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Artefactual cleavage of *E coli* H-NS by OmpT

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Summary — In the bacterium *Escherichia coli*, H-NS (H1, H1a) is a heat-stable protein with a molecular mass of 15.5 kDa involved in nucleoid organisation and gene regulation linked to certain signal transduction pathways. We have shown that, following addition of preparations of everted inner membrane vesicles, heat-stable cleavage products of approximately 10 kDa of H-NS are formed *in vitro* from newly synthesised, radio-labelled H-NS and from purified H-NS. The 15.5 kDa protein and its cleavage products were also recovered from a minicell system. These results raised the possibility that cleavage of H-NS is physiologically significant. However, the cleavage of H-NS observed appears to occur during cell breakage and to depend on the method of protein extraction and the presence of the outer membrane protease, OmpT. Nevertheless, the results indicate that H-NS may contain at least two separate domains with cleavage occurring between these domains at a preferred OmpT site. Failure to take account of H-NS cleavage in sample preparation and analysis can lead to serious underestimation of H-NS levels.

histone / H-NS / protease / OmpT / *Escherichia coli*

Introduction

The 15.5 kDa, heat-stable, DNA-binding protein H-NS (H1, H1a) is important in numerous aspects of the physiology of *E coli* and mutations in the genes encoding H-NS and its homologues in other bacteria are pleiotropic and affect the expression of many different genes [1]. Changes in transcription have been attributed to H-NS mutations altering either DNA supercoiling [2] (but see [3]) or binding to H-NS-specific sequences in promoters [4]. H-NS has been shown to bind preferentially to curved DNA [5, 6] (but see [7]) to form, it has been speculated, a nucleosome-like structure [8]. Recently, it has been reported that H-NS acts as a repressor of the *proU* operon by binding to the *proV* promoter [9] and as a repressor of its own expression by binding to its own promoter [10]. H-NS has also been reported to be a relatively non-specific DNA-binding protein, probably involved in the compaction of the bacterial nucleoid [11–13]. Finally, the presence of H-NS in an *oriC*-membrane complex has been reported [16].

H-NS is considered a key element in some signal transduction pathways and post-translational modification of this protein could enable bacteria to respond rapidly to environmental stimuli and modulate, for example, the expression of genes encoding virulence determinants [14]. The possibility of proteolysis of H-NS *in vivo* has been raised [13]. Specific proteolysis would assign H-NS to the important class of short-lived proteins involved in responses to stress and development [15]. We were therefore interested in the possibility that proteolysis might play a role in the regulation of H-NS action in response to environmental signals. Such signals can be transduced by changes in membrane structure and composition. We investigate in this paper the proteolytic cleavage of H-NS by membrane vesicles *in vitro* and show that this cleavage can be attributed to an outer membrane protease, OmpT. Proteolysis of a small number of proteins by OmpT may occur as an artefact when *E coli* cells are permeabilized or broken [17–19] and the conditions required for H-NS cleavage are described and the implications discussed.

Materials and methods

Bacterial strains

MC4100 $\Delta(\text{argF-lac})\text{U169}$, *rpsL*, *araD*, $\Delta(\text{lacIPOZYA})\text{U169}$, *flbB*, *deoC*, *rbsR*, *ptsF* [20], Q172 *HfrH*, *thi*, *relA*, *serB* [21], DS410

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Abbreviations: PBS, phosphate buffered saline; EGTA, ethyleneglycol-bis(*b*-aminoethylether)-*N,N,N',N'*-tetraacetic acid; PMSF, phenylmethylsulphonyl fluoride; TLCK, tosyl lysyl chloromethyl ketone; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis.

minB, *ara*, *thi*, *gal*, *xyl*, *mtl*, *tonA*, *rpsL*, *lacY*, *malA* [22]. N43 *acrA1*, *ara14*, Δ *lac-85*, *galK2*, *rpsL97*, *malA1*, *xyl-5*, *mtl-1* [23]. N43 *ompT::Tn5* (constructed by P1 transduction from MC4100 *ompT::Tn5*).

Plasmids

pLG339 [24], pLG701 derivative of pLG339, containing the genes *hns*, *galU* and *hnrG* (Goldberg *et al.* paper in preparation).

Cell preparation (heat-stable proteins)

An overnight culture of *E. coli* strain Q172 [21] was diluted (1:100) into fresh nutrient broth (Oxoid) and grown with aeration at 30°C to an A_{600} of 0.3–0.4. Cells were harvested from 4 mL volumes, resuspended in 200 μ L of hot (90°C) phosphate-buffered saline and boiled for 2 min. Cells were sonicated twice for 10 s at 5-min intervals using an MSE Soniprep 150 sonicator at a 10 micron amplitude setting. Sonicates were boiled for a further 2 min followed by centrifugation, 13000 g, at room temperature for 5 min. Supernatants were then analysed by SDS-PAGE (20% acrylamide).

Immunoblots

Heat-stable supernatant proteins electrophoresed on SDS-polyacrylamide (20%) gels were electrotransferred [25] to nitrocellulose and probed with either polyclonal rabbit anti-H-NS antibody and detected by the immunoperoxidase indirect method [26] using goat anti-rabbit IgG and rabbit-PAP (Dako) with 3-amino-9-ethylcarbazole (Sigma) as substrate, or by protein A conjugated horseradish peroxidase with luminol (Amersham, UK) as substrate. Detection with the monoclonal mouse anti-H-NS antibody used the alkaline phosphatase indirect method [27] consisting of alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulins (Dako) with 5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium (Sigma) as substrate. To achieve quantitative and reproducible detection of H-NS degradation products, the required modifications to the immunoblot procedure were: overnight transfer at 20 V in 25 mM phosphate buffer to polyvinylidene difluoride paper (Immobilon P, Millipore) in preference to nitrocellulose.

