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Hexamer to monomer equilibrium of *E. coli* Hfq in solution and its impact on RNA annealing

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Abstract

The bacterial Sm-like protein Hfq forms a ring shaped homo-hexamer that is necessary for Hfq to bind nucleic acids and to act in sRNA regulation. Using semi-native gels and fluorescence anisotropy, we show that Hfq undergoes a cooperative conformational change from monomer to hexamer around 1 μ M protein, which is comparable to the *in vivo* concentration of Hfq and above the dissociation constant of the Hfq hexamer from many RNA substrates. Above 2 μ M protein, Hfq hexamers associate in high molecular weight complexes. Mutations that impair RNA binding to the proximal face strongly destabilize the hexamer, while the mutation R16A near the outer rim prevents hexamer association. Stopped-flow FRET experiments showed that Hfq subunits interact within a few seconds, suggesting that Hfq monomers, hexamers and multi-hexamer complexes are in dynamic equilibrium. Finally, we show that Hfq is most active in RNA annealing when the hexamer is present. These results suggest that RNA binding is coupled to hexamer assembly, and that the biochemical activity of Hfq reflects the equilibrium between different quaternary structures.

Keywords

sRNAs; Sm/Lsm proteins; RNA chaperone; fluorescence anisotropy

Introduction

Hfq is a Sm/Lsm super-family protein abundant in most Gram positive and Gram negative bacteria¹. Hfq acts in a wide range of regulatory networks, such as stationary phase stress response (σ^S), sugar metabolism, low iron and quorum sensing, by facilitating anti-sense base pairing between small non-coding RNAs (sRNAs) and their target mRNAs in bacteria^{2; 3}. Hfq also helps regulate RNA stability and decay^{4; 5; 6}.

The core of Hfq, which forms a ring-shaped homo-hexamer, is evolutionarily conserved between amino acids 7 – 66⁷. Crystal structures show that the Sm core of Hfq invariably forms an α -helix in the N-terminus and then a five-strand anti-parallel β -sheet^{8; 9; 10}. The inner ‘proximal’ face of the Sm core preferentially binds single-stranded U-rich RNA⁸, while the ‘distal’ face binds A-rich RNA using 6 A-R-N motifs^{9; 11}. In contrast to the Sm core, the amino-acid composition of the Hfq C-terminus varies considerably among

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bacteria⁷. Recent studies suggest this region is structurally disordered, and extends outward from the hexamer core¹².

Although crystallographic and NMR studies show that Hfq forms a hexamer at the high (millimolar) concentrations used for those studies, Hfq binds with U-rich or A-rich RNA oligomers with nanomolar affinity^{9; 13}. Thus, whether Hfq forms a stable hexamer at low protein concentrations in solution is important for its function in sRNA regulation. Secondly, electron microscopy and ultracentrifugation have shown that Hfq and archaeal Sm proteins form high molecular weight complexes^{14; 15}, and multi-hexamer ($\geq 12:1$) complexes are observed when excess Hfq is added to short RNAs^{16; 17}. How such multi-hexamer complexes alter the function of Hfq is also poorly understood.

In this study, we used fluorescently labeled Hfq to measure the stability and dynamics of subunit interactions in the nanomolar to micromolar concentrations typical of biochemical assays. Our results show that Hfq monomers assemble as hexamers just below 1 μM concentration, becoming very stable above this concentration. At higher concentrations, Hfq predominantly forms multimers that inhibit its RNA annealing activity. Finally, we show that certain mutations which impair RNA binding also destabilize the hexamer, raising the possibility that changes in Hfq quaternary structure contribute to the loss of function associated with these mutations.

Results and Discussion

Hfq hexamer stability by semi-native PAGE

To visualize the quaternary structure of Hfq in dilute solutions (Fig. 1a), we analyzed Cy3 or Cy5-labeled Hfq using semi-native PAGE containing 0.5% w/v SDS. SDS was added to samples just before loading, so that hexamers were not denatured. Subunits labeled with Cy3, Cy5, or a 1:1 mixture of Cy3-Hfq + Cy5-Hfq were loaded in adjacent lanes of the gel (Fig. 1b). As the total Hfq monomer concentration was raised from 0.1 to 10 μM , the proportion of hexamer increased (Fig. 1c). At low concentration (~ 100 nM monomer), about half the Hfq migrated as the monomer and half migrated as a hexamer. In high concentration (10 μM), the majority of the population migrated as a high molecular weight complex (multimer). Therefore, Hfq exists in a mixture of quaternary structures at concentrations typically used in RNA annealing assays (0.1 to 1 μM Hfq subunits) and comparable to its intracellular concentration (~ 1 μM ; ¹⁸). (We refer to the concentration of Hfq subunits throughout this report.)

