Nature and mechanism of the *in vivo* oligomerization of nucleoid protein H-NS

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Two types of two-hybrid systems demonstrate that the transcriptional repressor, nucleoid-associated protein H-NS (histone-like, nucleoid structuring protein) forms dimers and tetramers *in vivo*, the latter being the active form of the protein. The H-NS 'protein oligomerization' domain (N-domain) is unable to oligomerize in the absence of the intradomain linker while the 'DNA-binding' C-domain clearly displays a protein–protein interaction capacity, which contributes to H-NS tetramerization and which is lost following Pro115 mutation. Linker deletion or substitution with KorB linker abolishes H-NS oligomerization. A model describing H-NS dimerization and tetramerization based on all available data and suggesting the existence in the tetramer of a bundle of four α -helices, each contributed by an H-NS monomer, is presented.

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Introduction

DNA-binding protein H-NS (histone-like, nucleoid structuring protein) (Lammi *et al*, 1984; Spassky *et al*, 1984; Falconi *et al*, 1988) plays an architectural role in the organization of the bacterial nucleoid and regulates the expression of a large number of genes (Ussery *et al*, 1994; Altung and Ingmer, 1997; Hommais *et al*, 2001; Dorman, 2004; Pon *et al*, 2005). Both *in vitro* and *in vivo* experiments indicated that H-NS oligomerization is critical for determining the capacity of this protein to bind curved DNA, to bend noncurved DNA and for its biological activity (Spurio *et al*, 1997; Pon *et al*, 2005). However, the type of quaternary structure acquired by H-NS and the nature of the interactions involved in its formation are somewhat controversial. In an early study (Gualerzi *et al*, 1986; Falconi *et al*, 1988), this problem was tackled by analyzing the products of protein–protein crosslinking obtained as a function of H-NS concentration; reaction with the bifunctional reagents dimethyl suberimidate (DMS) (8 Å

and dimethyl adipimidate (DMA) (6.4 Å) yielded the same amount of crosslinked products, with the amounts of these products remaining constant between 16.5 nM and 100 μ M H-NS. However, above 100 μ M H-NS, the yield of dimers, trimers and tetramers increased considerably. The results were interpreted to suggest the existence of an equilibrium: monomer $\rightleftharpoons$ dimer $\rightleftharpoons$ tetramer. Moreover, it was concluded that the protein surfaces responsible for monomer–monomer interactions are different from those implicated in dimer–dimer interaction and that, unlike with HU (designated NS at that time), the presence of DNA did not influence the yield of crosslinked products of H-NS (Losso *et al*, 1986; Falconi *et al*, 1988). In agreement with these conclusions, size-exclusion chromatography indicated that H-NS consists mainly of dimers and tetramers (Spurio *et al*, 1997; Ueguchi *et al*, 1997). More recently, large zone gel permeation chromatography, an approach that allows quantification of individual molecular species at equilibrium, led to the conclusion that monomers, dimers and tetramers coexist at equilibrium and that the prevalent form of H-NS (at $\geq 10^{-7}$ M) is the tetramer (Ceschini *et al*, 2000). The ionic strength and type of monovalent metal ion present in solution were also found to have a profound effect on the oligomerization equilibrium (Ceschini *et al*, 2000). However, in contrast with the conclusion shared by all the above-mentioned articles, results obtained by a variety of biophysical techniques (NMR spectroscopy, gel filtration, sedimentation equilibrium, CD spectroscopy) have led Smyth *et al* (2000) and Renzoni *et al* (2001) to conclude that wild type (wt) H-NS, like its isolated N-terminal domain, exists as a trimer. Furthermore, the same authors reported that when the H-NS concentration is increased to $\approx 30 \mu$ M, the trimers undergo further aggregation to yield a wide range of different and large heterodisperse oligomers. This type of disordered quaternary structure could account for the massive, concentration-dependent, 'linear polymerization' of DNA-bound H-NS held responsible for the extended and ill-defined footprints often produced by H-NS on some tracts of DNA (Rimsky and Spassky, 1990; Zuber *et al*, 1994; Jordi *et al*, 1997). However, a trimeric structure does not fit with either of the two alternative 3D models proposed for the N-terminal domain of *Escherichia coli* (residues 1–46) and *Salmonella typhimurium* (residues 1–64) of H-NS (Esposito *et al*, 2002; Bloch *et al*, 2003), both of which predict a dimeric structure for this 'oligomerization domain'.

Since oligomerization of H-NS is essential for the recognition of the DNA targets on which this protein exercises its (mostly repressor) activity, it is of interest to clarify the *in vivo* quaternary structure of H-NS, and to map the protein sites responsible for these interactions.

In this study, we have used two types of gene fusions to investigate H-NS oligomerization *in vivo*, to determine the effect of some H-NS mutations on this property. Our results have (a) shown that H-NS can form both dimers and tetramers within the cell, (b) demonstrated that, in addition to the N-domain, both linker and C-domain play an important role

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in H-NS oligomerization, and (c) allowed us to build a model of how dimers and tetramers are formed.

Results

The experimental systems

In a previous study (Spurio *et al*, 1997), the lytic cycle of phage λ was found to be repressed in *E. coli* cells expressing the chimerae H-NS:: λ cIN, but not the DNA-binding domain alone (λ cIN) of λ repressor. These data indicated that H-NS can replace the C-domain (λ cIC) of λ cI and induce the oligomerization of λ cIN, thus conferring transcriptional repressor function to this otherwise inactive repressor fragment. The oligomerization capacity of wt H-NS and of some of its mutants was quantified from the λ plaque forming units obtained on cells expressing wt λ cI, λ cIN or fusions between λ cIN and wt or mutated H-NS. These experiments demonstrated that H-NS P115 mutants retained the basal DNA binding capacity of wt H-NS, but were severely impaired in protein–protein interaction, which caused a drastic reduction of their capacity to bind intrinsically curved DNA and bend noncurved DNA (Spurio *et al*, 1997). Since P115 (indicated by yellow dot in Figure 4Ac) is located in a loop of the C-domain, which is regarded as the DNA-binding domain (Shindo *et al*, 1995, 1999; Ueguchi *et al*, 1996; Williams *et al*, 1996), the phenotypes of the P115 mutations were somewhat unexpected. Unfortunately, these experiments could not determine whether the protein–protein interaction affected by the P115 mutations was that responsible for dimerization, for tetramerization or for the formation of other types of quaternary structures. To elucidate the nature of the defect in protein–protein interaction caused by the P115 mutations, to solve the apparent paradox of protein oligomerization phenotypes caused by mutations in the DNA-binding domain and, more generally, to help clarify the somewhat controversial nature of the *in vivo* quaternary structure of H-NS, we have used two systems capable of detecting dimerization and tetramerization *in vivo*.

In the dimerization test (Di Lallo *et al*, 2001) schematically illustrated in Figure 1A, the expression of *lacZ* depends upon the activity of a promoter overlapped by two adjacent bipartite hybrid operator sites, Or₂₄₃₄Or₁_{P22} and Or₁₄₃₄Or₁_{P22}, both located within the promoter core. Each bipartite site is recognized by the DNA binding domain of two phage repressors (P22 and 434), which must undergo heterodimerization to yield a functional repressor. Thus, the simultaneous expression of different chimeric constructs consisting of the DNA binding domains of the P22 and 434 repressors fused to wt H-NS or to individual domains or variants of this protein allows the determination of the dimerization capacity of these molecules.