In vitro coupled transcription-translation experiments

Expression of the cloned *hns* gene was analysed by using the *E. coli* S30 *in-vitro* coupled transcription-translation kit, minus methionine, manufactured by Promega Corp. essentially as instructed by the manufacturer plus, where indicated, 0.15 A_{260} units *E. coli* everted inner membrane vesicles. The final reaction volume was adjusted to 12.5 μ L with distilled water, except where specified. The radio-labelled proteins were precipitated with 2.5 vol of acetone at 0°C for 15 min and pelleted by centrifugation. The labelled proteins were analysed by resuspending the dried protein pellets in 50 μ L of SDS-PAGE sample buffer (75 mM Tris-HCl, pH 6.8, 25% v/v glycerol, 0.00125% w/v SDS, 2.5% v/v 2-mercaptoethanol, 0.001% v/v bromophenol blue). The samples were boiled for 3 min and then 10 μ L of each sample were loaded onto a 13% SDS-polyacrylamide gel [28]. Following fixation in 10% v/v acetic acid and 12% v/v methanol, the gels were washed in distilled water, then in 1 M sodium salicylate, and were exposed overnight to Fuji RX X-ray film.

Preparation of membrane vesicles

Everted inner-membrane vesicles were prepared from the *E. coli* strain MC4100, essentially as described by Pratt [29], except that passage through a French press was replaced by sonication. After harvesting and washing, cells were suspended in 60 mL ice cold membrane buffer A (50 mM triethanolamine (Sigma), 5 mM magnesium acetate (FSA) and 1 mM dithiothreitol (Sigma)), sonicated for ten 30-s pulses separated by 30-s intervals, using a pre-chilled (–20°C) 19 mm diameter probe, attached to an MSE sonicator set at medium power, peak-to-peak amplitude, 8 μ s. After sonication, the debris was removed by centrifuging at 24000 g and the remainder of the procedure, involving separation of inner and outer membrane fractions on sucrose equilibrium gradients was as described by Pratt [29]. The vesicles were stored in aliquots at –20°C at a concentration of 0.15 A_{280} units/ μ L. Vesicles were shown to be fully competent for protein translocation as measured previously [29].

Cleavage of H-NS in minicells

The minicell producing strain, DS410, was transformed with plasmid pLG339 [24], which has a copy number of approximately five, and its *hns*-containing derivative, pLG701. Minicells containing these two plasmids were prepared using the procedure described by Stoker *et al.* [30]. After purification, the minicells were labelled with ³⁵S-methionine, washed three times with 10 mM K₂PO₄ buffer pH 7.4 before being finally resuspended in 200 μ L SDS-PAGE buffer, boiled 5 min, and analysed by autoradiography after SDS-PAGE (13% w/v polyacrylamide).

Heat stability of cleaved H-NS

The heat stability of cleaved and un-cleaved H-NS was investigated by boiling proteins synthesised in the *in vitro* transcription translation system. 2 μ g of pLG339 and pLG701 were added to the *in vitro* transcription-translation system with and without the addition of 2 μ L of everted inner membrane vesicles, to give a final volume of 25 μ L. After labelling, half of each sample was transferred to a second fresh tube, boiled for 5 min — conditions which denature and precipitate about 90% of *E. coli* proteins [31] — and centrifuged at 13000 g for 5 min. The supernatant was recovered and added to an equal volume of 2 \times SDS-PAGE sample buffer. The unboiled mixtures were precipitated with acetone, dried and resuspended in 50 μ L 1 \times SDS-PAGE buffer. The proteins were analysed by SDS-PAGE (13% acrylamide).

Preparation of purified H-NS

H-NS was obtained from *Salmonella typhimurium* strain LT2 (pGP1-2 pJSH, Kan^R Amp^R, 500 mL of this strain were grown at 30°C aerobically in Luria broth containing 50 μ g/mL kanamycin and 100 μ g/mL ampicillin to an A_{600} of 1.0. To induce the expression of H-NS, the culture was transferred to a 42°C shaking water bath and incubated for a further 45 min. The culture was then transferred to a 37°C shaking incubator and grown into stationary phase (A_{600} 2.2–2.4). Cells were harvested by centrifugation (6000 g for 20 min) at 4°C. The pellet was washed twice with 20 mM Tris-HCl, 1 mM EDTA and 1 mM DTT, pH 7.5 (TED), resuspended in 15 mL of TED and lysed by sonication (ten 15-s treatments at 1-min intervals) in an MSE Soniprep sonicator set at medium power (6–8 microns). Cell debris was removed by centrifugation (20 000 g for 45 min) at 4°C. The crude extract was then precipi-

tated sequentially with two additions of ammonium sulphate (0–40% and 40–60%). The 40–60% precipitate, containing H-NS, was re-dissolved in 2 mL of TED and dialysed against TED for 4 h at 4°C. Precipitated material was removed by centrifugation and the dialysate loaded onto a 10 mL double-stranded DNA cellulose column (Sigma) equilibrated in TED. H-NS was purified using a 150 mL gradient of 0–700 mM NaCl in TED, and eluted between 350 and 500 mM NaCl. Fractions containing the protein were pooled, re-dialysed as described, loaded onto a Mono Q 5/5 anion exchange column equilibrated in TED, and fractionated using a 25 mL 0–1 M NaCl (in TED) gradient. Pure H-NS was obtained in fractions which eluted between 400 and 500 mM NaCl.

