interactions underlying H-NS-mediated modulation of gene expression. Proteins of the Hha/YmoA family are small (approx. 8 kDa) and are moderately basic. They were reported to repress gene expression in different enterobacteria in response to changes

in temperature or osmolarity [16,17]. When analysing sequence similarity data, no apparent relationship with other families of nucleoid-associated proteins could be established. Studies focused to understand the mechanism underlying Hha-mediated repression of *E. coli* toxin α -haemolysin showed that, rather than exhibiting DNA-binding activity, Hha exhibited H-NS-binding activity [18]. A role for a Hha–H-NS complex repressing the *hly* operon at low temperature was then shown [11,18]. Since then, interactions of other members of the family (YmoA, YdgT) with members of the H-NS family have also been shown [19,20]. In spite of the genetic and biochemical evidence demonstrating protein–protein interactions between both families of proteins, molecular details of the process are lacking. A mutational analysis of the Hha protein failed to identify a specific region of Hha involved in binding to H-NS. Instead, mutations in different regions can inhibit Hha binding to H-NS, suggesting that most of the protein is involved directly or indirectly in the binding process [19].

H-NS consists of an N-terminal dimerization domain and a C-terminal DNA-binding domain separated by a linker domain that is involved in the formation of high-molecular-mass oligomers [4]. A truncated form of H-NS comprising residues 1–64 (H-NS₆₄) has been reported to form only dimers [21]. An alignment of the amino acid sequences of members of the Hha family of proteins with the N-terminal region of H-NS-like proteins showed groups of conserved residues, which suggests that Hha-like proteins may be functionally equivalent to the N-terminal domain of H-NS-like proteins [19].

In the present paper, we report fluorescence anisotropy and NMR studies of the interaction between Hha and H-NS₆₄ that allow the determination of the stoichiometry and stability of the complex formed and provide evidence for a conformational process in Hha associated with its interaction with H-NS₆₄, but that can be detected also in free Hha.

Abbreviations used: CPMG, Carr–Purcell–Meiboom–Gill; DTT, dithiothreitol; HSQC, heteronuclear single-quantum correlation NMR spectroscopy; IPTG, isopropyl β -D-thiogalactoside; Ni-NTA, Ni²⁺-nitrilotriacetate.

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MATERIALS AND METHODS

DNA clones

The clone encoding His₆-Hha has been described previously [22]. The expressed sequence is MGSS(H)₆SSGRENLYFQGH-(Hha)GS. H-NS₆₄, carrying a C-terminal His₆-purification tag, was prepared by PCR amplification from a full-length *E. coli* H-NS plasmid [19]. Restriction sites, His₆-tag and a C-terminal stop codon were introduced using PCR primers: sense primer, 5'-GA-TTACTACCATGGGCGAAGC-3' and antisense primer, 5'-CG-GGATCCTATTAATGGTGATGGTGATGGTGCGAGCATTTCG-CGA-3' (restriction sites are underlined). The PCR product was then subcloned into the NcoI/BamHI sites of plasmid pET-15b (Novagen) following standard protocols. All clones were sequenced before use.

Protein expression and purification

Transformed BL21(DE3) cells were grown at 37 °C in either LB (Luria-Bertani) or in M9 minimal medium containing ¹⁵NH₄Cl until a *D*₆₀₀ of 0.7 was reached. In both cases, Hha overexpression was induced overnight at 15 °C by the addition of IPTG (isopropyl β-D-thiogalactoside) (1 mM final concentration). Cell pellets were frozen, resuspended in 20 mM Tris/HCl, 800 mM NaCl, 15 mM 2-mercaptoethanol and 5 mM imidazole, pH 8.0, and lysed by sonication (six 10 s pulses). The lysate was centrifuged at 20 000 *g* for 30 min at 4 °C, and the supernatant was then treated with Ni-NTA (Ni²⁺-nitrilotriacetate)-agarose (Qiagen). The resin was washed extensively with the same buffer, and the protein was eluted using 400 mM imidazole. A final purification step on a Superdex 75 column in 20 mM sodium phosphate, 150 mM NaCl, 2 mM DTT (dithiothreitol) and 0.01 % (w/v) sodium azide, pH 7.0, yielded pure Hha as a monomer. MS of the purified protein gave a molecular mass of 11 202 Da, corresponding to the sequence expressed with the depletion of the N-terminal methionine residue. Oligomers were occasionally formed during purification or upon storage, but were cleanly separated by size-exclusion chromatography on a Superdex 75 column. The purity and aggregation state of Hha was checked by gel filtration, SDS/PAGE, MS and steady-state fluorescence anisotropy.