In the tetramerization test (Beckett *et al*, 1993) schematically illustrated in Figure 1B, expression of *lacZ* depends upon the activity of a promoter controlled by two adjacent operator sites, Or1 and Or2; Or1 is a high-affinity site upstream of the low-affinity site Or2, which partially overlaps the -35 element of the promoter. Dimeric repressor molecules bind to Or1 but not to Or2 and therefore fail to repress transcription. Instead, once Or1 has been occupied, tetramerization allows the binding of a second dimer to Or2 and causes transcriptional repression. Thus, unlike the dimerization test, this method specifically detects tetramer formation

Figure 1 Experimental systems used to study protein–protein interactions *in vivo*. (A) Test for protein homo- and heterodimerization. (a) The *lacZ* reporter gene is derepressed when the RNA polymerase has access to the *lacZ* promoter. This occurs if the DNA binding domains of 434cI and P22cI, which bind specifically to Or₂₄₃₄/Or₁₄₃₄ and to Or₁_{P22} sequences, respectively, fail to dimerize. (b) The *lacZ* reporter gene is repressed when efficient dimerization of the DNA binding domains of 434cI and P22cI is induced with consequent occupancy of all Or sites, which prevents the RNA polymerase from having access to the promoter. (B) Test for protein tetramerization. (a) The *lacZ* reporter gene is derepressed when, in the absence of tetramerization, the repressor can bind to the high-affinity site Or1 but not to the low-affinity site Or2 thereby allowing the RNA polymerase to have access to the -35 element of the *lacZ* promoter. (b) The *lacZ* reporter gene is repressed if tetramerization allows the repressor to bind to the low-affinity site Or2 thereby preventing the RNA polymerase to have access to the -35 element of the promoter.

but cannot distinguish between the formation of tetramers and the formation of larger aggregates.

Preliminary controls demonstrated that all the chimeric constructs were expressed at quantitatively similar levels and that none was selectively removed from equilibrium by precipitation or formation of inclusion bodies (results not shown).

In vivo dimerization and mutations affecting this activity

Chimerae were constructed by fusing the DNA-binding domain of the lambdoid phages 434cI(Δ 102–263) (repressor of phage 434) and P22cI(Δ 102–263) (repressor of phage P22) with different H-NS fragments. Control experiments showed

that the expression (and hence the presence) of a single complete chimera, in the absence of the other complete chimera that would allow the formation of the heterodimeric repressor, is not sufficient to block the transcription of the reporter gene. This indicates that a heterodimeric repressor must be formed and bound to each hybrid operator and that long-distance homodimerization (i.e. spanning the two hybrid operators) either does not occur or results in an inactive structure. Furthermore, the presence in the cells of wt H-NS and StpA, two proteins that could theoretically form heterodimers with H-NS fragments like those contained in the chimerae (for review see Dorman, 2004), does not interfere with the results of the *in vivo* test. In fact, changing the H-NS/chimera ratio by modulating the expression of the chimerae by addition of IPTG was found not to affect qualitatively the results; the level of repression of the reporter gene varied as a function of the variation of the above ratio, but the relative repressor activity of the various chimeric constructs remained unchanged (not shown).

In light of these results, the constructs were then used to measure the *in vivo* dimerization capacity of the H-NS fragments and of six mutants (L26P, L30P, L33P, P115A, Δ P115 and Δ GRTP115) some of which had been reported to be defective in protein-protein interactions (Spurio *et al*, 1997; Ueguchi *et al*, 1997).

As seen in Figure 2, the cells expressing constructs containing only the DNA-binding domains of the lambdoid repressors but completely lacking the dimerization domain (Figure 2A(12)) clearly show full derepression of *lacZ*, while in cells expressing dimerization-competent chimeric repressor pairs containing wt H-NS (Figure 2A(8) and B(10)) or the

dimerization domain of 434cI (Figure 2A(11)), the reporter gene is strongly repressed. The β -galactosidase expressed by cells in which *lacZ* is fully derepressed is ≈ 30 -fold higher than in cells in which this gene is fully repressed. The dimerization capacity displayed by a trimeric protein like chloramphenicol acetyl transferase (CAT) (Leslie *et al*, 1988), as judged from the intermediate level of repressor activity that it induces (Figure 2B(1)), is slightly reduced compared to that displayed by the oligomerization domain of 434cI (Figure 2A(11)) and by wt H-NS (Figure 2A(8) and B(10)).

Compared to the chimeric constructs containing wt H-NS, constructs containing only the N-terminal domain (residues 1–64) fused to the DNA binding domain of the two lambdoid repressors are essentially incapable of dimerizing (Figure 2A(7)). However, a fairly high dimerization capacity is conferred on the N-terminal domain of H-NS by the inclusion of the H-NS linker in the chimeric construct (Figure 2A(6)). Unlike the N-terminal domain, the C-domain of H-NS can confer a good dimerization capacity on the DNA binding domain of the two lambdoid repressors (Figure 2A(5)) and this capacity is further strengthened by the inclusion of the linker (Figure 2A(4)). The actual formation of a dimeric structure exclusively sustained by the C-domain of H-NS (Figure 2A(5)) is fully supported by the finding that a single amino-acid deletion (Δ P115), a mutation previously found to cause oligomerization defects of the otherwise intact H-NS molecule (Spurio *et al*, 1997), strongly reduces the dimerization capacity of the C-terminal domain (Figure 2A(3)). It should be noted that the dimerization capacity of H-NS P115A (Figure 2B(2)), H-NS Δ P115 (Figure 2B(3)) and H-NS Δ GRTP115 (Figure 2B(4)) is only marginally reduced

Figure 2 Dimerization capacity of H-NS domains (A) and mutants (B). Dimerization was measured from the β -galactosidase activity (Miller units) of cells each expressing a pair of chimeric constructs, containing the DNA binding domains (not shown in the scheme) of P22 (upper construct) and of 434 (lower construct) lambdoid repressors fused to the protein modules schematically represented to the left of each histogram bar. Each pair of chimerae is identified by the letter of the panel and by the number to the left of the scheme. In the chimera containing wt H-NS (i.e. A8 and B10), the three modules of this protein are schematically represented in dark gray (N-terminal), light gray (C-terminal) and white (linker); the positions of the mutations are indicated by white (deletion) or black (substitutions) bars. The precise composition of each construct is listed in Table I. The dimerization tests were performed three times taking triplicate experimental points in each case; thus, the error bars represent the deviation from the average of nine measurements.