Cleavage of purified H-NS *in vitro*

0.7 µg of purified H-NS was incubated with 0.5 µL (0.075 A₂₆₀ units) of everted inner membrane vesicles in 0.05 M sodium phosphate buffer (pH 7.4) and incubated at 37°C for 60 min. The mixture was then boiled for 10 min, centrifuged at 13000 g for 5 min (which removes most denatured vesicle proteins), and the supernatant added to an equal volume of 2 × SDS PAGE loading buffer. The entire sample was loaded onto a 13% polyacrylamide gel, together with 2.1 µg of uncleaved H-NS. The gel was stained with Coomassie blue.

Determination of the site of cleavage of H-NS by OmpT

To determine the site of cleavage by OmpT, purified H-NS (25 µg) was mixed with 15 µg of an outer membrane preparation containing OmpT (total volume 50 µL) and incubated at 37°C for 15 h. The digestion was terminated by addition of 25 µL of 2 × SDS-PAGE sample buffer containing 10 mM DTT followed by boiling at 95°C for 5 min. Cleaved H-NS was separated from uncleaved H-NS and outer membrane proteins by electrophoresis on a 16.5% (6% C) Tris tricine gel [32], followed by electroblotting onto fluorotrans PVE⁺ (Pall) in 25 mM Tris, 192 mM glycine, 0.037% w/v and 10% methanol. Blotted proteins were visualised with 0.1% Coomassie blue R250 in 50% methanol followed by destaining with 50% methanol and 10% acetic acid. The N-terminal sequences of the H-NS fragments were determined using an Applied Biosystems gas phase sequencer.

Determination of the concentration of benzamidine-HCl required to inhibit H-NS cleavage

The standard procedure for the *in vitro* transcription-translation kit (Promega) was used in the presence of everted vesicles and different concentrations of benzamidine-HCl (0.125–32 mM). The extent of H-NS cleavage was then evaluated after SDS-PAGE and autoradiography.

Results

Expression of the gene encoding H-NS *in vitro*

In studies of the expression of *hns* and adjacent genes in a coupled transcription/translation system [33], we were particularly interested to determine whether H-NS was subject to proteolysis by membrane vesicles. ³⁵S-methionine-labelled H-NS protein of the expected size, 15.5 kDa, was

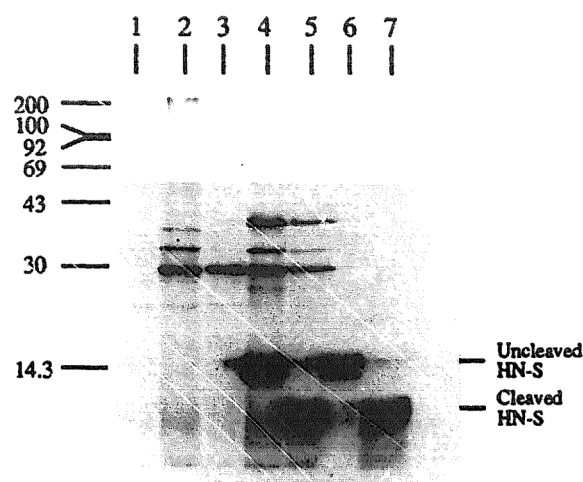


Fig 1. *In vitro* coupled transcription/translation of the *hns* gene. Lanes: 1, no DNA; 2, pLG339; 3, pLG339 with inner membrane vesicles; 4, pLG701; 5, pLG701 with vesicles; 6 pLG701, boiled; 7, pLG701 with vesicles, boiled.

observed when plasmid pLG701 was used as DNA template (fig 1, lane 4). However, when everted inner membrane vesicles (see *Materials and methods*) were included in the reaction mixture, a large proportion of the radio-labelled H-NS was then detected as a lower M_r form of 10 kDa (fig 1, lane 5). To enrich for 'heat stable' H-NS and to reduce proteolysis, the reaction mixture was boiled in some experiments for 2 min before mixing the supernatant, which contains proteins resistant to denaturation (see *Materials and methods*), with SDS-sample buffer. This boiling step did not affect the pattern of labelled H-NS on SDS-PAGE. It should be noted that the inner membrane vesicles used here were shown in other experiments to be fully competent in protein translocation *in vitro* [29] and are therefore predominantly everted, having the cytoplasmic surface outermost. However, such vesicles are inevitably contaminated with low levels of outer membrane fragments. Other control experiments showed that all forms of H-NS were fully susceptible to proteolytic degradation by subtilisin and hence their cleavage is not associated with translocation to the vesicle lumen (data not shown).