H-NS₆₄ was expressed using the same procedure, except that expression was initiated with 0.5 mM IPTG, lysis and purification steps were carried out in the presence of 1 M NaCl, 20 mM 2-mercaptoethanol and 0.5 mM EDTA, and the protein was eluted from the Ni-NTA resin with 50 mM EDTA. Ni-EDTA was removed by gel filtration on a Superdex 75 column. Compared with protein molecular-mass standards, H-NS₆₄ showed an apparent molecular mass consistent with a dimer. MS gave the expected mass for H-NS₆₄ monomer. Ellman analysis, after dialysis to remove DTT, confirmed that cysteine was in the reduced form.

Fluorescence spectroscopy

In contrast with full-length H-NS, H-NS₆₄ does not contain tryptophan, allowing for selective observation of Hha fluorescence during complex formation.

Hha samples used for fluorescence measurements were prepared directly from the isolated peak corresponding to the Hha monomer from a Superdex 75 column, quantified using the absorption intensity at 280 nm and diluted to the desired concentration. Samples stored for more than 1 week and showing higher anisotropy than expected were re-purified before use.

Fluorescence measurements were performed in 20 mM sodium phosphate, pH 7.0, 150 mM NaCl, 0.01 % (w/v) sodium azide and 1 mM Tris(2-carboxyethyl)phosphine. This reducing agent was

used in order to minimize both the absorption and the fluorescence background.

Fluorescence spectra were recorded in 1 cm path-length cells at 25 °C on an Aminco Bowman AB2 fluorimeter using an excitation wavelength of 295 nm to minimize excitation of tyrosine and an excitation bandwidth of 2 nm. Emission spectra were obtained as the ratio between observed and reference signals, and were corrected for instrument response and background fluorescence. Relative quantum yields of Hha or Hha-H-NS₆₄ complexes were obtained by comparing the integrated intensities of their fluorescence spectra with those of tryptophan measured in the same buffer (i.e. with the same refractive index) and the same total absorption. The ratios of quantum yields of Hha and tryptophan in one hand, and complexed and free Hha in the other, are both 0.95. The sample of complexed Hha is a 1:2.5 mixture with H-NS₆₄. The absorbance of the samples was kept below 0.05 absorbance units to avoid inner filter effects.

Fluorescence anisotropy

Steady-state fluorescence anisotropy is observed after excitation with polarized light if the correlation time for the re-orientation of the chromophore is long with respect to the fluorescence lifetime. Steady-state anisotropy is related to correlation time of an isotropic species through the Perrin equation (eqn 1) [23]:

$$\frac{1}{A} = \frac{1}{A_0} \left(1 + \frac{\zeta}{\tau} \right) \quad (1)$$

where *A*₀ is the anisotropy expected for a fixed macromolecule and includes intrinsic factors of the chromophore and local motion effects, ζ is the fluorescence lifetime, and τ is the isotropic rotational correlation time. Fluorescence anisotropy can be used to follow the increase in correlation time associated with the formation of protein-protein complexes.

Steady-state fluorescence anisotropy was determined at 344 nm with a bandwidth of 4 nm using an autopolarizer accessory with 'L' geometry, and is the average of 40 measurements, each with an independent determination of the G-factor. Experimental uncertainty was evaluated by comparing at least three duplicates of the complete measurement and was ± 0.001 .

