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Review Article

Post-translational modification of nucleoid-associated proteins: an extra layer of functional modulation in bacteria?

Ivar W. Dilweg[1] **and Remus T. Dame**[1,2]

[1]Leiden Institute of Chemistry, Leiden University, Leiden, The Netherlands; [2]Centre for Microbial Cell Biology, Leiden University, Leiden, The Netherlands

Correspondence: Remus T. Dame (rtdame@chem.leidenuniv.nl)

Post-translational modification (PTM) of histones has been investigated in eukaryotes for years, revealing its widespread occurrence and functional importance. Many PTMs affect chromatin folding and gene activity. Only recently the occurrence of such modifications has been recognized in bacteria. However, it is unclear whether PTM of the bacterial counterparts of eukaryotic histones, nucleoid-associated proteins (NAPs), bears a comparable significance. Here, we scrutinize proteome mass spectrometry data for PTMs of the four most abundantly present NAPs in *Escherichia coli* (H-NS, HU, IHF and FIS). This approach allowed us to identify a total of 101 unique PTMs in the 11 independent proteomic studies covered in this review. Combined with structural and genetic information on these proteins, we describe potential effects of these modifications (perturbed DNA-binding, structural integrity or interaction with other proteins) on their function.

Introduction

In eukaryotes, the importance of post-translational modification (PTM) has been firmly established. Such modifications provide an extra layer of flexibility and thus, complexity on the function of a protein. They regulate vast networks of cellular mechanisms, determine the retention or degradation of proteins and activate or inhibit enzymatic activity [1–5]. Among the most impactful PTMs in eukaryotes are those of histones, which are extensively and diversely modified at numerous residues [6–8]. Whether specific histone residues are for instance acetylated, methylated or ubiquitylated influences DNA replication, the stability of the chromatin fibre, the degree of DNA compaction and thus the level of DNA transcription [9–11]. Nucleoid-associated proteins (NAPs) are functionally similar proteins in bacteria forming the compactly organized bacterial nucleoid, by bridging, bending and aggregation of genomic DNA [12]. Until recently, PTMs were believed to occur primarily in eukaryotes. However, recent studies using newly developed mass spectrometry-based proteomic methods have increased the rate at which PTMs can be discovered and identified [13,14]. These approaches resulted in the demonstration that PTMs abundantly occur in bacteria as well [15–19]. Extensive modification occurs also in the case of NAPs, which may have — currently unappreciated — functional significance.

The four most abundant and best-characterized NAPs in *Escherichia coli* are H-NS, HU, IHF and FIS [20]. H-NS is a global regulator of transcription, genomic binding of which results in repression of 5–10% of genes [21]. Its C-terminal helix-turn-helix domain binds (preferentially AT-rich) DNA [22], while its N-terminal domain functions as oligomerization domain (yielding dimerization, as well as higher-order dimer–dimer interactions). The ability to multimerize is essential for genome compaction and gene silencing [23–25]. Owing to dimer–dimer interactions, the protein forms filaments along DNA [26]; its multivalency also facilitates bridging of two DNA duplexes [27,28], potentially

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yielding loops *in vivo* [29]. The DNA-binding properties of H-NS and its various roles are modulated by interaction with a paralogue, StpA, association partners (such as Hha and YdgT) and changes in physicochemical conditions (such as osmolarity) [24,30–32].

Different from H-NS, HU does not preferentially target specific regions of the bacterial genome, but is found scattered, binding DNA in a sequence-independent manner, but with a high affinity for structurally aberrant DNA [33–35] and RNA [36,37]. It exists primarily as a heterodimer of two homologous subunits HUα and HUβ, but homodimers may form as well [38]. The relative amounts of HU homo- and heteromers depend on growth phase [38]. Whether HUαα, HUαβ or HUββ multimerizes along DNA, determines if either HU-mediated stiffening, bridging or supercoiling of DNA occurs [39,40]. HU may also bend DNA and is thereby involved in genome compaction, as well as in stabilizing or facilitating regulatory DNA loops [40]. HU plays an additional role in genome folding due to its effects on DNA topology via genetic cross-talk with DNA gyrase and DNA topoisomerase I [41].