compared to wt H-NS (Figure 2A(8) and B(10)); thus, the strong effect of Δ P115 on the dimerization capacity is seen only in the isolated C-domain and not when the same or a similar mutation (P115A) is present within the entire H-NS molecule. Three other amino-acid substitutions, namely L30P (Figure 2B(6)), which was reported to reduce the oligomerization capacity of H-NS (Ueguchi *et al*, 1997), L26P (Figure 2B(5)) and L33P (Figure 2B(7)) were found to have only a modest effect on the dimerization capacity of intact H-NS, in spite of the fact that they interrupt the continuity of the long α -helix responsible for N-domain dimerization (Esposito *et al*, 2002; Bloch *et al*, 2003). The marginal effect of these six mutations on dimerization of intact H-NS, in contrast with their rather strong effect on the dimerization of H-NS fragments and on the tetramerization of intact H-NS, suggests that the H-NS fragments may dimerize in two different ways that rely on the protein surfaces involved in either dimerization or tetramerization of the native molecule (see below). The first type of protein-protein interaction surface, sensitive to the three L/P substitutions, would be that provided by two N-terminal domains plus linker, and the second type would be that provided by two C-domains and sensitive to the P115 mutations. This premise is fully supported by the finding that a double mutant, containing an amino-acid substitution in both N-domain (L30P) and C-domain (Δ P115) (Figure 2B(8)), is definitely less active in dimerization than either single mutation (Figure 2B(6) and (3)). That this interpretation is correct is even more clearly shown by the results obtained with a chimera containing another double mutation, namely L30P/ Δ GRTP115 (Figure 2B(9)). The latter mutation, consisting of the deletion of four amino-acid residues of loop 2 within the C-domain, causes a more severe defect than P115A and Δ P115 in the protein-protein interaction responsible for tetramerization (cf. construct 3 and constructs 1 and 2 in Figure 3), but still has a very efficient basal DNA-binding activity, and can induce some nucleoid compaction *in vivo* (Spurio *et al*, 1992) although it has lost the capacity to recognize bent DNA and to bend noncurved DNA (Spurio *et al*, 1997). As shown in Figure 2B(9), the double mutant bearing this deletion has essentially lost all dimerization capacity, displaying a much more severe phenotype than that caused by the L30P/ Δ P115 double mutant (Figure 2B(8)), while the single mutants L30P (Figure 2B(6)), Δ P115 (Figure 2B(3)) and Δ GRTP115 (Figure 2B(4)) retain substantial 'dimerization' activity due to either the presence of an intact dimerization surface (the latter two) or involvement of the tetramerization surface (the former) in assuring some protein-protein interaction activity.

Although the chimerae containing the linker alone are not active in dimerization (Figure 2A(10)) and, as shown later, in tetramerization (Figure 3(10)), the above data indicate that the linker plays a role in both dimerization and tetramerization. These premises are further supported by the finding that dimerization is almost completely lost upon deletion of the linker (Figure 2A(9)) and this linker cannot be functionally replaced by the KorB linker (Figure 2B(11)), a structure having size and function similar to that of the H-NS linker (Delbruck *et al*, 2002; Khare *et al*, 2004). Similar negative results were obtained when the KorB linker was fused at the N-terminus of the H-NS C-domain (not shown). The mechanism of the participation of the linker in the dimerization process is not clear in light of the reports that this part of

Figure 3 Tetramerization capacity of H-NS domains and mutants. Tetramerization was measured from the β -galactosidase activity (Miller units) of cells expressing the chimeric constructs containing the DNA binding domain (not shown in the scheme) of phage λ repressor fused to the protein modules schematically represented to the left of each histogram bar. Each chimera is identified by the number to the left of the scheme. In the chimera containing wt H-NS (i.e. #15), the three modules of this protein are schematically represented in dark gray (N-terminal), light gray (C-terminal) and white (linker); the positions of the mutations are indicated by white (deletion) or black (substitutions) bars. The precise composition of each construct is indicated in Materials and Methods. The tetramerization tests were performed three times taking triplicate experimental points in each case; thus, the error bars represent the deviation from the average of nine measurements.

the protein is unstructured (Renzone *et al*, 2001; Esposito *et al*, 2002; Bloch *et al*, 2003). However, it is possible that it may acquire an active structure upon interaction with the N-domain (highlighted by the black oval in Figure 4Bb). Furthermore, a linker/C-domain interaction (highlighted by the black circle in Figure 4Bc) could be surmised from the results obtained with the construct in which the DNA binding domains of the two lambdoid repressors are fused, one to the N-domain plus linker and the other to the C-domain (Figure 2A(1) and (2)). This combination (Figure 2A(2)) displays a fairly good *lacZ* repressor capacity. However, it should be noted that the reciprocal combination displays an extremely low repressor capacity (Figure 2A(1)). While it is likely that the different behavior of these two combinations of chimeric

Figure 4 Three-dimensional structures of H-NS domains and schematic models for H-NS dimerization and tetramerization. **(A)** 3D structures of isolated H-NS domains: dimeric N-domain of *S. typhimurium* (Esposito *et al*, 2002) (a) and *E. coli* (Bloch *et al*, 2003) (b); monomeric C-domain of *E. coli* (Shindo *et al*, 1995) with P115 being indicated by the yellow dot (c). A schematic representation of the two N-domains (Dorman, 2004) is shown to the right of the structures from which they are derived; the green dot indicates the position of the last residue (residue 49) of the structure solved in 'b'. **(B)** Hypothetical, schematic models of the H-NS structures: (a) monomer; (b) dimer; (c) tetramer and (d) an alternative 'dimer' generated by removing a monomer from each dimer of the tetramer. In the scheme, the protein-protein interactions found to be important for oligomerization are highlighted: in 'b', a black oval encircles the linker-N-domain interaction contributing to dimerization and in 'c', the large black circle encompasses the C-domain-C-domain (with a contribution of the linker) and the small black circles indicate the linker-linker interactions contributing to tetramerization. See text for further details.

constructs can be explained by their different orientation, it is not clear whether the lack of repressor activity of the combination shown in Figure 2A(1) arises from a failure to dimerize or from the generation of an inactive dimer.

***In vivo* tetramerization and mutations affecting this activity**

The *in vivo* tetramerization capacity of intact H-NS and some of its fragments and mutants was quantified. Positive and negative controls in these tests were the cells expressing wt λ cI (Figure 3(18)) and its N-domain λ cIN (Figure 3(19)), respectively. The β -galactosidase activity is at least 10-fold lower in cells expressing the active repressor than in those expressing a tetramerization-defective repressor. A fusion of wt H-NS with a λ cIN mutant (λ cIA57–263) defective in DNA binding proved to be completely inactive in repressing *lacZ* (not shown), thereby excluding the possibility that transcriptional repression caused by the H-NS-containing chimera (Figure 3(15)) could be due to H-NS itself. Furthermore, very little repression of the reporter gene was observed when the tetramerization test was carried out with a chimera containing the trimeric protein CAT (Figure 3(16)). This finding demonstrates, on the one hand, that protein tetra-

merization is required for transcriptional repression in this test and, on the other hand, that wt H-NS, which is very active in this test, is a tetramer and not a trimer.