Cleavage of purified H-NS

Although the above results are consistent with cleavage of a 15.5 kDa H-NS to yield a proteolytic fragment or fragments, they might also be considered consistent with translation from transcripts of different lengths. To demonstrate that H-NS is indeed cleaved, membrane vesicles were added to purified H-NS (see *Materials and methods*), and forms of 10 and 8 kDa were observed after boiling in sample buffer and analysis by SDS-PAGE (fig 2, lane 1). None of a variety

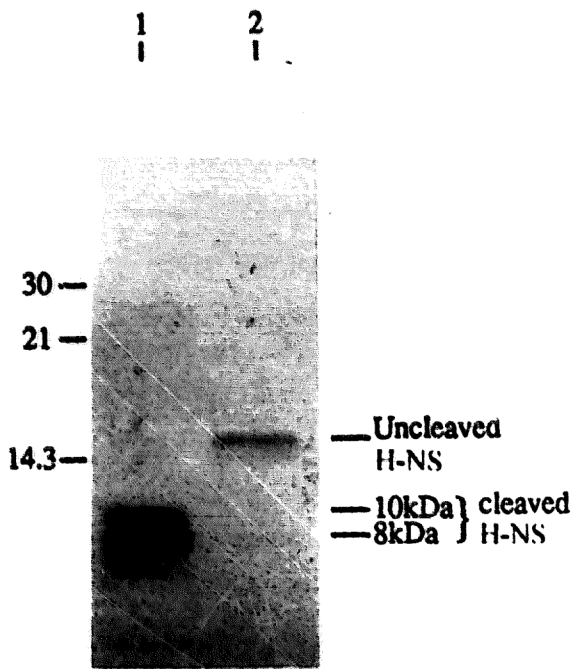


Fig 2. *In vitro* cleavage of purified H-NS. Purified H-NS incubated with everted inner membrane vesicles (lane 1); purified H-NS (lane 2). Samples were analysed following boiling (2 min) in SDS-sample buffer.

of protease inhibitors (including PMSF, EGTA, TLCK, chymostatin and elastinal) prevented cleavage of H-NS *in vitro*. However, the serine protease inhibitor benzamidine hydrochloride at a concentration of 4 mM did inhibit cleavage *in vitro* (data not shown).

Expression of the gene encoding H-NS in minicells

The possibility of cleavage of H-NS in near-physiological conditions was investigated. A minicell-producing strain, DS410, was transformed with the plasmid pLG701 which carries the *hns* gene (Goldberg *et al.* in preparation). ³⁵S-methionine labelling of minicells (see *Materials and methods*) containing the plasmid was carried out and the minicells then immediately boiled in sample buffer and analysed by SDS-PAGE. This again revealed the 15.5 kDa form of H-NS and a major band corresponding to a form of approximately 10 kDa (fig 3).

Apparent cleavage of H-NS in vivo

To extend the investigation of H-NS cleavage to *in vivo* conditions, cultures of laboratory strains of *E. coli* grown at 30°C were sampled in early exponential phase taking care to preserve possible proteolytic fragments. In order to enrich for H-NS in the extracts, advantage was taken of the known heat-resistance of H-NS. Heat stable proteins from

cell extracts were therefore prepared prior to final boiling in sample buffer (*Materials and methods*). Immunoblots were then performed on these supernatants using anti-H-NS monoclonal antibodies. These showed that H-NS is present in extracts as both a 15.5 kDa form and a set of heat-stable polypeptides around 8 kDa (fig 4); identical results were obtained with six laboratory strains. In most experiments, however, the lower *M_r* cleavage products were not visible when samples were prepared by more conventional methods involving either TCA precipitation of cells or direct addition of SDS-PAGE loading buffer to cell pellets (data not shown). This was the first indication that the method of sample preparation from whole cells (unlike minicells) might influence the level of cleavage of H-NS observed.

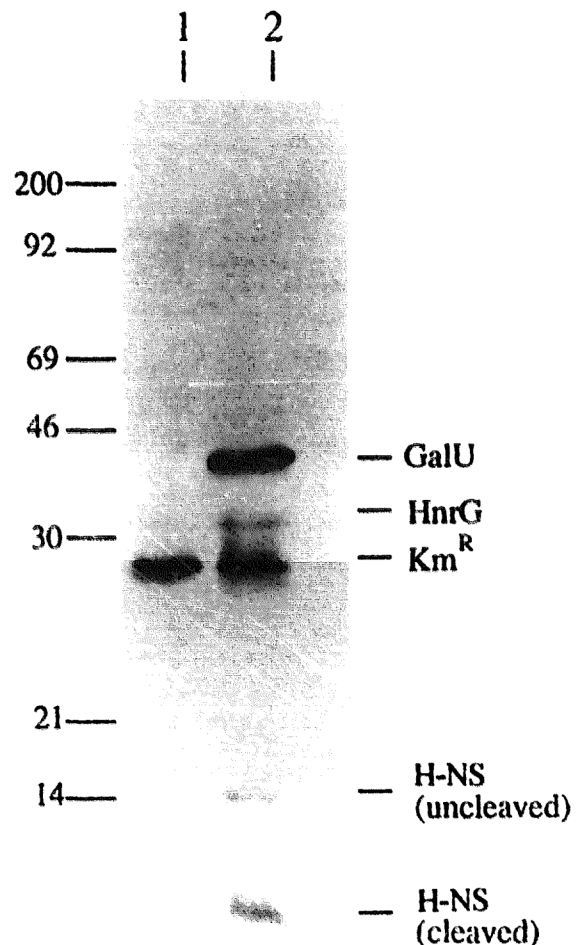


Fig 3. Expression of the gene encoding H-NS in minicells. Minicells containing plasmids pLG339 (lane 1) and pLG701 (lane 2) were labelled with ³⁵S-methionine for 60 min and samples immediately boiled in sample buffer and analysed by SDS-PAGE and autoradiography.