IHF, a homologue of HU, consists of α- and β-subunits. In addition to sequence-unspecific binding and bending of DNA [42], implying a generic HU-like role in genome compaction, IHF interacts with a specific, conserved nucleotide sequence [43,44], inducing a sharp, U-shaped bend, essential for its role in transcription regulation [45,46]. FIS, a homodimeric protein, plays a role in transcription initiation, genome organization and initiation of replication [47–49], binding to a degenerate consensus sequence [49–51]. The expression level of FIS is elevated during phases of fast growth [52,53]. The activity of NAPs is thus, to date, known to be modulated by changes in expression level, association with protein partners and physicochemical conditions, altering gene expression patterns to allow for environmental adaptation.

In this review, we investigate the possibility that the activity of NAPs is additionally modulated by PTMs signalling differences in growth conditions. By scrutiny of the findings of 11 recent bacterial proteomics studies, we detected 29, 24, 13, 14, 17 and 4 unique PTMs on *E. coli* H-NS, HUα, HUβ, IHFα, IHFβ and FIS, respectively, which are listed in the Supplementary Material [54–64]. An overview of all modifications of these proteins in context of their protein sequence is shown in Figure 1. The properties of different types of protein modifications are summarized in Table 1. Put in a structural perspective, and correlated with genetic studies, we predict that several of these PTMs influence (1) DNA-binding affinity, (2) oligomerization and (3) protein–protein interactions.

Modulation of DNA binding of NAPs by PTM

H-NS

In *E. coli* H-NS (see Figure 2a,b), a DNA-binding motif is present at residues 112–117 [65]. No modifications on H-NS at this particular sequence are observed. However, on two flanking lysines (Lys96 and Lys121) in close proximity to the bound DNA [65], PTMs do occur [57,62,64]. Both these lysines can be acetylated; Lys96 can also be succinylated. The canonical lysine positive charge is probably necessary for the association with negatively charged DNA, which would be disturbed by acetylation or succinylation, resulting in a neutral or negative charge, respectively. Moreover, a succinylation event imposes a large carbon moiety onto the protein, which could induce sterical hindrance. These PTMs would thus probably reduce H-NS binding affinity towards DNA. Also, five positive residues have been identified in the unstructured H-NS linker region as required for gene silencing by contacting DNA through electrostatic interactions [66]. Two of these residues, Lys83 and Lys87 can be acetylated [57,64], which would obstruct such interactions, thus reducing the DNA-binding properties of H-NS.

In the DNA-binding domain of H-NS paralogue and modulator StpA, the aforementioned DNA-binding motif is present as well. However, it harbours a lysine instead of an alanine at position 117, which could contribute towards the greater DNA-binding affinity of StpA over H-NS [67]. Interestingly, this lysine is subject to acetylation [64], which could reduce the DNA-binding affinity of StpA to resemble that of H-NS. In turn, such a modification could reduce the extent to which StpA enhances bridging through the formation of H-NS–StpA bridging filaments [30]. StpA outperforms H-NS in binding RNA as well, both being involved in post-transcriptional regulation of *malT* and *td* expression [68,69]. Intriguingly, an experiment comparing *td* RNA splicing by StpA and H-NS showed a 100-fold higher effectiveness of StpA over H-NS *in vitro* [69]. This was reduced to an 8-fold higher effectiveness *in vivo*, which may imply a role for PTM. However, currently structural and functional information on StpA, for instance in the form of the effect of single-amino acid residue mutations, is limited, complicating interpretation of these effects.