The correlation time of Hha in complexes can be estimated from the ratio of fluorescence anisotropies of free and complexed Hha using eqn (2), which is directly derived from eqn (1):

$$\tau_b = \frac{R\tau_f\zeta_b}{\tau_f(1-R) + \zeta_f} \quad (2)$$

where $R = A_b/A_f$, τ_b and τ_f are isotropic correlation times of the free and bound forms, and ζ_f and ζ_b are the corresponding fluorescence lifetimes.

The implicit assumption that *A*₀ in Perrin's equation is not significantly affected by complexation is supported by the similar emission maxima and quantum yields.

The correlation time for free Hha at 25 °C was estimated using the Stokes-Einstein equation [23] and the molecular mass of Hha. The fluorescence lifetimes were obtained from the relative quantum yields measured for tryptophan, free Hha and Hha in the presence of 2.5 equivalents of H-NS₆₄. Taking as a reference the lifetime of tryptophan (2.6 ns at pH 7.0 and 25 °C) the lifetimes of tryptophan in Hha and Hha-H-NS₆₄ complexes were calculated to be 2.47 and 2.35 ns respectively under our experimental conditions.

The fluorescence anisotropy of the complex was obtained from the fitting of a titration of Hha with H-NS₆₄ (see below).

H-NS₆₄ titrations

H-NS₆₄ titrations were carried out at 25 °C with freshly purified Hha and H-NS₆₄ samples. The sample initially contained 2 ml of 4 μ M Hha, and, for each point, 70 μ l of a H-NS₆₄ stock solution was added and incubated for 5 min with magnetic stirring before measurement. The accuracy of the volumes added was calibrated gravimetrically and was better than ± 0.2 μ l. The actual concentrations, corrected for the effects of dilution at each point, were used for the analysis. Titration curves were analysed using a model involving the formation of a 1:2 complex:

with a dissociation constant, K_d , given by

$$K_d = \frac{[\text{Hha}][\text{H-NS}_{64}]^2}{[\text{Hha} - (\text{H-NS}_{64})_2]} \quad (3)$$

The fraction of Hha bound to H-NS₆₄ can be determined from the fluorescence anisotropy, corrected by the relative quantum yields of Hha in the free (Φ_f) and bound (Φ_b) forms using eqn (4) [24].

$$f_b = \frac{A - A_f}{A_b - A + Q(A - A_f)} \quad (4)$$

where A is the observed anisotropy and A_f and A_b are the anisotropies of free Hha and the complex respectively and $Q = \Phi_b/\Phi_f$.

By combining eqns (3) and (4), the observed anisotropy A can be related to the known values of A_f , Q and the total concentrations of Hha and H-NS₆₄, and the unknown parameters: K_d and A_b . These were determined by non-linear minimization of the error function

$$\chi^2 = \sum \frac{(A_{\text{obs}} - A_{\text{th}})^2}{\sigma^2} \quad (5)$$

where A_{obs} is the observed fluorescence anisotropy, A_{th} is the value predicted by the model and σ^2 is the experimental uncertainty. The minimization was carried out using in-house scripts written in Mathematica (Wolfram Research). Alternative models, including the formation of an intermediate 1:1 complex, were compared using the fitting and statistical tools of DynaFit, without the inclusion of quantum yield corrections [25]. Alternative models were compared using F-statistics. Error estimates were obtained from DynaFit.

NMR spectroscopy

NMR samples used for variable temperature spectra and for titrations with H-NS₆₄ were 80–90 μ M uniformly ¹⁵N-labelled Hha in 20 mM sodium phosphate buffer, 150 mM NaCl, 2 mM DTT and 0.01 % (w/v) sodium azide, pH 7.0, in the presence of 10 % (v/v) ²H₂O. ¹H-¹⁵N HSQC (heteronuclear single-quantum correlation NMR spectroscopy) spectra were recorded on a Bruker Avance 600 using 1024 $\times$ 128 points and 48 accumulations. Assignments of ¹H-¹⁵N HSQC spectra at 25 °C were obtained from Yee et al. [22].