Importantly, efficient detection of the cleaved forms was also dependent on immunoblot conditions since the cleaved forms, which are of low M_r , pass readily through nitrocellulose (D Laoudj, C Anderson and J Canvin, unpublished) and the modified blotting conditions (see *Materials and methods*) were necessary to detect reproducibly the cleavage products. The exigencies of extraction and/or blotting may be responsible for the fragments of H-NS usually escaping detection [13] and this failure may lead to a serious underestimation of the level of H-NS in cell extracts analysed by immunoblotting. Since levels of H-NS present in cells have been reported to vary widely with the growth phase [12], it was also important to measure the production of cleavage products with respect to growth phase. The results of such an experiment are shown in figure 4 where it is clear that H-NS was only recovered intact from exponentially growing cells.

Cleavage of H-NS by OmpT

One unwelcome possibility was that the observed cleavage of H-NS was an artefact of preparation of samples. A membrane-associated protease might, for example, cleave H-NS only during particular conditions of extraction or growth phase. The outer membrane protein, OmpT, is one such candidate [34]. An *ompT* strain was therefore constructed by

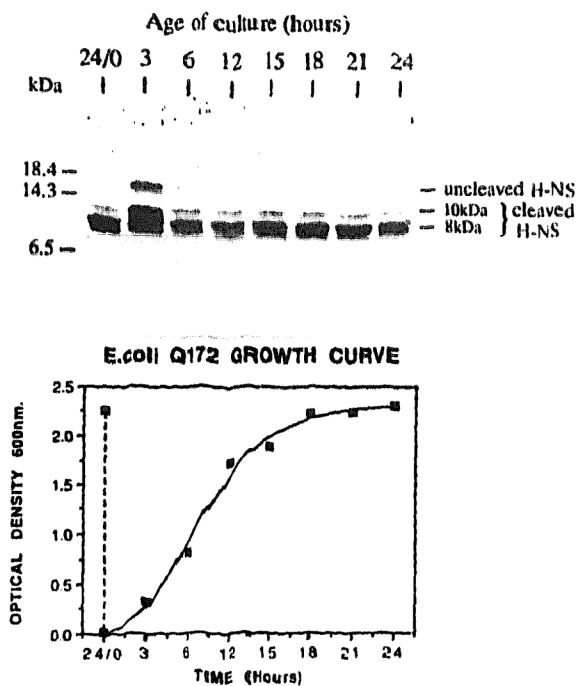


Fig 4. Changes in H-NS cleavage associated with growth phase. *E. coli* Q172 was grown at 30°C with samples taken at 0, 3, 6, 12, 15, 18, 21 and 24 h and H-NS detected with anti-H-NS polyclonal serum as described in *Materials and methods*.

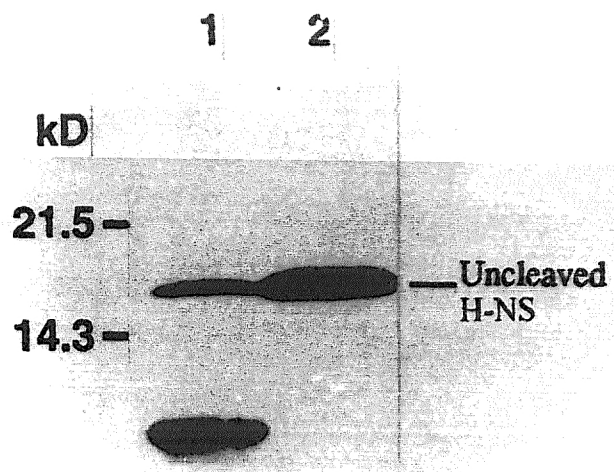


Fig 5. Dependence of H-NS cleavage on OmpT. Immunoblot analysis with anti-H-NS polyclonal antibodies of *E. coli* N43 (lane 1) and an isogenic *ompT* strain (lane 2) using anti-H-NS polyclonal serum as described in *Materials and methods*.

P1 transduction and heat-stable supernatants prepared from exponentially growing cultures of this strain and its isogenic parent, N43 (see *Materials and methods*). Immunoblots using anti-H-NS polyclonal antibodies showed that H-NS was cleaved in extracts from the parental strain but not from the *ompT* mutant (fig 5). Moreover, the quantity of intact H-NS was increased in extracts from the *ompT* mutant (fig 5) strongly suggesting that OmpT is responsible for the observed cleavage of H-NS and that levels of H-NS in cells will be underestimated unless cleavage is taken into account.

The site of *ompT* cleavage

H-NS contains four major predicted OmpT cleavage sites (adjacent basic residues, Arg, Lys), highly conserved in both *S. typhimurium* and *E. coli* (see fig 6). When a truncated version of H-NS was employed, arising from a *Tn10* insert at a position equivalent to approximately 11.5 kDa (P Moreno, personal communication), the size of the major OmpT cleavage fragment was identical to that generated from wild type H-NS (fig 6). This indicated that the major cleavage site was towards the C-terminus and probably at the position equivalent to 10.5 kDa. In order to confirm this, H-NS was purified from *S. typhimurium* LT2 (*Materials and methods*) and incubated with a preparation of outer membranes. Thus, incubation of intact H-NS with an outer membrane preparation from an *ompT*⁺ strain resulted in the cleavage of the H-NS protein into three fragments, two closely migrating proteins of 10 kDa and about 9 kDa and a smaller peptide of 4 kDa (fig 7). All fragments cross-reacted with polyclonal antisera to H-NS (data not shown). N-terminal sequence analysis of the two larger fragments

