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Snap Denaturation reveals dimerization by AraC-like protein Rns

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ABSTRACT

Here we show that the Rns regulator of *Escherichia coli* dimerises *in vivo* and *in vitro*. Furthermore, we demonstrate that Rns forms aggegrates *in vitro* and describe a methodology to ameliorate aggregation thus permitting the analysis of Rns by cross-linking.

KEYWORDS

Escherichia coli, virulence regulator, dimerization, DNA-binding protein, AraC, fimbriae

1. INTRODUCTION

The Rns protein of Enterotoxigenic *Escherichia coli* positively regulates the expression of CS1 fimbriae, which are required for host cell adhesion. Rns is a member of the AraC-like family of regulators [1, 2], a defining feature of which is a 100 amino acid region of homology that contains two predicted helix-turn-helix (HTH) motifs [1]. Insolubility is also a characteristic of these proteins, therefore only a fraction have been experimentally characterised. [1].

AraC-like proteins are functionally active as monomers or dimers. Family members MarA and Rob are monomers while several others that are involved in regulating the metabolism of sugars are active as dimers [2]. The AraC protein itself functions as a dimer in solution and binds to DNA as a dimer [3].

It is not yet known whether the AraC family members that regulate virulence act primarily as monomers or oligomers [2]. Some, such as ToxT [7] and RegA [8] have been found to dimerise, while a recent report suggested that it was not

possible to detect dimerization of the Rns N-terminal domain *in vivo* or of full-length Rns *in vitro* [6]. Amongst the Rns-related regulators it has been suggested that VirF of *S. flexneri* is a dimer [4] and PerA is a monomer [5]. However, aside from these examples, progress in the biochemical analysis of AraC-like virulence regulators has been hampered due to their insolubility and instability *in vitro*.

Here we show that Rns can dimerise *in vivo* and *in vitro.* Furthermore we show that Rns is prone to aggregation. In response to this we have defined the appropriate conditions to examine the protein:protein interactions of Rns *in vitro.*

2. MATERIALS AND METHODS

2.1 *In vitro* cross-linking. *In vitro* cross-linking was carried out with purified MBP-fusion proteins. The zero-length chemical cross-linker 1-ethyl-3-(3′-dimethylaminopropyl) carbodiimide (EDC) and the catalyst *N*-hydroxy-succinimide (NHS) were added to 1.25 μg of protein. The reaction volume was made up to 20 μl with 7 mM MES buffer (pH 6). The final concentrations of EDC and NHS were 50 mM and 200 mM, respectively [9]. The reaction mixture was incubated at room temperature for 60 min. The reaction was stopped by the addition of an equal volume of Laemmli buffer. The samples were heated at 100°C for 5 min prior to Western immunoblotting.

2.2 *In vitro* cross-linking and snap denaturation. Alternatively, and to prevent protein aggregation, cross-linking reactions were stopped by treating the samples with urea using a modification of the method of Soulié *et al*. [10, 11]. Briefly, a master mix was prepared in which 15 μl (per sample) of a mixture containing 6.7% (w/v) SDS and 4.6 M β-mercaptoethanol was added to 12.5 mg of crystalline ultra-pure urea. The mixture was then vortexed for 2 min. 22.5 μl of this master mix was added to each cross-linking reaction and the samples were heated at 100°C for 70 s. Crucially, the entire process from dissolving the urea until loading the gel was <10 min duration.

2.3 Gel filtration chromatography. MBP-Rns was passed through a Superdex 200 10/30 column and eluted with PBS at a flow rate of 0.4 ml/min. The protein content of each fraction was measured at 280_{nm}. The column was calibrated with six high and low molecular weight standards ranging from 29 to 669 kDa to

obtain a calibration graph for determination of the molecular weight of the eluted proteins. Eluted fractions were analysed by immunoblotting with an anti-Rns sera.

2.4 Construction of a LexA-Rns fusion. The *rns* ORF was amplified by PCR from plasmid pSS2192 [12]. The PCR product was digested and inserted into pSR660 [13] resulting in the plasmid pRns660. Repression of a chromosomal *sulA*::*lacZ* fusion was measured in *E. coli* SU101 by β-galactosidase assay using the method of Miller [14].