¹H-¹⁵N HSQC experiments containing a relaxation compensated CPMG (Carr–Purcell–Meiboom–Gill) filter [26] were recorded at 37 °C on a Bruker Avance 800 MHz. Two experiments with an identical duration of the CPMG train (32 ms), but with different CPMG refocusing delays (250 and 500 μ s), were collected.

Temperature coefficients ($\delta\Delta_{\text{NH}}/\Delta T$) in parts per billion per K, where $\delta\Delta_{\text{NH}}$ is the difference of the HN chemical shift, were measured from a series of four ¹H-¹⁵N HSQC experiments recorded between 25 and 37 °C, and were obtained by plotting the

Figure 1 Fluorescence anisotropy titration of Hha with H-NS₆₄

Titration was carried out at 25 °C with an initial Hha concentration of 4 μ M.

amide chemical shift versus the temperature. Chemical-shift values were referenced considering the temperature-dependence of HDO chemical shifts [27].

CD

CD spectra were measured on a JASCO J-810 instrument, equipped with a Peltier accessory for temperature control, using a 1 cm path-length cell. CD measurements were performed at far UV (200–250 nm) with a scan speed of 50 nm/min, 4 nm bandwidth and an average response time of 4 s.

RESULTS

Fluorescence anisotropy provides the stoichiometry of the complex and its dissociation constant

H-NS₆₄ has no tryptophan residues and it is therefore possible to observe the formation of Hha–H-NS₆₄ complexes using the intrinsic fluorescence of Hha. Emission spectra of Hha were measured with excitation at 295 nm to minimize light absorption by tyrosine. The emission maximum at 25 °C is at 344 nm, typical of a partially solvent exposed tryptophan side chain. In the presence of 2.5 equivalents of H-NS₆₄, the emission maximum is shifted to 340 nm.

Figure 1 shows the change in fluorescence anisotropy of Hha caused by the addition of H-NS₆₄. The fluorescence anisotropy of Hha increases and levels off after the addition of approx. 2 equivalents of H-NS₆₄ to Hha, suggesting the formation of a discrete complex. A good fitting of the curve was achieved assuming the formation of a 1:2 complex with a dissociation constant of 0.45 μ M² at 25 °C in 20 mM sodium phosphate buffer and 150 mM NaCl, pH 7.0. The uncertainty estimated from the fitting was ± 0.08 . The actual precision could be lower, as the concentration of free H-NS₆₄ was determined indirectly in an iterative procedure.

Small deviations between computed and experimental curves are not due to interference from intrinsic monomer–dimer equilibria for H-NS₆₄. In gel-filtration experiments in the absence of Hha, H-NS₆₄ elutes at a constant volume, consistent with the molecular mass of a dimer, at concentrations between 4 and 470 μ M (T. N. Cordeiro and J. Garcia, unpublished work). A truncated form of H-NS containing only the first 46 residues forms dimers with a dissociation constant lower than 5 nM, as determined by fluorescence anisotropy [28]. Dissociation of H-NS₆₄ dimers induced by Hha cannot be ruled out. A model assuming an intermediate 1:1 species is compatible with titration data and gives a lower χ^2 . However, the fitting requires two additional adjustable

Figure 2 ^1H - ^{15}N HSQC spectra of Hha in the presence or the absence of H-NS₆₄

(A) ^1H - ^{15}N HSQC spectrum of 90 μM uniformly ^{15}N -labelled Hha at 25 °C under the conditions described in the Materials and methods section. (B) ^1H - ^{15}N HSQC spectrum under the same conditions after addition of 45 μM unlabelled H-NS₆₄. Broadening causes a decrease in intensity of signals. Cross-peak intensities were normalized with respect to the one showing the highest intensity in each spectrum. Residues that show a decrease in normalized intensity below 25 % of the value observed in free Hha are labelled. Side-chain NH groups are indicated by 'sc' after the residue number.