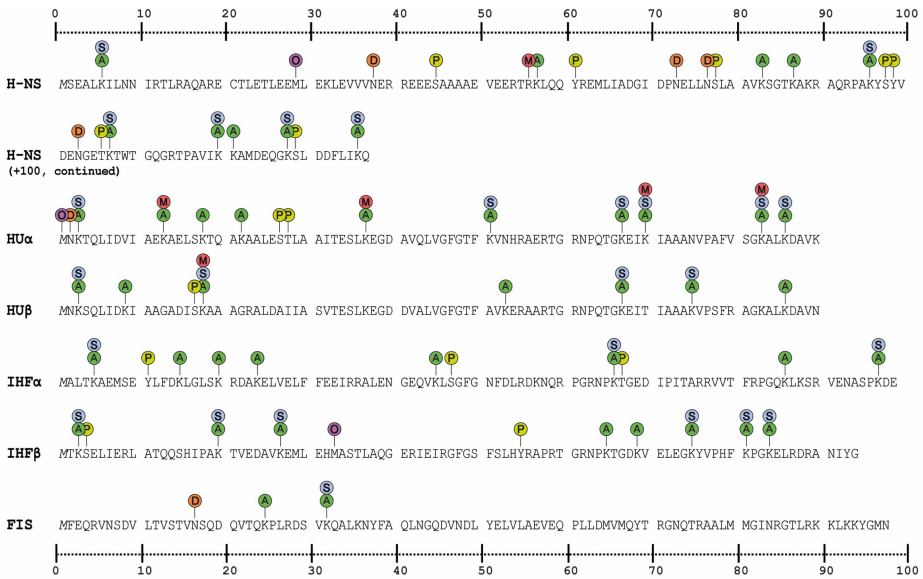

Figure 1. Amino acid sequences of *E. coli* NAPs H-NS, HUα, HUβ, IHFα, IHFβ and FIS.

Labelled residues depict PTMs that have been discovered by any of the proteomic studies regarded in this review (A, acetylation; S, succinylation; M, methylation; P, phosphorylation; D, deamidation; O, oxidation). Initiator methionines are shown in italics to indicate their post-translational cleavage.

Another PTM that may influence the DNA-binding ability of H-NS targets the C-terminal hydrophobic core. This is formed by several aliphatic and aromatic amino acid side chains and stabilizes the flanking secondary structures [70]. One of the side chains involved in this hydrophobic core, Tyr99, can be phosphorylated [55]. Such a phosphorylation event would introduce a polar moiety, which may either prevent correct hydrophobic collapse, rendering a misfolded protein, or could impose structural changes in the DNA-binding loop, potentially reducing DNA-binding activity.

Following oligomerization of H-NS along the DNA, the unbound DNA-binding domain may be either blocked through internal interactions, or exposed to the solvent, allowing for DNA-H-NS-DNA bridges [24]. Mg^{2+} ions have been shown to modulate the balance between the 'closed' and 'open' state through interactions with amino acid regions 42–45 and 98–105. Within these regions, four residues have been demonstrated to undergo phosphorylation (Ser45, Ser98 and Tyr99) or deamidation (Asn103) [54,55,58,64]. Such PTMs generate negatively charged residues, potentially allowing for stronger interactions with the Mg^{2+} ion, repulsion of the DNA-binding domain towards the solvent, and therefore more H-NS-mediated DNA bridging.

HU

In *E. coli* HU (see Figure 2c), the conserved residues HUα-Lys3 and HUβ-Lys3 form internal salt bridges with HUα-Glu26 and HUβ-Asp26, respectively [72]. Both lysines can be either acetylated or succinylated according to five independent proteomic studies, perturbing such interactions [56,57,61,62,64]. Interestingly, HU homologues that lack a glutamate or aspartate at this position — such as IHFβ — have their conserved lysine exposed to the solvent [73,74], which may then contact DNA several bp downstream or upstream from the primary bending location. This additional interaction enlarges the binding site [75], probably enhancing the DNA-binding affinity, as well as increasing bending. In a similar fashion, the abovementioned PTMs may have effects on HU as well.

In the HU heterodimer, several residues neighbour a charged surface consisting of several β-sheets from both monomers. This cradle-like surface contains conserved salt bridge-forming residues, which are present in IHF as well [44], and which are implicated in DNA binding. Since the Lys86-residues of both subunits flank this domain and are heavily conserved, they may be involved in the bending of DNA, 'pulling' on both ends of the DNA duplex, while the DNA in between rests on the cradle. HUα-Lys86 and HUβ-Lys86 can both be

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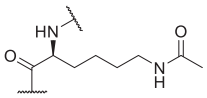
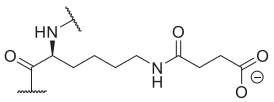
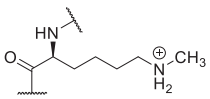
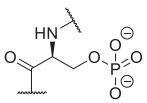
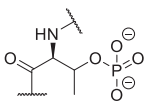
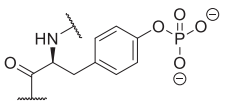
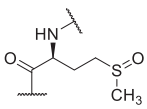
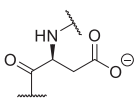
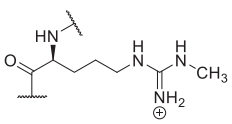
Table 1 Properties of amino acid PTMs

Amino acid	Modification	Structure	Change of charge at physiological pH	Mass shift (Da)
Lysine	Acetylation		+1 to 0	+42
Lysine	Succinylation		+1 to −1	+100
Lysine	Methylation		no change	+14
Serine	Phosphorylation		0 to −2	+80
Threonine	Phosphorylation		0 to −2	+80
Tyrosine	Phosphorylation		0 to −2	+80
Methionine	Oxidation		no change	+16
Asparagine	Deamidation		0 to −1	+1
Arginine	Methylation		no change	+14

acetylated [64], while HUα-Lys86 can also be succinylated [56]. Acetylating these residues could decrease HU-binding affinity towards DNA and result in reduced DNA bending; succinylation would yield a similar, if not stronger effect, due to electrostatic repulsion. This notion coincides with the severely reduced DNA-binding capacity shown for the homodimer of a *Bacillus subtilis* HU K86A mutant [76].