Tetramerization tests carried out with cells expressing λ cI(Δ 161–263)::H-NS (Figure 3(15)) demonstrated that wt H-NS has a remarkable capacity (i.e. up to 70%) to replace functionally the tetramerization domain of wt λ cI (Figure 3(18)). The tetramerization capacity of various domains of H-NS was then tested. It was found that chimeras containing either the N-domain (Figure 3(12)) or the C-domain (Figure 3(13)) are incapable of repressing efficiently the expression of *lacZ*, the β -galactosidase expressed by the corresponding cells being $\geq 50\%$ that expressed by cells in which the reporter gene is completely derepressed. Inclusion of the H-NS linker in the chimera containing the H-NS N-domain improved considerably the tetramerization/repressor activity, reducing the β -galactosidase level (Figure 3(11)) to that of cells expressing the chimera containing wt H-NS (Figure 3(15)). Inclusion of the linker in the chimera containing the C-domain also improved somewhat the tetramerization capacity of this construct (Figure 3(14)). As with dimerization, also tetramerization is abolished by deletion of the linker leading to complete derepression of *lacZ* (Figure 3(9)); the

KorB linker, whether fused to the N-terminus (Figure 3(17)) or C-terminus (not shown) of the H-NS N-domain, cannot functionally replace the homologous linker. H-NS tetramerization, unlike dimerization, was very sensitive to mutations in the C- and N-domain; in fact, the chimerae containing the L26P, L30P or L33P substitutions (Figure 3(4–6)) as well as the HNSΔGRTP115 deletion (Figure 3(3)) are totally inactive in *lacZ* repression. As expected from the previous data (Spurio *et al*, 1997), also the P115 mutations (Figure 3(1) and (2)) display a substantially reduced activity, albeit not as much as the H-NSΔGRTP115 deletion. Finally, also the L30P/ΔP115 and the L30P/ΔGRTP115 double mutants are essentially inactive in tetramerization (Figure 3(7) and (8)).

Discussion

In this article, we have studied the *in vivo* oligomerization properties of H-NS and of some of its mutants and domains; two experimental systems to detect dimerization and tetramerization *in vivo* were used. Both systems are based on the transcriptional repression of a reporter gene (*lacZ*) caused by chimeric constructs containing the DNA-binding domain of λ or lambdoid phage repressors and specific H-NS fragments or mutants to be tested. Although both tests rely on the binding of the repressors to two sets of operator sites (two hybrid operators in the case of the dimerization test), the two systems are clearly different and have different requirements. In the tetramerization test (Beckett *et al*, 1993), the two operators are located between –56 and –73 and between –32 and –49 so that there is only a partial overlap with the core elements of the promoter. This fact and the fact that the downstream operator is a very weak target for the repressor makes the formation of a tetrameric repressor mandatory for a successful competition with RNA polymerase. On the other hand, in the dimerization test (Di Lallo *et al*, 2001), the two hybrid operators both overlap the recognition site of the sigma subunit of RNAPol. In turn, in this system, binding of two heterodimeric repressors is necessary and sufficient for competition with RNA polymerase, even in the absence of dimer–dimer interaction, which occurs only as a consequence of the binding of the dimers to DNA (Ciubotaru and Koudelka, 2003). All the results obtained in the controls and in the test samples confirm the premise that the requirements of the tetramerization assay are much more stringent than those of dimerization. In fact, the chimerae containing the trimeric protein CAT repress the β -gal activity by 90% in the dimerization test but <20% in the tetramerization test, and several other constructs proved to be active in dimerization but inactive in tetramerization.

From the results obtained in this study, we can conclude that oligomerization of H-NS *in vivo* gives rise to both dimers and tetramers. Compared to the native molecule, the N-domain, which is regarded as the dimerization domain of H-NS, performs very poorly in both dimerization and tetramerization. On the other hand, the C-domain, which is responsible for DNA binding, although inefficient in tetramerization, is able to sustain a fairly high level of dimerization. Furthermore, the results obtained suggest an important role of the H-NS linker in protein–protein interaction. In fact, although the linker is inactive in promoting dimerization and tetramerization of the chimerae containing this domain alone, its inclusion at the C-terminus of the N-domain or at

the N-terminus of the C-domain improved both dimerization and tetramerization of the corresponding chimerae. The importance of the linker in protein–protein interactions is also confirmed by the finding that its deletion produces a protein almost completely inactive in both dimerization and tetramerization and that it cannot be replaced in its function by a linker of similar size and, at least superficially, similar function, such as that of the KorB transcriptional regulator of plasmid RP4 (Delbruck *et al*, 2002; Khare *et al*, 2004).

One of the most interesting and somewhat unexpected findings of the present study is the proficiency by which the C-domain of H-NS can support the dimerization of the DNA-binding domains of the lambdoid repressors. This can be taken as direct evidence that this domain is involved not only in DNA binding (Shindo *et al*, 1999) but also in protein–protein interaction. Previous mutational analysis had clearly indicated that substitutions or deletions of residues within this domain, P115 in particular, could affect H-NS oligomerization and, with that, abolish the most characteristic H-NS functions such as the preference for bent DNA, the capacity to bend DNA and selectively repress transcription (Williams *et al*, 1996; Spurio *et al*, 1997). However, these earlier results had not clarified whether the effects of the C-domain mutations were directly or indirectly responsible for the defects in H-NS oligomerization and whether they concerned dimerization or tetramerization. The present findings that disruption of the dimerization capacity is caused by ΔP115 within the C-domain alone clearly support the premise that this domain is directly involved in protein–protein interaction. Furthermore, our data demonstrate that both ΔP115 and P115A mutations, when present within the entire H-NS molecule, interfere with tetramerization much more than with dimerization; this indicates that the phenotypes of these mutations such as loss of preferential binding to bent DNA and DNA bending (Spurio *et al*, 1997) are due to a tetramerization defect. Thus, taken together, present and previous findings clearly support the notion that the biologically active form of H-NS is the tetramer. If the spatial organization of an H-NS tetramer resembles that schematically outlined in Figure 4Bc (see below), then the structural basis for the H-NS-induced lateral condensation of separate DNA tracts, which is believed to be at the basis of the repressor activity of this protein (Falconi *et al*, 1996, 1998; Dame *et al*, 2000, 2002), could be the bridging of two duplexes each bound to a dimeric DNA-binding domain.

Information concerning the 3D structure of H-NS is available from NMR spectroscopy but only for individual domains of the protein; surprisingly, alternative structures have been proposed by Esposito *et al* (2002) and Bloch *et al* (2003) for the N-domain dimers. These structures are shown in Figure 4Aa and b and the schemes presented to their right (Dorman, 2004) illustrate their different mechanisms of dimerization. As seen from the figures, both structures contain three α -helices (1, 2 and 3) of increasing length going from the N-terminus toward the C-terminus, but their topological orientation in space is completely different, the two longest α -helices (H3) being parallel in one case and antiparallel in the other. Our finding that the N-domain without the linker is incapable of sustaining either dimerization or tetramerization is inconsistent, at least superficially, with both models. Since we have shown that the chimera containing this fragment does not precipitate or form inclusion bodies *in vivo* more

