Biochimie 94 (2012) 2058–2061

Contents lists available at SciVerse ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Short communication

Snap denaturation reveals dimerization by AraC-like protein Rns

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ARTICLE INFO

Article history:
Received 9 March 2012
Accepted 14 May 2012
Available online 22 May 2012

Keywords:
Escherichia coli
Virulence regulator
Dimerization
DNA-binding protein
AraC
Fimbriae

ABSTRACT

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

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 *Shigella 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

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doi:10.1016/j.biochi.2012.05.014

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. 1A). Cross-linked MBP-Rns did not enter the separating gel and formed large aggregates in the stacking gel (data not shown). Using the urea-based snap denaturation procedure, however, detection of each of the proteins after cross-linking was much improved (Fig. 1B). 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

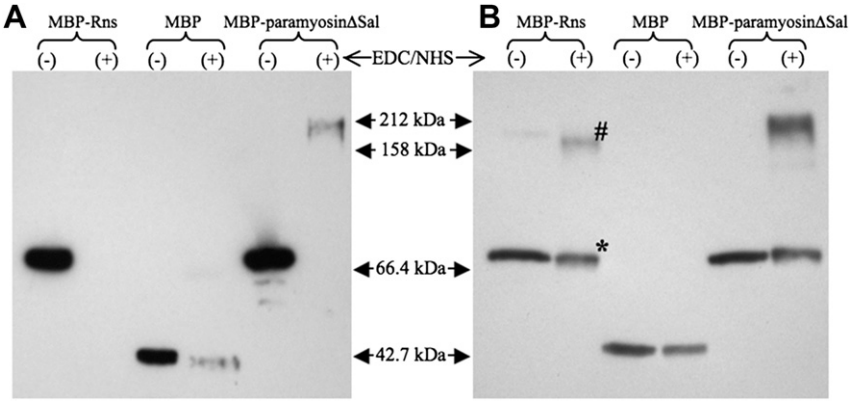

Fig. 1. Cross-linking of MBP fusions *in vitro*. Western immunoblot analysis (using anti-MBP antiserum) of MBP or MBP fusions, as indicated at the top of each panel, incubated with (+) or without (−) EDC-NHS cross-linking reagents. Prior to electrophoresis the proteins were denatured either by heating 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.