In addition to binding DNA, HU is involved in post-transcriptional regulation through binding mRNA. For example, it specifically recognizes and binds *rpoS* mRNA, thus promoting its translation [36]. Interestingly, the affinity of HU for *rpoS* mRNA is comparable to its affinity for nicked DNA or 3′ ssDNA overhangs, being ∼1000-fold stronger than for regular dsDNA [33,34,77]. Although concrete structural information of HU binding RNA is lacking, Balandina et al. postulated that HU may bind dsRNA with a 3′ ssRNA overhang in a fashion comparable to analogous DNA constructs [37,78]. The proposed model requires HU β-sheet arms to contact the double-stranded part, while the single-stranded part associates with the cradle-like surface formed on the HU body. In this light, an interesting question to pursue is whether the residues that are subject to PTM on the cradle (see Figure 2c) influence HU specificity for DNA versus RNA.

IHF

The sequence specificity of IHF (see Figure 3a) can be explained not only by its primary electrostatic interactions with specific bases, but also by the recognition of minute differences within DNA structure. The IHF

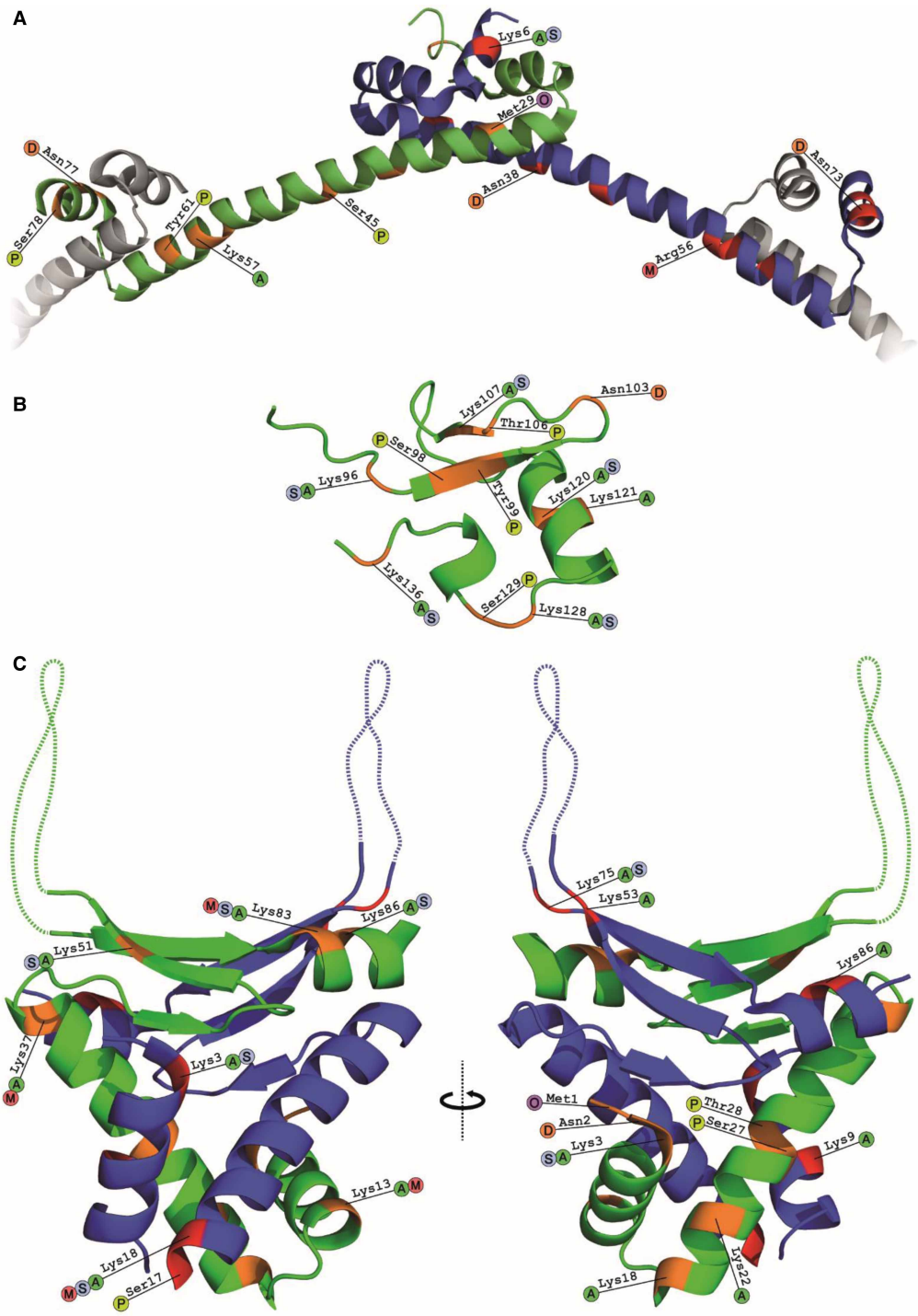


Figure 2. Protein structures depicting the position of residues (in orange and red) that have been shown to undergo a PTM event by proteomic studies regarded in this review. Part 1 of 2

Structures are based on PDB files (**A**) 3NR7 [71], showing the N-terminal domain (residues 1–82) from an antiparallel H-NS dimer (green and blue depict individual monomers), in association with two other H-NS dimers (in grey) via 'tail-to-tail' interactions. Note that this structure represents an H-NS homodimer with identical chains, but that for the