parameters (two binding constants and two anisotropy values). A comparison of the 1:2 and 1:1 models gives an F value of 2.17, which is lower than the limit value of 2.72 (90 % confidence, and 9 and 7 degrees of freedom). One should conclude that the 1:1 model, although it cannot be excluded, does not provide a statistically significant improvement in the explanation of the present experimental data.

The anisotropy derived from the fitting provides an estimation of the correlation time of the complex which depends strongly on the reference correlation time assumed for free Hha. Reference values of 4 and 5 ns provide estimates of 7.6 and 11.0 ns for the correlation time of the complex using eqn (2). The correlation time calculated for a sphere with a mass corresponding to the molecular mass of the Hha-(H-NS₆₄)₂ complex is 10.1 ns.

Addition of excess H-NS₆₄ causes a marked increase in fluorescence anisotropy, indicating that higher-molecular-mass species containing Hha are being formed (results not shown). In the presence of more than 2.5 equivalents (8 μM) of H-NS₆₄, species with longer correlation times containing Hha are formed. In the absence of Hha, H-NS₆₄ forms only dimers even at much higher concentrations. High-molecular-mass Hha_m-(H-NS₆₄)_n (where *m* and *n* are variables) hetero-oligomers are thus induced by the presence of Hha, probably by the addition of further H-NS₆₄ molecules to the initially formed Hha-(H-NS₆₄)₂ complex.

Titration with H-NS₆₄ affects residues in the hydrophobic core of Hha

The three-dimensional structure of Hha at 25 °C has been solved by NMR [22]. The structure of Hha consists of four α -helical segments: helix 1 (residues 8–16), helix 2 (residues 21–34), helix 3 (residues 37–55) and helix 4 (residues 65–70). The four helical segments are separated by loops. The architecture of Hha is constructed around the long helix 3. Helices 1 and 2 are packed against the N-terminal half of helix 3. Helix 4 is very short and interacts with the C-terminal part of helix 3.

^{15}N -labelled Hha gives sharp well-resolved HSQC spectra at 25 °C. In the presence of 1 equivalent of H-NS₆₄, severe broaden-

ing of Hha signals is observed at 25 °C, leading to a general decrease in intensity of the whole spectra (results not shown). However, when only a 0.5 equivalent of H-NS₆₄ is added, most of the signals are still visible, but differential broadening of a set of residues is clearly observed. Figure 2 shows HSQC spectra of 90 μM ^{15}N -labelled Hha before and after the addition of 0.5 equivalents of unlabelled H-NS₆₄.

The broadening observed at 25 °C results from exchange between two sites in the intermediate regime, i.e. at a rate comparable with the difference in the chemical-shift frequencies of the two sites. This is clearly demonstrated by lowering the temperature to 7 °C. At this temperature, the rate of exchange becomes lower than the frequency difference, and duplicate signals are observed for the free and bound forms. Figure 3 shows an expansion of signals from Ala⁴⁶ of Hha in a series of HSQC spectra measured at 7 °C in the presence of increasing amounts of H-NS₆₄. A decrease of signals from free Hha and an increase in the intensity of a new signal corresponding to the formation of a complex are clearly observed.