than the other chimerae, the failure of the N-domain alone to yield a chimera that is capable of dimerizing cannot be attributed to its hydrophobic nature and to the formation of large polydisperse aggregates. Instead, since coiled-coils of two α -helices are common building blocks within protein domains but are generally insufficient to form complete domains (Branden and Tooze, 1999), it is likely that under the *in vivo* conditions (e.g. at protein concentrations much lower than that (≥ 1 mM) used in the above-mentioned NMR spectroscopy studies) and in the absence of stabilizing interactions provided by the linker, the α H3- α H3 interaction is not sufficient to yield a stable quaternary structure that is able to sustain the assembly of an active dimeric or tetrameric transcriptional repressor. That the oligomerization properties of the N-terminal domain can be profoundly influenced by the presence of the linker is clearly indicated by the different oligomerization properties acquired by this domain in the presence of this part of the molecule (Renzoni *et al*, 2001). Thus, we propose an alternative schematic model for the dimerization and tetramerization of H-NS (Figure 4B). Although graphically derived from the 3D structure of Bloch *et al* (2003), this model does not pretend to provide a high-resolution description of the interactions occurring at the atomic level between H-NS monomers and dimers, which is beyond the scope of this article. Instead, this model is meant to accommodate, in a single scheme, all the available data and to highlight interactions postulated by the present analysis but which have been overlooked so far. Obviously, the level of resolution of this model is by necessity limited to that reflected by the behavior of the H-NS domains and mutants contained in the chimerae constructed and analyzed here. As seen in Figure 4B, our model takes into account not only the well-established protein-protein interactions provided by α -helices 1 and 3 of the N-domain but also attributes, in agreement with the experimental findings, a direct role in H-NS dimerization to an interaction of the linker of one monomer with the α -helix of another (Figure 4Bb). Furthermore, the model indicates the protein-protein interactions between the C-domains (strengthened by the linker) postulated (Spurio *et al*, 1997) but never directly demonstrated before, and the linker-linker interaction involved in H-NS tetramerization (Figure 4Bc). In agreement with the data of Ueguchi *et al* (1997), the L30P mutation in the N-terminal domain was found to cause an almost complete loss of the tetramerization capacity of H-NS but did not affect more than marginally the dimerization. Virtually identical results were obtained also with L26P and L33P. These mutations are expected and, at least in the case of the L26P mutation, shown to cause a helix-random coil transition of α -helix (H3) (Esposito *et al*, 2002). Since this helix is responsible for (Esposito *et al*, 2002; Bloch *et al*, 2003) or simply contributes to (according to our model of Figure 4Bb) H-NS dimerization, the loss of the tetramerization capacity of these mutants can be easily explained if tetramerization is, as demonstrated by Ceschini *et al* (2000) and shown in Figure 4Bc, a 'dimer of dimers'. On the other hand, the residual dimerization activity of the L26P, L30P or L33P mutants could appear to be a paradoxical finding. However, it is likely that, in the absence of the active dimerization surface disrupted by the L/P mutations, the dimerization activity of the chimeric constructs bearing these substitutions is sustained by the H-NS surface normally involved in tetramerization and

which includes the C-domain. That this explanation is correct is indicated by the essentially complete loss of dimerization capacity displayed by the L30P/ Δ P115 double mutant (Figure 2B(9)) and by L30P substitution within a truncated H-NS molecule missing the C-domain (not shown). In fact, in both cases, dimerization and tetramerization surfaces are compromised by the L30P mutation and by deletion of the entire C-domain or just loop 2, respectively.

As mentioned above, the tetramer model presented in Figure 4Bc was constructed by juxtaposing two dimers in which the H-NS monomers are topologically oriented as in the structure of Bloch *et al* (2003) with the inclusion of the additional dimerization and tetramerization interactions emerging from the present data. However, it should be noted that if one subtracts a monomer from each of the two dimers constituting this tetramer, the resulting dimer has the same topological orientation as that seen in the structure of Esposito *et al* (2002) (cf. Figure 4Aa and Bd). Thus, our schematic model seems to be able to reconcile, at least for what concerns the topology of the two long α -helices, the 'parallel' (Figure 4Aa) and the 'antiparallel' (Figure 4Ab) structures proposed by Esposito *et al* (2002) and Bloch *et al* (2003) for essentially the same H-NS domain.

Thus, if our model is correct, the most probable structure for the 'core' of an H-NS tetramer would be that of a bundle of four α -helices, two parallel and two antiparallel, each contributed by a single H-NS monomer. Indeed, this is a very likely occurrence in light of the fact that a four-helix bundle, which can occur in different arrangements as far as handedness of interhelical packing and topology is concerned, is a stable structural domain, very common in nucleic acid binding proteins such as Rop, TMV coat protein, p53, TFIIA, Mnt and the tetramerization domain of Lac repressor (Branden and Tooze, 1999; Nooren *et al*, 1999). The stability of the four-helix bundle, due to the presence of a hydrophobic core contributed by the hydrophobic side chains of the residues of each α -helix, is often strengthened by the contribution of ionic interactions between hydrophilic, charged side chains of amino acids bordering the hydrophobic core. All these structural elements (hydrophobic helix-helix interactions and salt bridges between charged residues) have been described in both published structures (Esposito *et al*, 2002; Bloch *et al*, 2003) and therefore it can be assumed that they are compatible with either topological orientation of the helices. Furthermore, the likelihood of the formation of a four-helix bundle is supported by the finding that the parallel, coiled-coil α -helices, which characterize one of the structures, are in fact a four-helix bundle, at least for the portion in which the two pairs of small helices H1 and H2 fit, in an antiparallel orientation, within the groove between the two α -H3 helices (Esposito *et al*, 2002). Thus, our schematic model seems to reconcile, at least from the topological point of view, the two published structures suggesting that both could be correct within an H-NS tetramer.

Starting from the most reasonable assumption that both published structures were correctly solved, the fact that both NMR studies have yielded two dimeric structures and neither has shown a four-helix bundle is in complete agreement with the behavior of the chimerae reported here and can easily be explained by the lack of important protein-protein interacting surfaces (provided by the distal portion of the linker and by the C-domain) in the samples analyzed. On the other hand,

many reasons could have contributed to the formation of two dimers of different topology from essentially the same material. First of all, the starting material, although similar, is not identical for the presence/absence of a portion of the linker in one of two samples and this difference could have led to the formation of alternative structures *in vivo*, during the preparation of the samples. In addition, small but significant differences in the ionic composition and concentration and a 10°C temperature difference could have played a role in favoring the preferential formation of one or the other structure. Last but not least, it should be recalled that individual α -helices are very versatile and 'promiscuous' as far as their capacity to interact and to orient each other in space as indicated by the fact that α -helices can 'accept' a large number of mutations since similar functional structures can result from α -helices having very little sequence homology and that the same α -helix can be the interacting partner of several other different helices (Branden and Tooze, 1999). The fact that the very same H-NS N-terminal domain, in addition to yielding the two different dimeric structures discussed so far, has also been found in a trimeric form by another NMR study (Renzoni *et al*, 2001) represents, if not the best, for sure the most pointed example of this premise.

A large proportion of the numerous genes directly or indirectly controlled by H-NS are involved in bacterial adaptation to changes in environmental conditions (Hommais *et al*, 2001). Thus, since the present data have shown that the tetramer is the active form of H-NS, it could be hypothesized that changes of the tetramerization efficiency, triggered by changes of the external environment, could modulate the function of H-NS both as an architectural component of the nucleoid and as a transcriptional regulator. In agreement with this premise, results of separate studies (that will be reported in detail elsewhere) obtained using these same two-hybrid systems have demonstrated that environmental parameters, such as temperature and osmolarity, can indeed influence H-NS tetramerization (but not dimerization) *in vivo*.