purpose of clarity PTMs are labelled either on one or the other chain. (**B**) 1HNR [70], showing the C-terminal domain (residues 91–127) from an H-NS monomer. The unstructured linker between the N-terminal and C-terminal domains of H-NS has not been resolved. (**C**) 2O97 [72], showing HUαβ structure from the front and back (HUα, green; HUβ, blue). In this structure, HU residues 55–74

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Figure 2. Protein structures depicting the position of residues (in orange and red) that have been shown to undergo a PTM event by proteomic studies regarded in this review. Part 2 of 2

(HUα) and 56–74 (HUβ) are not resolved, but their structure has been approximated with dashed lines, based on atomistic models generated by Hammel et al. [39].

consensus binding site is A/T-rich, which straightens DNA and narrows the minor groove [79]. As IHF interacts with DNA, several water molecules are encapsulated between the A-tract and the IHF dimer, forming a 'spine of hydration' [73]. Among other interactions, backbone amides from amino acids flanking IHFα-Ser47 form hydrogen bonds with trapped water molecules, which undergo a similar interaction with phosphates in the DNA backbone. Ser47 itself forms hydrogen bonds with water molecules that reside deeper within the minor groove, stabilizing the interaction as such [73]. Two proteomic studies have shown that this Ser47 can be phosphorylated [58,63]. Such phosphorylation would introduce a negatively charged moiety into the minor groove, probably causing repulsion from the negatively charged DNA phosphate backbone. Similarly, Lys5, Lys24, Lys45, Lys86 from IHFα, and Ser4 and Lys75 from IHFβ are residues that undergo modifications which