Exchange broadening depends on the exchange rate, the frequency difference between exchanging sites and the product of the relative populations of the sites. At low concentrations of H-NS₆₄, only those residues showing the largest frequency differences between exchanging sites show extensive broadening. Figure 4 shows the location of affected residues in the structure of Hha. Residues whose NH signals are most perturbed by the addition of H-NS₆₄ are located in the four helices of Hha: residues Lys⁸, Thr⁹, Asp¹⁰ and Leu¹² are at the N-terminus of helix 1. Helix 3 has eight residues perturbed by H-NS₆₄: Leu⁴⁰, Val⁴², Ser⁴⁵, Ala⁴⁶, Ala⁴⁷, Asp⁴⁸, His⁴⁹ and Ala⁵². Five of them, i.e. more than one complete helix turn, are consecutive (Ser⁴⁵–His⁴⁹). Val⁴², Ser⁴⁵, Ala⁴⁶, His⁴⁹ and Ala⁵² are on one side of the helix, and Ala⁴⁶ is at the interface between helices 1 and 3. Leu⁴⁰ and Ala⁴⁷ are in the opposite side of helix 3, and are flanking two aromatic residues (Phe⁴³ and Tyr⁴⁴) that form the interface with helix 2. Helix 4 has three residues broadened by the addition of H-NS₆₄: Val⁶⁷, Trp⁶⁸ and Phe⁷⁰. Ile²⁸ is the only perturbed residue in helix 2 and is located at the contact interface with helix 3.

Figure 3 ^1H - ^{15}N HSQC spectra at 7 °C of Hha with increasing amounts of H-NS₆₄

Expansions of the ^1H - ^{15}N HSQC spectra recorded at 7 °C illustrating the decrease in intensity of the Ala⁴⁶ cross-peak in the presence of increasing concentrations of H-NS₆₄. A new peak corresponding to the complex appears simultaneously. The Hha/H-NS₆₄ ratio is indicated on the right.

Temperature effects on Hha conformation

Exchange effects observed in core residues of Hha when it binds to H-NS₆₄ suggest that Hha changes its conformation, and we have tried to determine whether, in the absence of H-NS₆₄, alternative conformations of Hha are also accessible. The presence of exchange in free Hha could be revealed by non-linear changes in chemical shifts or by the observation of exchange-broadening effects similar to those observed in the presence of H-NS₆₄ induced by a small temperature increase.

Figure 5 shows a comparison of ^1H - ^{15}N -HSQC spectra of 80 μM Hha at 25 and 37 °C. In spite of the lower correlation time at higher temperatures that is expected to give narrower NMR line widths, at 37 °C, exchange broadening is observed for all residues, but is especially significant for six residues which show intensity below 25 % of that observed at 25 °C: Lys⁸, Ile²¹, Ile²⁸, Tyr⁴⁴, Tyr⁶⁰ and Ile⁶³. An exchange contribution to most of the NH peaks could be confirmed by comparing the intensities of ^1H - ^{15}N HSQC spectra filtered with relaxation-compensated CPMG periods of identical duration, but differing in the interpulse delay (Figure 6). Exchange effects are minimized when interpulse delays are shorter and signal intensity in these spectra will be higher.

Temperature coefficients represent the dependence of chemical shift on temperature. In rigidly structured proteins, amide protons usually show negative temperature coefficients with values that depend on solvent exposure or hydrogen bond formation. Positive temperature coefficients are usually indicative of conformational exchange [29]. Four Hha residues show positive temperature coefficients in their amide proton signals (Figure 6): Ala⁴¹, Ala⁴⁶, Val⁶⁷ and Trp⁶⁸. In addition, Val⁶⁷ changes its ^{15}N chemical shift between 25 and 37 °C. The aliphatic region of ^1H -NMR spectra obtained at 25 and 37 °C also present frequency shifts and variations

Figure 4 Overview of Hha residues affected by H-NS₆₄ binding and temperature changes

(A) Ribbon and surface representation of Hha structure. Residues most affected by broadening in the presence of a 0.5 equivalent of H-NS₆₄ are coloured yellow. The affected residues are identified along the sequence in (C, row 4). (B) Solvent accessibility of Hha. Residues are coloured according to the percentage of solvent-exposed surface: red (< 5%), yellow (5–20%) and blue (> 20%). The sequence location of buried residues is indicated in (C, row 3) using the same colour code. (C) Secondary structure (row 1), solvent exposure (row 3), H-NS₆₄-induced perturbations (row 4) and temperature-induced exchange effects (row 5) are compared along the Hha sequence (row 2). In row 5, residues that are selectively broadened at 37 °C are indicated in yellow and those whose temperature coefficients are positive in red.