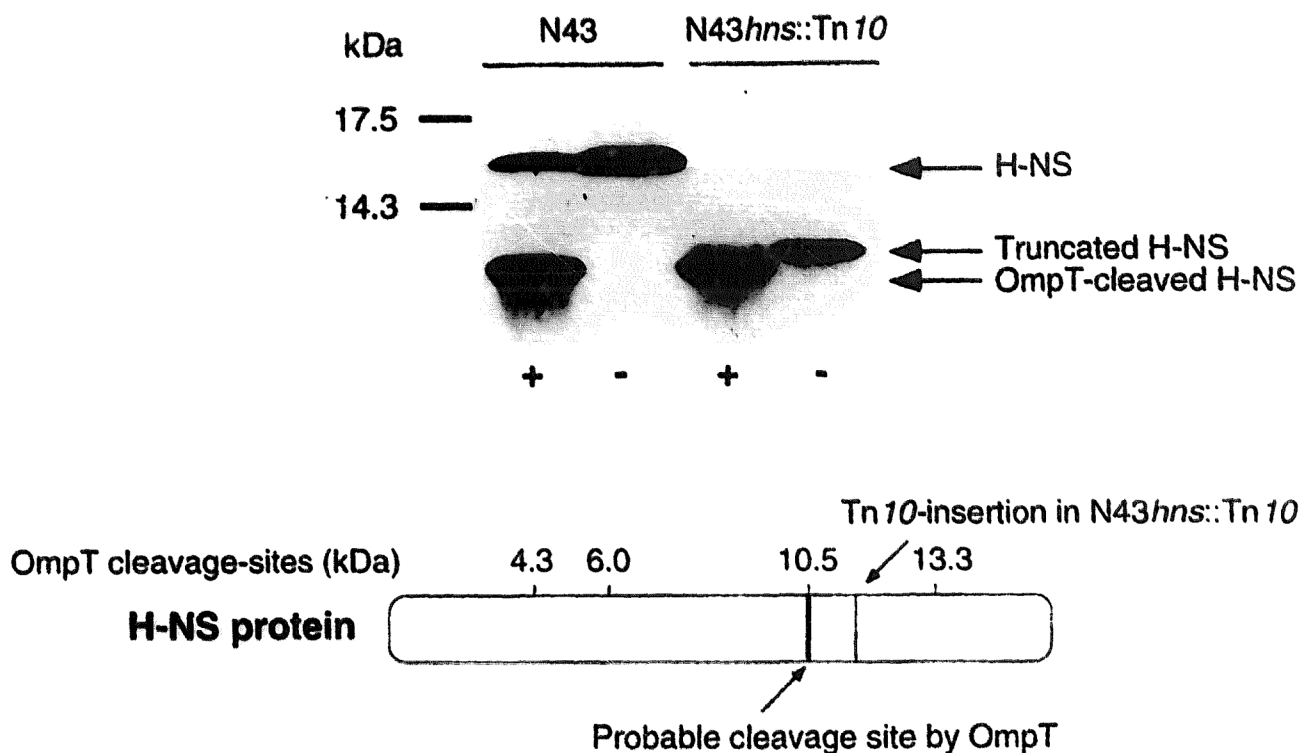


Fig 6. Predicted cleavage sites in H-NS for OmpT protease and analysis of H-NS cleavage products by Western blotting. Top panel, cell samples prepared from strain N43 or its derivatives as described in *Materials and methods*, were analysed by SDS-PAGE and Western blotting. N43 *verA* is deleted for *hns* (Goldberg *et al.* in preparation). Lower panel, the position with respect to kDa, measured from the N-terminal, of predicted OmpT cleavage sites in the H-NS molecule.

revealed identical sequences of SEALKI, which correspond to amino acids 1–6 of the N-terminal portion of the purified H-NS protein. The slight differences in the molecular mass of these two fragments may be the result of an additional cleavage by enzymes other than OmpT. These results provided further evidence that OmpT cleaves preferentially at a C-terminal site. Finally, sequencing of the smallest H-NS fragment yielded RAARPA, a sequence which begins at position 89 in the C-terminus of the intact protein and which is preceded at position 88 by a basic lysine residue. Thus, we conclude that under our conditions OmpT primarily cleaves H-NS between residues at positions 88–89 of the protein (fig 6).

Discussion

H-NS has been proposed to play a major role in signal transduction pathways involving virulence, pH and osmotic regulation in *E coli* and other bacteria. Hence, modification of H-NS by a membrane-bound protease is a potentially important regulatory mechanism that could couple the activity of H-NS to environmental or other signals *via*, for

example, changes in membrane composition. H-NS cleavage is readily detected in crude cell extracts or specifically *in vitro* following addition of membrane vesicles. However, our analysis provides strong evidence that the observed H-NS cleavage is the result of the action of the outer membrane protein, OmpT [35], with cleavage occurring *in vitro* during sample preparation. The efficiency of these cleavage reactions varied and was particularly favoured, for example, by boiling the cell extracts (to isolate the heat-resistant fraction that includes H-NS) before addition of SDS sample buffer. The exact conditions of immunoblotting constituted a major factor in the detection of cleavage products since the small fragments passed readily through nitrocellulose filters during transfer. This is important since, for example, it is generally assumed that transposon disruptions of *hns* completely eliminate the entire protein. As shown in figure 6 we detected large quantities of the N-terminal fragment of H-NS encoded by an *hns* gene disrupted by a transposon insertion using the modified immunoblotting conditions described in this report. Whilst this manuscript was in preparation, Dersch *et al* [36] described similar results. This effect, together with the likelihood of OmpT cleavage occurring under the standard conditions of extraction de-