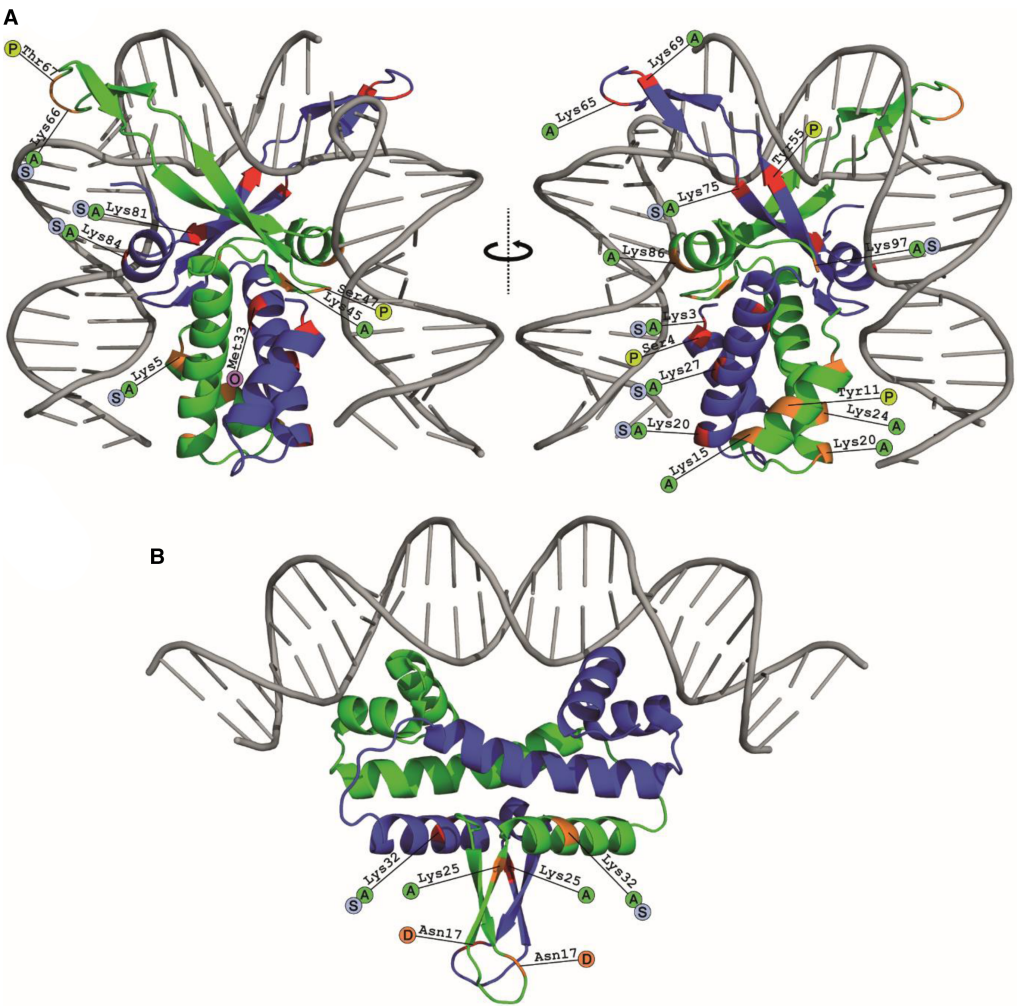

Figure 3. Protein structures depicting the position of residues (in orange and red) that have been shown to undergo a PTM event by proteomic studies regarded in this review.

Structures are based on PDB files (**A**) 1IHF [73], showing IHFαβ bound to 35 bp DNA, from the front and back (IHFα, green; IHFβ, blue) and (**B**) 4IHV [80], showing FIS bound to 27 bp DNA (green and blue depict individual monomers).

disturb electrostatic interactions with DNA [73], either directly or through hydrogen bonding with H_2O associated with the phosphate backbone. As a consequence, these modifications may relax IHF sequence specificity and reduce DNA-binding affinity.

The flexible β-ribbon arm from IHFβ that wraps itself around the minor groove contains a lysine at position 75 that is involved in an interaction with the DNA phosphate backbone [73]. This IHFβ-Lys75 may be either acetylated or succinylated [56,64]. Both modifications would inhibit salt bridge formation and could therefore reduce the binding affinity of IHF. In binding DNA, both β-arms use prolines (IHFα-Pro65 and IHFβ-Pro66) to intercalate between base pairs, which is key to the large bend angle induced by IHF. These prolines are flanked by lysines (IHFα-Lys66 and IHFβ-Lys65) which can be acetylated [64]. These modified lysines may restrain the protrusion of hydrophobic prolines within the negatively charged DNA and between its bases, thus attenuating the bend angle IHF may induce.