Figure 5 Comparison of ^1H - ^{15}N HSQC spectra of Hha at 25 °C and 37 °C

^1H - ^{15}N HSQC expansions of Hha (80 μM) under analogous conditions to those described in the Materials and methods section recorded at 25 °C (A) or 37 °C (B). Exchange between different conformations causes variations in line width of most of the cross-peaks. Residues that show the largest effects have been labelled.

Figure 6 NMR evidence for temperature-induced conformational exchange in Hha

(A) Temperature coefficients of NH signals of Hha. Residues showing positive temperature coefficients, indicative of exchange, are highlighted. (B) Distribution of intensity ratios between equivalent cross-peaks in two CPMG-filtered HSQC experiments with 250 (exp 1) and 500 μs (exp 2) interpulse delays, but identical CPMG length. Ratios (exp 1/exp 2) higher than 1 are indicative of chemical exchange. The higher sensitivity of CPMG experiments show that exchange affects most residues of Hha, as expected for a global conformational change. The temperature was 37 °C, and only well-resolved signals visible at this temperature are included.

in line width, suggesting exchange between different conformations (results not shown). All changes are reversible.

We have used CD to determine the effect of temperature in the secondary structure of Hha. CD spectra of Hha (results not shown) are typical for a helical protein, with minima at 205 and 222 nm. The change in ellipticity between 6 °C and 40 °C is linear. The ellipticity at 222 nm measured at 37 °C is 96 % of the value observed at 25 °C, and the change is fully reversible. Therefore the secondary structure of Hha is not substantially affected by the increase in temperature from 25 to 37 °C. These results suggest that temperature-induced changes in Hha correspond to a change in tertiary structure leading to a modification of helical packing.

DISCUSSION

Hha is known to bind to H-NS, and the presence of Hha affects the expression of genes under H-NS control. Although the overall homology between Hha and H-NS is low, Hha shows groups of conserved residues with the dimerization domain of H-NS present in a truncated form containing only the first 64 residues of H-NS. Our fluorescence and NMR results clearly show that Hha interacts strongly with the dimerization domain of H-NS. The stoichiometry of the complex formed under the experimental conditions used in the present study (20 mM sodium phosphate and 150 mM NaCl, pH 7.0, 25 °C) suggests that Hha interacts with two H-NS₆₄ molecules.

The interaction between Hha and H-NS₆₄ causes important changes in the NMR spectra of Hha that affect residues that are solvent-accessible in the published three-dimensional structure [22], as well as some deeply buried residues including residues located in the interface between helices 1, 2 and 3 in Hha.

At 25 °C, conformational-complexation-induced exchange causes broadening of a number of residues. At 7 °C, the rate of exchange is reduced and separate signals are observed for free and bound forms. The relative intensities change by the addition of H-NS₆₄ and, in the presence of approx. 2 equivalents of H-NS₆₄, only signals from the complex are observed, in agreement with

the stoichiometry determined from fluorescence results. However, under our present experimental conditions, the sensitivity is still too low to attempt the complete determination of the structure of the complex.

Perturbed residues include both surface and buried residues. While surface residues could be directly in contact with H-NS₆₄, perturbation of buried residues suggests a major conformational rearrangement induced by complexation. Some of the perturbed residues are completely buried in the hydrophobic core of Hha with a very low percentage of its surface exposed to solvent (Figure 4). Some of these residues are located at the interface between helices: Ala⁴⁶ (0.1 % accessibility) between helices 1 and 3, and Ile²⁸ (accessibility 13.6 %) between helices 2 and 3. This observation indicates that Hha experiences a conformational change upon complexation and suggests a model in which Hha may exist in a 'closed' form, corresponding to the structure determined by NMR for the isolated molecule, and an 'open' form that is stabilized by interaction with H-NS₆₄.