Materials and methods

Construction of λ cl repressor fusions for the tetramerization test

E. coli JH607 (F'128 lacI_q lacZ::Tn5 λ 112O₅P₅) (Beckett *et al*, 1993), kindly provided by Dr J Hu (University of Texas), was transformed with the previously described plasmids (Spurio *et al*, 1997): pBF21 expressing wt λ cl (Figure 3(18)), pBF22 expressing λ cl(Δ 161–263) (Figure 3(19)), pBF23 expressing the chimera λ cl(Δ 161–263)::H-NS (Figure 3(15)), and the plasmids expressing the latter chimeric construct bearing the H-NS mutations shown in Figure 3 (constructs 1, 2 and 3 respectively): H-NS P115A (pBF25), H-NS Δ P115 (pBF26) and H-NSAGRTPI15 (missing four residues G112 through P115) (pBF42).

Plasmids expressing chimeras consisting of the N-domain of λ cl fused to various H-NS fragments were obtained by PCR amplification. The amplified fragments corresponding to the desired regions of *hns* and having *Hind*III or *Bam*HI restriction sites at one end were digested with the appropriate endonucleases and inserted in the corresponding sites of pBF21. The primers used are listed in Table I and the chimeras constructed with them are identified by the numbers reported in Figure 3: T1 and DT2 for pBF32, which expresses λ cl(Δ 161–263)::H-NS(Δ 65–136) (#12); T1 and DT2 for pBF33 expressing λ cl(Δ 161–263)::H-NS(Δ 90–136) (#11); T3 and DT1 for pBF35 expressing λ cl(Δ 161–263)::H-NS(Δ 1–89) (#13); T2 and DT1 for pBF36 expressing λ cl(Δ 161–263)::H-NS(Δ 1–65) (#14); T2 and DT3 for pBF42 expressing λ cl(Δ 161–263)::H-NS(Δ 1–65 Δ 90–136) (#10). To construct pBF37 encoding λ cl(aa Δ 161–263)::H-NS(Δ 64–89) (#9), two pairs of primers (T1-DT7 and DT8-DT1) were

Table I Desoxyoligonucleotides used in this study

T1	5'-cccaagctttatgagcgaagcac
T2	5'-cccaagctttatgctgacgctgacggtattg
T3	5'-cccaagctttacgtgctcagcgctcg
T4	5'-cccaagctttaaggccgcgcatcc
DT1	5'-tattaaattgctggatccggacaataaaa
DT2	5'-cgggatccttaacagcatttcgcatattg
DT3	5'-cgggatccttaacgttttagctttgtgc
DT4	5'-ggaagaaatgccggaataatagaag
DT5	5'-ttgaaacgcgcggaagaaatgct
DT6	5'-atgctggaaaaaccagaagtgtgctt
DT7	5'-atgagcgaagcagttataaatttc
DT8	5'-aagtgtcttcgtcatGGCCTTTCCTTGG
DT9	5'-ctgcagcaatcgcgaaatgCGTGCTCAGCGTCCGGCAA
DT10	5'-TTGCCGGACGCTGAGCACGatcttcgctattgctgcag
D1	5'-acggtcgacaatgagcgaagcacttaaaattc
D2	5'-acggtcgacaatgagcgaagcacttaaaattc
D3	5'-acggtcgacacgtgctcagcgctcg
D4	5'-acggtcgacactaaggccgcgcatcc

The oligonucleotides used in the genetic constructions are designated with D, T and DT, depending upon whether they were used to construct chimeras tested for dimerization, tetramerization or both. Regions of sequence complementarity exploited in the PCR reactions used to prepare the various chimeric constructs described in Materials and methods are indicated by uppercase letters while the underlined sequences represent restriction sites. Translational stop codons introduced in the constructs are indicated by bold letters, the underlined sequences are recognized by the different restriction enzymes and italicized bold letters indicate the nucleotide substitutions introduced.

used. The two partially complementary amplification products thus obtained were purified, mixed, allowed to anneal and subjected to a second amplification using T1 and DT1 as primers. Digestion with *Hind*III and *Bam*HI yielded the DNA fragment to be inserted into pBF21. The same strategy was used for the construction of pBF38 expressing the triple chimera λ cl(Δ 161–263)::KorB(residues 253–259)::H-NS(Δ 65–136) (#17). In this case, the primers initially used were T4-DT6 to amplify the fragment encoding the KorB linker (residues 253–295) encoded by *korB* carried by pMS51-1 (Balzer *et al*, 1992) and T1 and DT3 to amplify the desired *hns* fragment. Primers T4 and DT2 were used in the second amplification. Additional constructs, pBF39, pBF34 and pBF40 encoding λ cl(Δ 161–263)::H-NS with L26P (#4), L30P (#5) and L33P (#6) substitutions, were generated by oligonucleotide-directed mutagenesis (Spurio *et al*, 1997) using the mutagenic oligonucleotides DT4, DT5 and DT6, respectively (Table I). The mutated *hns* sequence thus obtained was amplified using T1 and DT1 as primers and cloned into pBF21 as a *Hind*III-*Bam*HI fragment. The pBF41 encoding λ cl(Δ 161–263)::H-NS with L30P and Δ P115 double mutation (#7) was generated by oligonucleotide-directed mutagenesis (Spurio *et al*, 1997) using pBF26 as DNA template and the mutagenic oligonucleotides DT4 (Table I). The pBF43 encoding λ cl(Δ 161–263)::H-NS with L30P and Δ (GRTPI15) double mutation (#8) was generated by oligonucleotide-directed mutagenesis (Spurio *et al*, 1997) using pBF42 as DNA template and the mutagenic oligonucleotides DT4 (Table I).

The DNA sequences of all constructs were verified before use (Sanger *et al*, 1977).

In vivo tetramerization test

Saturated cultures grown overnight in LB broth containing ampicillin (100 μ g/ml) were diluted to $A_{600} \approx 0.035$ with ampicillin-containing LB broth. After incubation at 37°C, cell growth was monitored spectrophotometrically and the β -galactosidase activity was assayed (Miller, 1972) when cell density reached $A_{600} \approx 0.5$.

Construction of repressor fusions for the dimerization test

E. coli R721 (*glpT*::O-P_{434/P22} *lacZ*), an *E. coli* 17/18 derivative, was the host strain for the plasmids expressing the chimeras listed in Table II. The plasmids for the dimerization test were constructed as described above for the pBF plasmids. However, in the place of the T-series of oligonucleotides, which bear a *Hind*III site at their 5' end, the corresponding oligonucleotides of the D-series (Table I) bearing

Table II Primers and templates used in the construction of the chimerae used in the dimerization test