Perturbation of oligomerization of NAPs by PTM

H-NS

H-NS Tyr61 is involved in a hydrophobic core that stabilizes tail-to-tail association of two H-NS dimers [71]. Recently, an H-$NS_{Y61DM64D}$ mutant has been shown to be incapable of forming multimeric structures and DNA-H-NS-DNA bridges [24]. Intriguingly, three separate proteomic studies have identified phosphorylation events on Tyr61 [55,58,63], presenting a negative charge, comparable to the Y61D mutation. These render the hydrophobic core more hydrophilic and could thereby perturb H-NS oligomerization. As H-NS dimers oligomerize along the DNA, a salt bridge between Lys57 and Asp68 from a flanking H-NS fixates the coiled-coil formed by anti-symmetrical α-helices from both dimers [71]. According to Kuhn et al., this lysine is acetylated [57], blocking such a salt bridge and compromising H-NS multimerization ability. Interestingly, Hong et al. engineered an H-NS variant (K57N) which exhibited induction of cell lysis, prophage excision and reduced formation of biofilms. These effects are proposed to be due to indirectly enhanced interaction with Hha, YdgT or StpA [81]. The previously mentioned Lys57 acetylation neutralizes the residue as well, and may thus induce similar phenotypes, enhancing association of H-NS with heterologous association partners indirectly, due to reduced multimerization. Strikingly, Asp68 and Lys57 are present on StpA as well, and its lysine has been shown to undergo acetylation [64]. If interaction between H-NS and StpA involves a comparable salt bridge, such a PTM may decrease the possibility of an H-NS–StpA interaction. Modulation of oligomerization along DNA and DNA bridging are key aspects of H-NS-mediated modulation of gene expression [24,30]. Thus, these PTMs could allow bacteria to regulate transcription, thereby adapting to their environment.

HU

HUαα and HUαβ organize DNA differentially through co-operative multimerization along DNA [39]. Dimer–dimer interactions between either homo- or heterodimers differ in nature as they require several, distinctive salt bridges. HUα-Lys37-HUα-Glu34 and HUα-Lys83-HUα-Glu26 salt bridges occur between two homodimers. In contrast, heterodimers utilize HUβ-Lys37-HUβ-Glu34 and HUα-Lys83-HUβ-Asp26 interactions. HUα-Lys37 and HUα-Lys83 can be acetylated or methylated; HUα-Lys83 can be succinylated as well [56,57,62,64]. Such PTMs prevent formation of these salt bridges, thus impeding dimer coupling. Acetylation is more likely to occur in the stationary phase due to increased levels of acetyl-phosphate [64]. Since association of HUαα to DNA is proposed to play a larger role in the exponential phase, while HUαβ is more important in the stationary phase [39], the acetylation of HUα could shunt formation of DNA-HU complexes towards utilizing more HUαβ as these would require only HUα-Lys83 to be unmodified. These PTMs may provide selectivity towards HU regulons as a function of different growth phases [82,83], granting a level of regulation additional to varying HUα and HUβ expression [38]. Mechanistically, this could be related to distinct co-operative binding modes used by HU to organize DNA [39].

Perturbation of interactions between NAPs and other proteins by PTM

H-NS-modulator Hha

H-NS-mediated transcriptional silencing of certain genes requires the association of two Hha-molecules with the N-terminal dimerization domain of an H-NS dimer [84]. NMR studies revealed a moderate role for H-NS-Lys6 in binding Hha [85]. Moreover, Lys6 resides in close proximity to an Hha-α-helix [86]. This lysine

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residue can undergo either acetylation or succinylation [56,57,61,64]. Succinylation of Lys6 could induce sterical hindrance, reducing the strength of the interaction, while acetylation could improve a potential interaction with the proximal hydrophobic residues in the Hha α-helix. These PTMs may thus influence H-NS-mediated silencing of genes that require Hha as a cofactor associated with H-NS. A positively charged surface on Hha opposite of the Hha–H-NS interaction interface has been proposed to enhance H-NS binding to DNA [86]. This surface harbours Hha-Lys32, which is subject to acetylation [64]; acetylation of this residue could reduce its affinity for DNA, which would in turn reduce the impact of Hha on DNA binding and DNA bridging by H-NS [24].

HU-looping partner GalR

HU is responsible for DNA looping in the GalR-driven repressosome, inhibiting the *gal* operon [87]. HU canonically acts sequence-independently, but at the *gal* operon it binds at a 'specific' site between two *gal* operators due to the co-operativity of HU-binding and GalR-looping mediated by HU–GalR interaction [88]. The HUα residues responsible for the GalR–HU interaction are Ser17, Lys18 and Thr19, of which Lys18 has been shown to undergo acetylation [57,64]. Such a neutralizing PTM, comparable to a K18A substitution [88], may therefore induce similar phenotypes, i.e. reduced GalR–HU interaction and therefore, derepression of the *gal* operon. Conversely, alanine-substitution at Lys22, a residue that also undergoes acetylation [57,64], did not affect HU function in the repressosome [88]. This indicates potential redundancy for this PTM, or a function in another HU-mediated mechanism.