3. RESULTS

3.1 Cross-linking of MBP-Rns *in vitro.* The Rns protein has previously been expressed as a maltose-binding protein (MBP) fusion, which is functional both *in vivo* and *in vitro* [12]. MBP is a monomer [15], and was previously shown not to interfere with cross-linking of an MBP fusion of XylS, an AraC family member [16]. MBP-Rns protein was used in NHS-catalysed EDC cross-linking reactions *in vitro*. The purified proteins MBP-paramyosinΔSal and MBP (NEB) were included as positive and negative oligomerization controls, respectively. After cross-linking, one set of protein samples was denatured using the standard method of heating at 100°C for 5 min in the presence of Laemmli buffer. Another set of samples was denatured using a modified urea-based method which was demonstrated to reduce protein aggregation [10, 11]. When the samples were denatured by heating in Laemmli buffer, cross-linking was found to result in a reduced amount of the MBP monomer, the detection of an oligomeric form of MBP-paramyosinΔSal and a lack of detection of any form of MBP-Rns (Fig. 1 A). Using the urea-based denaturation procedure, however, detection of each of the proteins after cross-linking was much improved (Fig. 1 B). Under these conditions the EDC-NHS treatment was found to have no effect on monomeric MBP but to result in the appearance of bands corresponding to oligomers of MBP-paramyosinΔSal. For cross-linked samples of MBP-Rns, in addition to the band corresponding to monomeric protein, a species migrating with an apparent molecular weight more than two-fold greater than that of the monomer was present. This species was reliably detected after EDC-NHS cross-linking of MBP-

Rns and is likely to be a dimer of the fusion protein. Therefore these cross-linking studies revealed that MBP-Rns is capable of dimerizing.

Further support for this notion was gained by gel filtration studies of affinity-purified MBP-Rns. When this preparation was subjected to gel filtration MBP-Rns eluted as a single peak with a molecular mass of 134 kDa (confirmed by immunoblotting of fractions with anti-Rns antiserum) (results not shown). This does not correlate exactly with the molecular mass predicted for a dimer of MBP-Rns (~146 kDa). However, as only perfectly globular proteins migrate precisely according to size during gel filtration it was still likely that this represented a dimeric form of MBP-Rns.

3.2 A LexA-based genetic system indicates that Rns dimerises *in vivo*

The LexA DNA-binding domain (DBD) alone can recognise the *sulA* operator, but the repressor is functional only as a dimer [13]. Thus the ability of a protein to dimerise can be evaluated by determining whether a fusion of the protein and the LexA DBD is capable of repressing transcription of a chromosomal *sulA*::*lacZ* fusion in the *E. coli* reporter strain SU101. The vector pSR660, which encodes the DBD of LexA alone, was used to construct pRns660, a plasmid encoding a fusion of Rns and the LexA DBD.

Proteins corresponding to the LexA DBD alone and the LexA DBD-Rns fusion were detected in induced cultures of *E. coli*/pSR660 and *E. coli*/pRns660 respectively (Fig. 2A), thus confirming that the fusion protein was expressed. Furthermore, this protein fusion retained the transcriptional activation characteristics of Rns, as introduction of pRns660 into *E. coli* bearing a CS1

promoter-*gfp* fusion (pCoo-GFP) [12] resulted in activation of that fusion (result not shown).

As the LexA DBD-Rns fusion had been shown to be both expressed and active, it was possible to assess whether it formed homodimers. The p660AraC plasmid encodes a LexA fusion to the known homodimer AraC and therefore serves as a positive control. The reporter strain SU101 constitutively transcribes *lacZ* from the strong *sulA* promoter. The LexA DBD-AraC fusion and the LexA DBD-Rns fusion were found to repress β-galactosidase activity by 97%, and 67% with strong statistical significance respectively, compared to *E. coli* SU101 with the LexA DBD alone (Fig. 2B). Therefore, this implies that the Rns protein homodimerises *in vivo*.

4. DISCUSSION

4.2 Rns dimerises *in vitro*

MBP-Rns was subjected to NHS-catalysed EDC cross-linking reactions after which it was denatured using a urea-based treatment and then analysed by immunoblotting. Under these conditions protein complexes corresponding in size to dimers of MBP-Rns were consistently detected. This differs from the findings of Basturea *et al*. who reported that MBP-Rns dimers were not observed following SDS-PAGE analysis of denatured samples of MBP-Rns that had been cross-linked with glutaraldehyde [6]. However, as the work performed herein illustrates, the denaturation method is significant as it can affect the results of cross-linking. Basturea *et al*. do state that the mobility of MBP-Rns was reduced

following cross-linking. Indeed the amount of monomeric MBP-Rns present decreased significantly as the concentration of cross-linker increased [6]. Intriguingly, glutaraldehyde cross-linking of Rns resulted in the presence of large complexes that did not enter the gel suggesting aggregation of Rns protein [6].

