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E. coli DNA associated with isolated Hfq interacts with Hfq's distal surface and C-terminal domain

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ABSTRACT

The RNA-binding protein Hfq has been studied extensively for its function as a modulator of gene expression at the post-transcriptional level. While most Hfq studies have focused on the protein's interaction with sRNAs and mRNAs, Hfq binding to DNA has been observed but is less explored. During the isolation of Hfq from *Escherichia coli*, we found genomic DNA fragments associated with the protein after multiple steps of purification. Sequences of 41 amplified segments from the DNA fragments associated with Hfq were determined. A large fraction of the DNA segments were predicted to have significant helical axis curvature and were from genes associated with membrane proteins, characteristics unexpected for non-specific binding. Analysis by analytical ultracentrifugation indicated that rA_{18} binding to Hfq disrupts Hfq-DNA interactions. The latter observation suggests Hfq binding to DNA involves its distal surface. This was supported by a gel mobility shift assay that showed single amino acid mutations on the distal surface of Hfq inhibited Hfq binding to duplex DNA, while six of seven mutations on the proximal surface and outer circumference of the hexamer did not prevent Hfq binding. Two mutated Hfq which have portions of their C-terminal domain removed also failed to bind to DNA. The apparent K_d for binding wild type Hfq to several duplex DNA was estimated from a gel mobility shift assay to be ~400 nM.

1. Introduction

The Hfq protein of *Escherichia coli* (also known as HF-1) is an RNA-binding protein and a key factor in post-transcriptional gene regulation [1–6]. The pleiotropic phenotypic effects that results from inactivating the Hfq gene in *E. coli* [7] and other gram negative bacterial species has been linked to Hfq's role in facilitating the interaction of small regulatory RNAs (sRNAs) with messenger RNAs (mRNAs) and as a modulator of sRNA and mRNA stability. Hfq's role in regulating mRNA translation by sRNAs is an important feature of bacterial adaptation to stress and the establishment of virulence [8–12]. Many studies of Hfq have been directed towards understanding its interaction with sRNAs and mRNAs in both *in vitro* and *in vivo* contexts. However, several studies on proteins associated with the *E. coli* nucleoid DNA suggested that Hfq binding to DNA may also have a functional role [13,14].

Hfq was among the ten most prevalent proteins associated with nucleoid DNA isolated from *E. coli* [14]. In exponentially growing cells, it was the third most prevalent of the ten proteins. *In situ* immunofluorescence studies indicated that most Hfq appears to be in the cytoplasm (80–90%), however a portion of this protein was found in the DNA nucleoid region of the cell (10–20%) [13,15]. Recent electron microscopy studies have confirmed the presence of Hfq in the cytoplasm and nucleoid and demonstrated that Hfq is also localized close to the inner membrane [16]. Plasmid DNAs grown in *E. coli* were shown to bind Hfq *in vivo* and *in vitro* [17]. Apparent equilibrium dissociation constants (K_d) for Hfq binding to two 60-bp DNA duplexes were reported as 125 and 250 nM [14]. Greater affinity was exhibited for the curved DNA than for the mixed sequence DNA. Although the above range of affinities is weaker than Hfq affinity for sRNAs (K_d ~ 20–50 nM), it is tight enough to suggest that Hfq binding to DNA may play a functional role *in vivo*, particularly in light of Hfq's μM-level cellular abundance [1].

In the current work, we have characterized *E. coli* genomic DNA fragments found associated with Hfq purified from lysed cells and investigated the nature of the Hfq–DNA interaction. Several lines of evidence indicate that Hfq binding to DNA involves the protein's distal surface and C-terminal domain. The sequences of amplified segments of the genomic DNA exhibit several interesting characteristics. Over half are predicted to have helical axis curvature and are predominantly from genes coding for membrane proteins.