FIS-inversion partner Hin

In *Salmonella* sp., FIS (see Figure 3b) is involved in the initiation of DNA inversion by Hin [89]. Within the invertasome, FIS interacts with Hin through its N-terminal β-hairpin motif. Mutational analyses have been carried out on the ability of FIS to activate DNA inversion reactions, which suggested several crucial residues within the flexible N-terminal domain, besides a possible role for solvent-exposed residues from α-helix A [90]. One residue within this region undergoes acetylation, Lys25 [57,64]. In an FIS dimer, this lysine engages in a hydrogen bond with Thr15, stabilizing the β-hairpin. Acetylation of Lys25 obstructs this fixation and thereby increases N-terminal domain flexibility. However, an analogous hydrogen bond-inhibiting K25C mutation only showed a moderate decrease in DNA inversion activation by FIS [90]. Its acetylation is therefore also unlikely to inhibit this reaction completely.

Discussion

This meta-analysis identified multiple PTMs of bacterial NAPs that probably influence important cellular processes. To date, none of the NAPs discussed here are known to bind any signalling ligands, a fact that may be compensated for by PTM. Of the four major NAPs, it is noteworthy that FIS is substantially less subject to functional modulation by PTM than the other NAPs. This may be explained by the fact that, different from the other three NAPs, FIS expression is much more dependent on growth phase and growth rate [53]. Moreover, extensive cross-regulation occurs between FIS, DNA topoisomerase I and gyrase, using changes in negative supercoiling to either activate or repress *fis* transcription [91–93]. Such a system may render an additional layer of FIS regulation, in the form of PTM, unnecessary.

Lysine acetylation contributes towards a large portion of the PTMs identified in the present study. In eukaryotes, various lysine acetyltransferases and deacetylases have been discovered alongside many other PTM 'writers' and 'erasers' [94]. In contrast, very few protein-modifying enzymes have been discovered in bacteria. In *E. coli*, acetylation and deacetylation seem to be almost completely regulated by acetyltransferase Pat and deacetylase CopB [64]. Therefore, the abundant occurrence of acetylation may point towards non-enzymatic reactions. Owing to an accumulation of acetyl-phosphate, indeed overall acetylation levels in *E. coli* are increased in growth-arrested cells, compared with growing cells [64]. This suggests an ancient form of protein function modulation in which primordial organisms exploited non-enzymatic acetylation to respond to changing physicochemical circumstances and nutrient availability. It seems likely that enzymatic modification evolved as a way to act upon the environment in a more efficient and active manner. Since PTMs within bacteria have only been considered for a brief time, additional prokaryotic protein-modifying enzymes may remain to be identified.

In the mid-1980s, Spassky et al. [95] identified three isoforms of H-NS, varying in their isoelectric points in *E. coli* cell lysates. However, the nature and biological function of these isoforms have remained obscure. Since

we have shown that H-NS may undergo several biologically relevant modifications, which may affect its isoelectric point, the existence of such different H-NS species in the cell is not surprising. Conversely, one could argue that with that many possible modifications, more isoforms would be present, which either may have carried a similar isoelectric point, or were not abundant enough to show up on the gel. Therefore, it is important to be aware that the scrutinized proteomic studies do not provide information on the relative abundance of post-translationally modified derivatives of the described NAPs. This asks for the application of quantitative mass spectrometry approaches [96].

Only 2 out of 12 proteomics studies covered in this meta-analysis were aimed at identifying a wide range of PTMs, while others targeted exclusively acetylation, phosphorylation or succinylation. Therefore, data including other PTMs are currently less substantial. Furthermore, most studies employed a single growth condition, with samples taken at a single, fixed time point. PTMs may therefore not be consistently detected due to their occurrence in different conditions. To effectively assess the function of PTMs, more extensive research is required. The effect of environmental signals can be scrutinized through systematic variation of experimental conditions. Furthermore, modification-specific antibodies may become a valuable tool in demonstrating the presence and, for instance, genomic distribution of modified NAPs. Moreover, constitutive NAPs carrying PTMs described in the present study can be constructed through chemical modification or genetic code expansion, to assess their function experimentally [97].

While the functions of NAPs are hardly set in stone, a new level of complexity makes an entrance. Owing to insufficient tools and lack of interest, PTMs on prokaryotic proteins have, until recent years, remained unexplored. Now, extensive PTM shown by several proteomics studies and the widespread functionality that they present in *E. coli* underline the need for the consideration of such factors within structural and functional investigations of NAPs and other prokaryotic proteins in general.

Abbreviations

NAPs, nucleoid-associated proteins; PTM, post-translational modification.

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Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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