Aggregation is a general characteristic of many AraC-like proteins. AraC itself, RhaS, ToxT and XylS have been reported to aggregate and this has impeded the analysis of these proteins [17-20]. There has been conjecture that AraC may be incompletely folded prior to binding to DNA; thus the exposure of an excessive number of hydrophobic might explain its propensity to aggregate [17]. The tendency of Rns to aggregate appeared to increase when the protein was heated in the presence of Laemmli buffer, the standard method of denaturation used prior to SDS-PAGE. Thermal denaturation can cause proteins to unfold and expose buried hydrophobic patches. Aggregation then occurs as a consequence of intermolecular interactions between these hydrophobic protein surfaces [21]. Hydrophobic interactions are abolished in the presence of high concentrations of urea. Therefore, snap-denaturation of MBP-Rns enabled the observation of protein cross-linking in the absence of aggregation.

4.2 Rns dimerises *in vivo.*

The finding that transcription from the *sulA* promoter was repressed by a fusion of the LexA DBD to full length Rns inferred that Rns is capable of dimerising *in vivo*. This LexA-based gene fusion system has previously been used to assess the dimerization of several AraC family members [5, 16, 18, 22, 23]. It was recently reported that dimerization of Rns could not be demonstrated

using an alternative gene fusion system based on the λ phage repressor, cI [6]. In that report, a fusion of the N-terminal 154 amino acids (of 265 amino acids) was used and found to be unable to restore repressor function to the cI DBD [6]. The findings that a fusion of full length Rns to the LexA DBD is functional, and therefore a dimer, while a fusion of Rns (1-154) to the cI DBD is not, suggests that the dimerization domain of Rns reside within residues 155-265.

4.3 Conclusion.

Here we show that Rns is capable of forming dimers *in vitro* and *in vivo.* The use of cross-linking followed by snap denaturation may be useful in the analysis of other AraC-like proteins that have been heretofore intractable to this procedure.

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Fig. 1

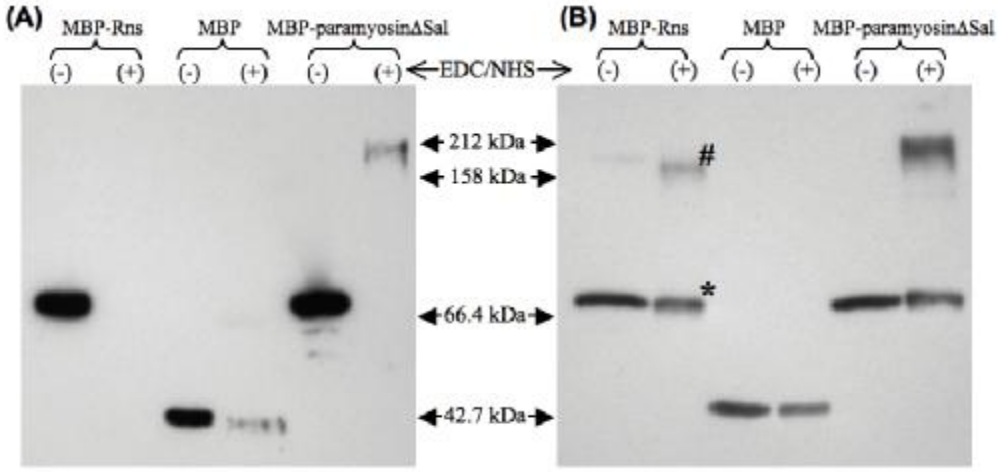
(A)
MBP-Rns
MBP
MBP-paramyosinΔSal
(-) (+) (-) (+) (-) (+)
EDC/NHS
212 kDa
158 kDa
66.4 kDa
42.7 kDa
(B)
MBP-Rns
MBP
MBP-paramyosinΔSal
(-) (+) (-) (+) (-) (+)
#
*