Primers	Template	Chimerae	Reference	Position
		434cl wt	Di Lallo <i>et al</i> (2001)	Figure 2A(11)
		P22cl(Δ 102–236)::434cl(Δ 1–101)		
		434cl(Δ 102–236)	Di Lallo <i>et al</i> (2001)	Figure 2A(12)
		P22cl(Δ 102–236)		
		434cl(Δ 102–236)::CAT	Di Lallo <i>et al</i> (2001)	Figure 2B(1)
		P22cl(Δ 102–236)::CAT		
D1/DT1	pBF23	434cl(Δ 102–236)::H-NS	This study	Figure 2A(8) and B(10)
D1/DT1	pBF23	P22cl(Δ 102–236)::H-NS		
D1/DT1	pBF25	434cl(Δ 102–236)::H-NS P115A	This study	Figure 2B(2)
D1/DT1	pBF25	P22cl(Δ 102–236)::H-NS P115A		
D1/DT1	pBF26	434cl(Δ 102–236)::H-NS Δ P115	This study	Figure 2B(3)
D1/DT1	pBF26	P22cl(Δ 102–236)::H-NS Δ P115		
D1/DT1	pBF34	434cl(Δ 102–236)::H-NS L30P	This study	Figure 2B(6)
D1/DT1	pBF34	P22cl(Δ 102–236)::H-NS L30P		
D1/DT1	pBF39	434cl(Δ 102–236)::H-NS L26P	This study	Figure 2B(5)
D1/DT1	pBF39	P22cl(Δ 102–236)::H-NS L26P		
D1/DT1	pBF40	434cl(Δ 102–236)::H-NS L33P	This study	Figure 2B(7)
D1/DT1	pBF40	P22cl(Δ 102–236)::H-NS L33P		
D1/DT1	pBF41	434cl(Δ 102–236)::H-NS L30P Δ P115	This study	Figure 2B(8)
D1/DT1	pBF41	P22cl(Δ 102–236)::H-NS L30P Δ P115		
D1/DT3	pBF23	434cl(Δ 102–236)::H-NS(Δ 65–136)	This study	Figure 2A(7)
D1/DT3	pBF23	P22cl(Δ 102–236)::H-NS(Δ 65–136)		
D1/DT2	pBF23	434cl(Δ 102–236)::H-NS(Δ 90–136)	This study	Figure 2A(6)
D1/DT2	pBF23	P22cl(Δ 102–236)::H-NS(Δ 90–136)		
D2/DT1	pBF23	434cl(Δ 102–236)::H-NS(Δ 1–65)	This study	Figure 2A(4)
D2/DT1	pBF23	P22cl(Δ 102–236)::H-NS(Δ 1–65)		
D2/DT3	pBF23	434cl(Δ 102–236)::H-NS(Δ 1–65 Δ 89–136)	This study	Figure 2A(10)
D2/DT3	pBF23	P22cl(Δ 102–236)::H-NS(Δ 1–65 Δ 89–136)		
D3/DT1	pBF23	434cl(Δ 102–236)::H-NS(Δ 1–89)	This study	Figure 2A(5)
D3/DT1	pBF23	P22cl(Δ 102–236)::H-NS(Δ 1–89)		
D1/DT2	pBF23	434cl(Δ 102–236)::H-NS(Δ 90–136)	This study	Figure 2A(1)
D3/DT1	pBF23	P22cl(Δ 102–236)::H-NS(Δ 1–89)		
D3/DT1	pBF23	434cl(Δ 102–236)::H-NS(Δ 1–89)	This study	Figure 2A(2)
D1/DT2	pBF23	P22cl(Δ 102–236)::H-NS(Δ 90–136)		
D3/DT1	pBF26	434cl(Δ 102–236)::H-NS(Δ 1–89) Δ P115	This study	Figure 2A(3)
D3/DT1	pBF26	P22cl(Δ 102–236)::H-NS(Δ 1–89) Δ P115		
D1/DT1	pBF37	434cl(Δ 102–236)::H-NS(Δ 64–89)	This study	Figure 2A(9)
D1/DT1	pBF37	P22cl(Δ 102–236)::H-NS(Δ 64–89)		
D1/DT1	pBF42	434cl(Δ 102–236)::H-NS(Δ 112–115)	This study	Figure 2B(4)
D1/DT1	pBF42	P22cl(Δ 102–236)::H-NS(Δ 112–115)		
D1/DT1	pBF43	434cl(Δ 102–236)::H-NS L30P(Δ 112–115)	This study	Figure 2B(9)
D1/DT1	pBF43	P22cl(Δ 102–236)::H-NS L30P(Δ 112–115)		
D4/DT2	pBF38	434cl(Δ 102–236)::KorB(residues253–295)::H-NS(Δ 65–136)	This study	Figure 2B(11)
D4/DT2	pBF38	P22cl(Δ 102–236)::KorB(residues253–295)::H-NS(Δ 65–136)		

The sequence of the pairs of primers listed in the first column are reported in . The plasmids used as templates in the PCR reaction are described in Materials and methods. The chimerae listed in the third column are schematically represented in Figure 2 and are identified in the fifth column of the table by the corresponding letter and number present in each panel.

a *SalI* site at the 5' end were used. Additional information concerning the dimerization test and p22/434 plasmids can be found in Di Lallo *et al* (2001).

In vivo dimerization tests

E. coli R721 were grown in LB broth containing ampicillin (100 µg/ml) and kanamycin (25 µg/ml) at 37°C, and harvested at $A_{600} = 0.6$ for the β -galactosidase assay (Miller, 1972).

References

- Atlung T, Ingmer H (1997) H-NS, a modulator of environmentally regulated gene expression. *Mol Microbiol* **24**: 7–17
- Balzer D, Ziegelin G, Pansegrau W, Kruft V, Lanka E (1992) KorB protein of promiscuous plasmid RP4 recognizes inverted sequence repetitions in regions essential for conjugative plasmid transfer. *Nucleic Acids Res* **20**: 1851–1858
- Beckett D, Burz DS, Ackers GK, Sauer RT (1993) Isolation of lambda repressor mutants with defects in cooperative operator binding. *Biochemistry* **32**: 9073–9079

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- Bloch V, Yang Y, Margeat E, Chavanier A, Auge MT, Robert B, Arold S, Rimsky S, Kochoyan M (2003) The H-NS dimerization domain defines a new fold contributing to DNA recognition. *Nat Struct Biol* **10**: 212–218
- Branden C, Tooze J (1999) *Introduction to Protein Structure*. New York: Garland Publishing Inc.
- Ceschini S, Lupidi G, Coletta M, Pon CL, Fioretti E, Angeletti M (2000) Multimeric self-assembly equilibria involving the histone-like protein H-NS. A thermodynamic study. *J Biol Chem* **275**: 729–734