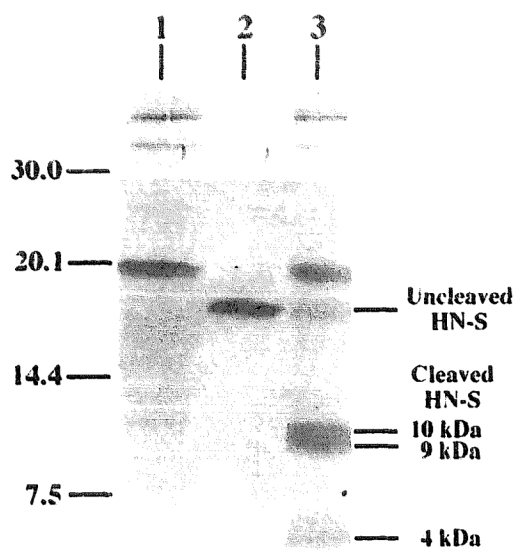


Fig 7. Protein blot showing H-NS cleavage following incubation with outer membrane proteins containing OmpT; lane 1, 15 mg of protein; lane 2, uncleaved H-NS control (10 mg of H-NS); lane 3, H-NS cleavage fragments (25 mg H-NS).

scribed here, may be responsible for serious underestimations of H-NS levels in cells using immunoblotting.

OmpT cleaves proteins between a pair of positively charged amino acids [37] which occur at residues 40, 56, 89 and 120 of the H-NS protein. Cleavage at these sites will yield a variety of fragments of which some may be useful in structure-function studies of H-NS. In fact, under our conditions cleavage occurs primarily at position 89 to yield a 10.5 kDa fragment and a small C-terminal fragment which previous studies have indicated is implicated in DNA-binding [38]. Indeed, a fragment of a very similar size is released by trypsin treatment of H-NS [39], supporting the idea that the H-NS molecule forms at least two separate structural domains. Our results also provide further information about the regulation of the *ompT* gene and/or its product [35]. Marked differences in the expression of *ompT* or the activity of OmpT in exponential and stationary phase cells would be consistent with our finding that intact H-NS could only be recovered from heat-stable extracts of laboratory strains in exponential phase. Moreover, differences in OmpT regulation between clinical isolates, where we never detected cleavage of H-NS (J Canvin and V Norris, unpublished data), and laboratory strains would be consistent with our finding that only intact H-NS was recovered from the seven clinical isolates examined. In summary, the simplest explanation for our results is artefactual cleavage of H-NS by heat resistant OmpT in certain conditions of sample preparation. Such cleavage by OmpT of a number of proteins —

including cytoplasmic ones — has indeed been reported [17–19].

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References

- Higgins CF, Hinton JCD, Hulton CSJ, Owen-Hughes T, Pavitt GD, Seirafi A (1990) Protein H1: a role for chromatin structure in the regulation of bacterial gene expression and virulence? *Mol Microbiol* 4, 2007–2012
- Hulton CSJ, Seirafi A, Hinton JCD, Sidebotham JM, Waddell L, Pavitt GD, Owen-Hughes T, Spassky A, Buc H, Higgins CF (1990) Histone-like protein H1 (H-NS), DNA supercoiling, and gene expression in bacteria. *Cell* 63, 631–642
- Ramirez RM, Villarejo M (1991) Osmotic signal transduction to *proU* is independent of DNA supercoiling in *Escherichia coli*. *J Bacteriol* 173, 879–885
- Rimsky S, Spassky A (1990) Sequence determinants for H1 binding on *Escherichia coli lac* and *gal* promoters. *Biochemistry* 29, 3765–3771
- Yamada H, Muramatsu S, Mizuno T (1990) An *Escherichia coli* protein that preferentially binds to sharply curved DNA. *J Biochem* 108, 420–425
- Tanaka KI, Muramatsu S, Yamada H, Mizuno T (1991) Systematic characterization of curved DNA segments randomly cloned from *Escherichia coli* and their functional significance. *Mol Gen Genet* 226, 367–376
- Lucht JM, Dersch P, Kempf B, Bremer E (1994) Interactions of the nucleoid-associated DNA-binding protein H-NS with the regulatory region of the osmotically controlled *proU* operon of *Escherichia coli*. *J Biol Chem* 269, 6578–6586
- Tupper AE, Owen-Hughes TA, Ussery DW, Santos DS, Ferguson DJP, Hulton CSJ, Sidebotham JM, Hinton JCD, Higgins CF (1994) The chromatin-associated protein H-NS alters DNA topology *in vitro*. *EMBO J* 13, 258–268
- Ueguchi C, Mizuno T (1993) The *Escherichia coli* nucleoid protein H-NS functions directly as a transcriptional repressor. *EMBO J* 12, 1039–1046
- Ueguchi C, Kakeda M, Mizuno T (1993) Autoregulatory expression of the *Escherichia coli hns* gene encoding a nucleoid protein: H-NS functions as a repressor of its own expression. *Mol Gen Genet* 236, 171–178
- Cukier-Kahn R, Jacquet M, Gros F (1972) Two heat-resistant, low molecular weight proteins from *Escherichia coli* that stimulate DNA-directed RNA synthesis. *Proc Natl Acad Sci USA* 69, 3643–3647
- Spassky A, Rimsky S, Garreau H, Buc H (1984) Hla, an *E. coli* DNA binding protein which accumulates in stationary phase, strongly compacts DNA *in vitro*. *Nucleic Acids Res* 12, 5321–5340