Fig. 2

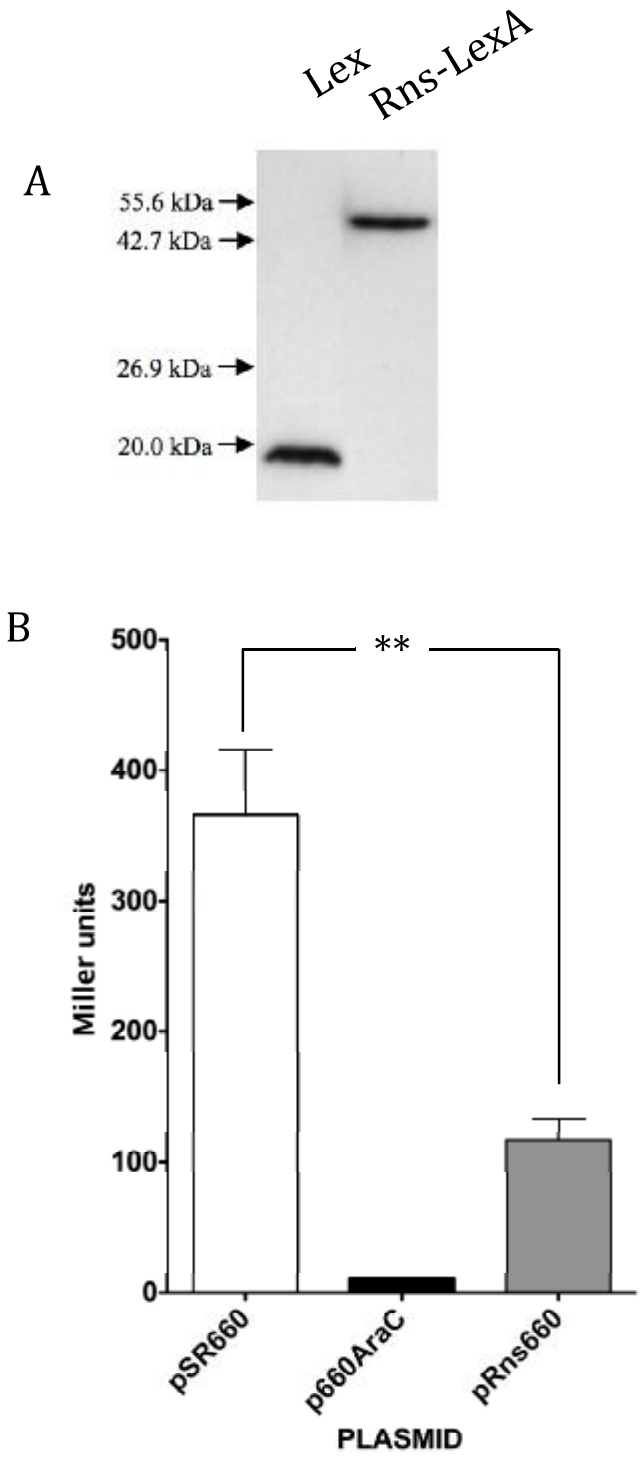
A
Lex
Rns-LexA
55.6 kDa
42.7 kDa
26.9 kDa
20.0 kDa
B
**
500
400
300
200
100
0
Miller units
pSR660
p660AraC
pRns660
PLASMID

FIGURE LEGENDS

Fig. 1 Cross-linking of MBP fusions *in vitro*. Western immunoblot analysis (using anti-MBP antiserum) of MBP or MBP fusions, as indicated at the top of each panel, incubated with (+) or without (-) EDC-NHS cross-linking reagents. Prior to electrophoresis the proteins were denatured either by heating at 100°C for 5 min in the presence of Laemmli buffer **(A)** or by snap denaturation **(B)** as described in section 2.2. Molecular mass markers are indicated. The positions of bands corresponding to monomeric and potentially dimeric MBP-Rns are indicated with an asterix or a hash sign, respectively

Fig. 2 Analysis of the LexA DBD-Rns fusion. (A) Western immunoblot analysis (with an anti-LexA) of lysates of induced cultures of *E. coli* pSR660 and pRns660 demonstrating that the LexA DBD and LexA DBD-Rns fusion (respectively) are expressed. **(B)** Regulation of the *sulA* promoter by LexA DBD or LexA DBD fusions**.** β-galactosidase activities of *E. coli* SU101 carrying LexA, LexA DBD-AraC) or LexA DBD-Rns were measured. Measurements were performed independently three times; a representative data set is shown. Statistical significance is indicated by ******, where $P < 0.005$.

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