Abbreviations: nt, nucleotides; sRNA, small RNA; bp, base pairs; PAGE, polyacrylamide gel electrophoresis; MWCO, molecular weight cut-off; FAM, 6-carboxyfluorescein

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2. Materials and methods

2.1. Purification and characterization of wt Hfq and mutant Hfq

The Impact-CN intein system (New England Biolabs) was used to produce and purify Hfq proteins as previously described [18]. The *E. coli hfq* gene was cloned into the pTYB11 plasmid to create the expression plasmid pTYB11-wt Hfq. Hfq was expressed from this plasmid in *E. coli* strain ER2566 using the recommendations of the manufacturer. Cells were lysed using a French press in 0.5 M NaCl, 20 mM Tris (pH 8.3), 0.1 mM EDTA, 0.1% Triton X100, and 5% glycerol. The cell lysate was centrifuged and the supernatant loaded onto a chitin column. The column was extensively washed (15 to 20 bed volumes) with the wash buffer that consisted of 20 mM Tris (pH 8.3) and 0.5 or 1.0 M NaCl with or without 0.1% Triton X100 (all variations gave similar outcomes). The column was then incubated with 0.5 M NaCl and 20 mM Tris buffer plus 40 mM dithiothreitol. Eluted protein was concentrated and buffer-exchanged to 0.5 M NaCl and 20 mM Tris at pH 8.3 using 30 kD MWCO centrifugation filtration units. The protein preparation at this stage is referred to as Hfq-NA. Hfq was further purified by either a DEAE column or more commonly by a nuclease treatment to remove 250–260 nm absorbing material.

The nuclease treatment of Hfq-NA preparations was carried out by adding 7.5 U of micrococcal nuclease (Worthington Biochemical Corporation) to 1 ml of 0.2–0.4 OD_{274nm} units of the protein sample in a solvent of 0.2 M NaCl, 20 mM Tris (pH 8.3) and 5 mM $CaCl_2$. We note that micrococcal nuclease activity is absolutely dependent on Ca^{2+}. Reactions were incubated at 37 °C for 1 h and terminated by adding 10 µl of 0.5 M Na_2EDTA. Reactions were then extensively buffer exchanged with 0.5 M NaCl and 20 mM Tris (pH 8.3) and their volumes reduced to ~1 ml using a 15 ml 30 kD centrifugation filter. This approach was more consistent than a DEAE column in giving a high A_{275}/A_{250} absorbance ratio (Fig. 1A).

Plasmids containing mutant *hfq* genes were generated from pTYB11-wtHfq using the QuikChange Mutagenesis Kit from Stratagene Inc [19]. In addition to the previously described mutations F42A, F39A, Q8A, R16A, K31A, and Y25A [18], *hfq* genes with single residue mutations R19A, R17A, and F11A were constructed and their proteins expressed. Two additional mutant *hfq* genes were constructed by creating stop codons at residues 76 and 66, respectively. These plasmids yielded truncated Hfq designated Hfq-65 and Hfq-75. The wt Hfq and mutant Hfqs were characterized for purity by SDS-PAGE and UV spectroscopy [18].

2.2. Analytical ultracentrifugation

Sedimentation velocity studies were performed using a Beckman Optima XLA analytical ultracentrifuge equipped with absorbance optics and an An60 Ti rotor at 19.7 °C. Temperature was calibrated as described previously [20]. Velocity data were typically collected at appropriate speeds using 274 nm to monitor Hfq and Hfq-NA, and 495 nm when FAM-A_{18} was added. Spacing of 0.002 cm was employed with one flash at each point in a continuous-scan mode. All experiments were initially analyzed with Sedfit to produce $c(s)$ distributions [21] and with $DCDT^{+2}$ to produce $g(s)$ distributions and weight average S values [22]. Direct boundary fitting of velocity data to discrete models were also performed with the program Sedanal [23]. Analysis with Sedanal requires input of MW, extinction coefficients, and density increments (typically estimated from 1-vbar*rho values). The buffer solution density for 0.5 M NaCl and 20 mM Tris (8.3) was estimated in Sednterp to be 1.01920 gm/ml at 19.7 °C. The vbar of Hfq was estimated with Sednterp [24] to be 0.7248. The vbar of FAM-A_{18} is assumed to be 0.55. The extinction coefficient for FAM-A_{18} at 495 nm is 75,000 M^{-1} cm^{-1}; the extinction coefficient of Hfq at 274 nm is 4350 M^{-1} cm^{-1} [18]. Parameter uncertainty is calculated with an Fstat routine within Sedanal at the 95% confidence interval and reported in a < , > format.