- 13 Durrenberger M, La Teana A, Citro G, Venanzi F, Gualerzi CO, Pon CL (1991) *Escherichia coli* DNA-binding protein H-NS is localized in the nucleoid. *Res Microbiol* 142, 373-380
- 14 Dorman CJ (1991) DNA supercoiling and environmental regulation of gene expression in pathogenic bacteria. *Infect Immun* 59, 745-749
- 15 Gottesman S, Maurizi MR (1992) Regulation by proteolysis: energy-dependent proteases and their targets. *Microbiol Rev* 56, 592-621
- 16 Kaidow A, Wachi M, Nakamura J, Magae J, Nagai K (1995) Anucleate cell production by *Escherichia coli* Dhns mutant lacking a histone-like protein, H-NS. *J Bacteriol* 177, 3589-3592
- 17 Akiyama Y, Ito K (1990) SecY protein, a membrane-embedded secretion factor of *E coli*, is cleaved by the OmpT protease *in vitro*. *Biochem Biophys Res Commun* 167, 711-715
- 18 Baneyx F, Georgiou G (1990) *In vivo* degradation of secreted fusion proteins by the *Escherichia coli* outer membrane protease OmpT. *J Bacteriol* 172, 491-494
- 19 Henderson TA, Dombrosky PM, Young KE (1994) Artefactual processing of penicillin-binding proteins 7 and 1b by the OmpT protease of *Escherichia coli*. *J Bacteriol* 176, 256-259
- 20 Casadaban MJ (1976) Transposition and fusion of the *lac* genes to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu. *J Mol Biol* 104, 514-555
- 21 D'Ari R, Jaffe-Brachet A, Touati-Schwartz D, Yarmolinski MB (1975) A *dnaB* analog specified by bacteriophage P1. *J Mol Biol* 94, 341-366
- 22 Dougan G, Sherrat D (1977) The transposon Tn1 as a probe for studying ColE1 structure and function. *Mol Gen Genet* 151, 151-160
- 23 Nakamura H, Sugunuma A (1972) Membrane mutation associated with sensitivity to acriflavine in *Escherichia coli*. *J Bacteriol* 110, 329-335
- 24 Stoker NG, Fairweather NF, Spratt BG (1982) Versatile low-copy number plasmid vectors for cloning in *Escherichia coli*. *Gene* 18, 335-341
- 25 Towbin H, Gordon J (1984) Review article. Immunoblotting and dot immunoblotting. *J Immunol Methods* 72, 313-340
- 26 Nakane PK, Pierce GB (1966) Enzyme-labelled antibodies: preparation and application for the localization of antigens. *J Histochem Cytochem* 14, 929-931
- 27 Mason TC, Phifer RF, Spicer SS, Swallow RA, Dreskin RB (1969) An immunoglobulin-bridge method for localizing tissue antigens. *J Histochem Cytochem* 17, 563-569
- 28 Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685
- 29 Pratt JM (1984) In: *Transcription and Translation - a Practical Approach* (Hames BD, Higgins SJ, eds) IRL Press, Oxford, 179-209
- 30 Stoker NG, Pratt JM, Holland IB (1984) In: *Transcription and Translation - a practical approach* (Hames BD, Higgins SJ, eds) IRL Press, Oxford, 153-177
- 31 Laoudj D, Andersen CL, Bras A, Goldberg M, Jacq A, Holland IB (1994) EGTA induces the synthesis in *Escherichia coli* of 3 proteins that cross-react with calmodulin antibodies. *Mol Microbiol* 13, 445-457
- 32 Schagger H, von Jagow G (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem* 166, 368-379
- 33 Zubay G (1973) *In vitro* synthesis of protein in microbial systems. *Annu Rev Genet* 7, 267-298
- 34 White CB, Chen Q, Kenyon GL, Babbitt PC (1995) A novel activity of OmpT proteolysis under extreme denaturing conditions *J Biol Chem* 270, 12990-12994
- 35 Kaufmann A, Stierhof Y-D, Henning U (1994) New outer membrane protease of *Escherichia coli* K-12. *J Bacteriol* 176, 359-367
- 36 Dersch P, Kneip S, Bremer E (1994) DNA-binding protein H-NS is required for the efficient adaptation of *E coli* K-12 to a cold environment. *Mol Gen Genet* 245, 255-259
- 37 Baneyx F, Georgiou G (1992) Expression of proteolytically sensitive polypeptides in *Escherichia coli*. In: *Stability of protein pharmaceuticals, part A: chemical and physical pathways of protein degradation*. (Ahern TJ, Manning MC, eds) Plenum Press, New York
- 38 Ueguchi C, Suzuki T, Yoshida T, Tanaka K, Mizuno T (1996) Systematic mutational analysis revealing the functional domain organization of *E coli* nucleoid protein H-NS. *J Mol Biol* 263, 149-162
- 39 Shindo H, Iwaki T, Ieda R, Kurumizaka H, Ueguchi C, Mizuno T, Morikawa S, Nakamura H, Kuboniwa H (1995) Solution structure of the DNA binding domain of a nucleoid-associated protein, H-NS, from *E coli*. *FEBS Lett* 360, 125-131