Conformational plasticity is present in Hha, even in the absence of H-NS₆₄. Major changes in line widths and chemical shifts are observed when the temperature is increased from 25 to 37 °C. At 37 °C, the exchange rate and, probably, the relative populations of the closed and open conformations change, leading to the observed broadening and chemical-shift effects. Figure 4 shows that residues that are affected by changes in temperature and by the addition of H-NS₆₄ at 25 °C partially overlap. In particular, residues Ala⁴⁶ and Ile²⁸ located at helix interfaces are perturbed both by H-NS₆₄ binding and by temperature perturbation. Ile²⁸ is the only perturbed residue of helix 2 and provides an example of a residue that is probably affected mainly by conformational effects, but is not interacting directly with H-NS₆₄. CD spectra are nearly unaffected by changing the temperature from 25 to 37 °C, indicating that the helix content does not change significantly between the two temperatures. The observed conformational change therefore seems to imply a change in the helix packing of Hha. A similar effect has been observed for the apo regulatory domain of skeletal muscle troponin C [30].

Interaction with H-NS₆₄ causes a stabilization of the open form. H-NS₆₄-induced broadening observed at 25 °C may contain a contribution from exchange between free and complexed forms, as well as exchange between open and closed conformation that are still possible in the complex. At 25 °C, broadening is still observed in the presence of 3 equivalents of H-NS₆₄. This suggests that the observed broadening is not just the result of the equilibration between free and bound species. Further work is still needed to separate both possibilities.

A similar effect has been reported for calmodulin. The N-terminal domain of calmodulin is in equilibrium between an open and a closed form that is modulated by calcium complexation [31]. In the apo-form, the interaction with a target peptide causes broadening of a number of residues that are not concentrated in a discrete binding interface and has been interpreted as a modulation of the equilibrium between open and closed forms by the peptide [32].

Our NMR results show that Hha experiences a conformational change, affecting the packing of the helices, when it binds to H-NS₆₄. A similar conformational equilibrium takes place in free Hha and is strongly affected by changing the temperature from 25 to 37 °C. It is suggestive that temperature acts as a signal for bacterial colonization of warm-blooded hosts, and the temperature sensor involved should be sensitive to changes in temperature from a lower ambient temperature to 37 °C. It is now well-established that the H-NS system is involved in mediating changes in gene expression regulated by temperature [5,6]. The conformational equilibrium of Hha may provide a sensor mechanism

for modulating H-NS-regulated gene expression in response to environmental changes such as temperature and osmolarity.

Fluorescence anisotropy demonstrates the formation of additional Hha species with longer correlation times in the presence of excess H-NS₆₄. This can be interpreted as an indication that Hha can also nucleate the formation of hetero-oligomers of H-NS₆₄ under experimental conditions in which H-NS₆₄ only forms dimers. The observed 1:2 complex is probably a first step in this nucleation. These results are consistent with the fact that, in the presence of Hha, complexes between full-length H-NS and its target DNA sequences in the operon encoding for the toxin α -haemolysin, migrate slower than those obtained in the absence of Hha [18] suggesting the formation of Hha_m-(H-NS₆₄)_n hetero-oligomers of molecular mass higher than those generated by H-NS alone.

The H-NS system is composed of H-NS itself, which is able to interact with DNA and to oligomerize the DNA regions where H-NS interacts, which may have characteristic bending capabilities, and H-NS-binding proteins that modulate H-NS's ability to oligomerize. Our results lend support to the role of Hha as a modulator of H-NS oligomerization and identify the N-terminal domain of H-NS as an interaction site. Our results also suggest a possible mechanism by which environmental changes affecting conformational equilibria in Hha may cause changes in the expression of genes under H-NS control. Our groups are actively pursuing this line of research.

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