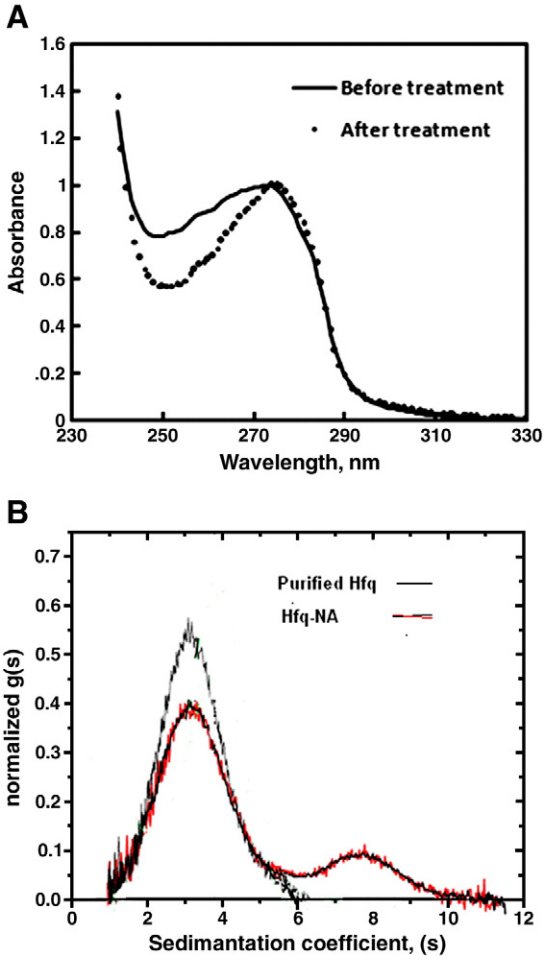

Fig. 1. A. UV spectra of Hfq-NA, prior to nuclease treatment (—), and after nuclease treatment (·····). B. Normalized g(s) distribution of sedimentation velocity runs monitored at 274 nm of purified Hfq (black line), and Hfq-NA samples (red/black line).

Sedimentation equilibrium studies were carried out on Hfq-NA at 1.75, 3.5, and 7 µM. The solvent for most studies was 0.5 M NaCl and 20 mM Tris (8.3). Employing a buffer of 0.2 M NaCl and 20 mM Tris (8.3) gave similar results. Samples were centrifuged at 12,000 rpm and 16,000 rpm at a temperature of 19.7 °C in six channel double sector cells. Data were collected at 274 nm. Equilibrium at each speed was judged with the software utility WinMATCH (http://www.biotech.uconn.edu/auf/?i=aufftp). This program makes a least-square comparison of successive scans to establish that equilibrium has been achieved. Values for density, vbar and extinction coefficients were as described above for sedimentation velocity measurements. Non-linear least squares fit of sedimentation equilibrium profiles to a model of two independent non-interacting components gave a much better fit than to a single species model (Fig. 2B). The six data sets from three concentrations and two speeds were best fit to a two species model using Sedanal. Molecular weight uncertainty is calculated with Fstat as described above.

2.3. Characterization of nucleic acid in Hfq-NA

The nucleic acid associated with Hfq in Hfq-NA was characterized by examining the aqueous phase after phenol-chloroform extraction. 30 µl of ~10 µM Hfq-NA was phenol-chloroform extracted and then analyzed on a 1% agarose gel, staining with ethidium bromide. The effect of RNase A on Hfq-NA was examined by adding 1 µg of RNase A (Promega) to 30 µl of Hfq-NA and incubating for 30 min at 37 °C prior to phenol-chloroform extraction. The influence of DNase on Hfq-NA