- Ciubotaru M, Koudelka GB (2003) DNA-stimulated assembly of oligomeric bacteriophage 434 repressor: evidence for cooperative binding by recruitment. *Biochemistry* **42**: 4253–4264
- Dame RT, Wyman C, Goosen N (2000) H-NS mediated compaction of DNA visualised by atomic force microscopy. *Nucleic Acids Res* **28**: 3504–3510
- Dame RT, Wyman C, Wurm R, Wagner R, Goosen N (2002) Structural basis for H-NS-mediated trapping of RNA polymerase in the open initiation complex at the *rrnB* P1. *J Biol Chem* **277**: 2146–2150
- Delbruck H, Ziegelin G, Lanka E, Heinemann U (2002) An Src homology 3-like domain is responsible for dimerization of the repressor protein KorB encoded by the promiscuous IncP plasmid RP4. *J Biol Chem* **277**: 4191–4198
- Di Lallo G, Castagnoli L, Ghelardini P, Paolozzi L (2001) A two-hybrid system based on chimeric recognition for studying protein homo-heterodimerization in *Escherichia coli*. *Microbiology* **147**: 1651–1656
- Dorman CJ (2004) H-NS: a universal regulator for a dynamic genome. *Nat Rev Microbiol* **2**: 391–400
- Esposito D, Petrovic A, Harris R, Ono S, Eccleston JF, Mbabaali A, Haq I, Higgins CF, Hinton JC, Driscoll PC, Ladbury JE (2002) H-NS oligomerization domain structure reveals the mechanism for high order self-association of the intact protein. *J Mol Biol* **324**: 841–850
- Falconi M, Brandi A, La Teana A, Gualerzi CO, Pon CL (1996) Antagonistic involvement of FIS and H-NS proteins in the transcriptional control of *hns* expression. *Mol Microbiol* **19**: 965–975
- Falconi M, Colonna B, Prosseda G, Micheli G, Gualerzi CO (1998) Thermoregulation of *Shigella* and *Escherichia coli* EIEC pathogenicity. A temperature-dependent structural transition of DNA modulates accessibility of *virF* promoter to transcriptional repressor H-NS. *EMBO J* **17**: 7033–7043
- Falconi M, Gualtieri MT, La Teana A, Losso MA, Pon CL (1988) Proteins from the prokaryotic nucleoid: primary and quaternary structure of the 15-kD *Escherichia coli* DNA-binding protein H-NS. *Mol Microbiol* **2**: 323–329
- Gualerzi CO, Lammi M, Losso MA, Friedrich K, Pawlik RT, Canonaco MA, Gianfranceschi G, Pingoud A, Pon CL (1986) Proteins from the prokaryotic nucleoid. Structural and functional characterization of *Escherichia coli* DNA-binding proteins NS (HU) and H-NS. in *Bacterial Chromatin*, Gualerzi CO, Pon CL (eds) pp 101–134. Heidelberg: Springer-Verlag
- Hommais F, Krin E, Laurent-Winter C, Soutourina O, Malpertuy A, Le Caer J-P, Danchin A, Bertin P (2001) Large-scale monitoring of pleiotropic regulation of gene expression by the prokaryotic nucleoid-associated protein, H-NS. *Mol Microbiol* **40**: 20–36
- Jordi BJ, Fielder AE, Burns CM (1997) DNA binding is not sufficient for H-NS-mediated repression of *proU* expression. *J Biol Chem* **272**: 12083–12090
- Khare D, Ziegelin G, Lanka E, Heinemann U (2004) Sequence-specific DNA binding determined by contacts outside the helix-turn-helix motif of the ParB homolog KorB. *Nat Struct Mol Biol* **11**: 656–663
- Lammi M, Paci M, Pon CL, Losso MA, Miano A, Pawlik RT, Gianfranceschi GL, Gualerzi CO (1984) Proteins from the prokaryotic nucleoid. Biochemical and ¹H-NMR studies of three bacterial histone-like proteins. in *Proteins Involved in DNA Replication*, Hubscher U, Spadari S (eds) pp 467–477. New York: Plenum Press
- Leslie AG, Moody PC, Shaw WV (1988) Structure of chloramphenicol acetyltransferase at 1.75-Å resolution. *Proc Natl Acad Sci USA* **85**: 4133–4137
- Losso MA, Pawlik RT, Canonaco MA, Gualerzi CO (1986) Proteins from the prokaryotic nucleoid. A protein-protein cross-linking study on the quaternary structure of *Escherichia coli* DNA-binding protein NS (HU). *Eur J Biochem* **155**: 27–32
- Miller JH (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press
- Nooren IMA, Kaptein R, Sauer RT, Boelens R (1999) The tetramerization domain of the Mnt repressor consists of two right-handed coiled coils. *Nat Struct Biol* **6**: 755–759
- Pon CL, Stella S, Gualerzi CO (2005) Repression of transcription by curved DNA and nucleoid protein H-NS: a mode of bacterial gene regulation. In *DNA Conformation and Transcription*, Ohshima T (ed). Austin: Landes Bioscience (in press)
- Renzoni D, Esposito D, Pfuhl M, Hinton JCD, Higgins CF, Driscoll PC, Ladbury JE (2001) Structural characterization of the N-terminal oligomerization domain of the bacterial chromatin-structuring protein, H-NS. *J Mol Biol* **306**: 1127–1137
- Rimsky S, Spassky A (1990) Sequence determinants for H1 binding on *Escherichia coli* lac and gal promoters. *Biochemistry* **29**: 3765–3771
- Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**: 5463–5467
- Shindo H, Iwaki T, Ieda R, Kurumizaka H, Ueguchi C, Mizuno T, Morikawa S, Makamura H, Kuboniwa H (1995) Solution structure of the DNA-binding domain of a nucleoid-associated protein, H-NS, from *Escherichia coli*. *FEBS Lett* **360**: 125–131
- Shindo H, Ohnuki A, Ginba H, Katoh E, Ueguchi C, Mizuno T, Yamazaki T (1999) Identification of the DNA-binding surface of H-NS protein from *Escherichia coli* by heteronuclear NMR spectroscopy. *FEBS Lett* **455**: 63–69
- Smyth CP, Lundbäck T, Renzoni D, Siligardi G, Beavil R, Layton M, Sidebotham JM, Hinton JCD, Driscoll PC, Higgins CF, Ladbury JE (2000) Oligomerization of the chromatin-structuring protein H-NS. *Mol Microbiol* **36**: 962–972
- Spassky A, Rimsky S, Garreau H, Buc H (1984) H1a, an *E. coli* DNA-binding protein which accumulates in stationary phase, strongly compacts DNA *in vitro*. *Nucleic Acids Res* **12**: 5321–5340
- Spurio R, Durrenberger M, Falconi M, La Teana A, Pon CL, Gualerzi CO (1992) Lethal overproduction of the *Escherichia coli* nucleoid protein H-NS: ultramicroscopic and molecular autopsy. *Mol Genet* **231**: 201–211
- Spurio R, Falconi M, Brandi A, Pon CL, Gualerzi CO (1997) The oligomeric structure of nucleoid protein H-NS is necessary for recognition of intrinsically curved DNA and for DNA bending. *EMBO J* **16**: 1795–1805
- Ueguchi C, Seto C, Suzuki T, Mizuno T (1997) Clarification of the dimerization domain and its functional significance for the *Escherichia coli* nucleoid protein H-NS. *J Mol Biol* **274**: 145–151
- Ueguchi C, Tomomi S, Yoshida T (1996) Systematic mutational analysis revealing the functional domain organization of *Escherichia coli* nucleoid protein H-NS. *J Mol Biol* **263**: 149–162
- Ussery DW, Hinton JCD, Jordi BJAM, Granum PE, Seirafi A, Stephen RJ, Tupper AE, Berridge G, Sidebotham JM, Higgins CF (1994) The chromatin-associated protein H-NS. *Biochimie* **76**: 968–980
- Williams RM, Rimsky S, Buc H (1996) Probing the structure, function, and interactions of the *Escherichia coli* H-NS and StpA proteins by using the dominant negative derivatives. *J Bacteriol* **178**: 4335–4343
- Zuber F, Kotlarz D, Rimsky S, Buc H (1994) Modulated expression of promoters containing upstream curved DNA sequences by the *Escherichia coli* nucleoid protein H-NS. *Mol Microbiol* **12**: 231–240