When FRET between Cy3-Hfq and Cy5-Hfq subunits was measured in the gel by exciting Cy3 and recording the Cy5 emission channel, we observed a considerable FRET signal in lanes containing a mixture of labeled subunits, over the background intensity of lanes containing only Cy3-Hfq or Cy5-Hfq (Fig. 1b, right). FRET was observed in both the hexamer and multimer bands, but not the monomer band, as expected. FRET in the multimer band can be explained by association of hexamers labeled with Cy3 and Cy5. However, FRET in the hexamer band suggested that hexamers either exchange subunits or assemble from monomers during our experiment.

Hexamer stability by steady-state anisotropy

To further investigate the stability of Hfq hexamers in solution, we labeled wild type Hfq with dansyl chloride and measured the change in fluorescence anisotropy while varying the total protein concentration. This method can be used to measure protein-protein interactions because the anisotropy reflects the hydrodynamic radius of the complex.¹⁹ The specific fluorescence intensity was constant during our titrations, indicating that the local environment of the dansyl group did not change with protein concentration. Dansyl-labeled

Hfq was mixed with unlabeled Hfq in a 1:5 ratio in potassium phosphate buffer (pH 7.5) at an initial concentration 10 μ M, and then diluted with the same buffer. The decrease in steady-state anisotropy as the protein was diluted (Fig. 1d) revealed at least two conformational transitions, which we assigned to the monomer, hexamer and multi-hexamer species based on our electrophoretic results in Fig. 1 and the difference in fluorescence polarization.

The roughly similar concentration dependence of the transitions observed in solution and by semi-native PAGE suggested that 0.5% SDS in our native gels did not grossly perturb the Hfq interactions. If anything, the hexamer was observed at lower concentrations by semi-native PAGE than predicted by the anisotropy experiments. Because the subunits dissociate upon dilution, the interactions are reversible. MALDI-TOF mass spectra confirmed that the interactions involve only Hfq subunits (Fig. S1), although relatively little hexamer was detected, as previously reported.²⁰ As discussed below, anisotropy and DLS experiments on mutant subunits support the mass differences among these complexes.

The monomer to hexamer transition occurred with a midpoint of 0.8 μ M, while the transition from hexamer to multimer had a midpoint of 4.9 μ M (Fig. 1d), roughly consistent with the semi-native PAGE results in Fig. 1c. The data were simulated well by a statistical model that assumed modest interactions between monomers ($\kappa = 2 \cdot 10^5 \text{ M}^{-1}$), strong cooperativity within each hexamer and weak interactions between hexamers (Fig. S2). The strong cooperativity of hexamer assembly is consistent with previous analytical ultracentrifugation experiments, which also indicated all-or-none ($6\text{H} \leftrightarrow \text{H}_6$) behavior²¹.

Effect of point mutations on hexamer stability

We next asked whether amino acid substitutions in the RNA binding sites of Hfq affect hexamer stability. Hfq subunits containing mutations in the proximal inner ring (Q8A, Y55A, K56A), the distal face (Y25D) or the outer rim (R16A) were analyzed by semi-native SDS-PAGE (Fig. 2a). As expected, 50 μ M wild type protein migrated as a multi-hexamer complex when analyzed by semi-native SDS-PAGE (Fig. 2b). However, Hfq subunits containing mutations in the proximal U-rich RNA binding site (Q8A, Y55A, K56A) migrated only as monomers, indicating that these mutations, which lie near the subunit interface, strongly destabilize the hexamer. This was supported by the low anisotropy of dansyl chloride-labeled Hfq:K56A (blue, Fig. 1d). The importance of this region of the subunit interface was previously demonstrated by amino acid substitutions of H57 in *Pseudomonas aeruginosa* Hfq, which were also highly destabilizing²². Our results raise the possibility that the RNA recognition is impaired in proximal face mutants in part because the hexamer itself is unstable.

The Y25D mutation in the A-rich RNA binding site on the distal face of Hfq had little effect on its quaternary structure at this concentration, apart from slightly disfavoring the multimer (Fig. 2b). Surprisingly, the R16A mutation near the rim of the Sm core completely blocked the formation of multimers, although the hexamer remained stable. Consistent with this conclusion, the dansyl-labeled R16A protein reached the intermediate anisotropy of the hexamer, but did not exhibit the second transition which we attribute to the formation of multi-hexamer complexes (red, Fig. 1d). That the R16A mutation prevents hexamer association implies that these complexes are due to specific protein interactions, and not random aggregation, as suggested by a recent structure showing that an RNA can bridge two hexamers along this surface.²⁴ In the absence of RNA, the R16 mutation could perturb edge to edge interactions between hexamers seen in certain EM studies¹⁵, or alter the orientation of the C-terminal peptide. Whether interactions between Hfq hexamers have a biological function will require further study.

By dynamic light scattering (10 μ M subunits; Malvern Zetasizer Nano-ZS90), we also observed that WT Hfq produced much larger particles than Hfq:R16A, which were in turn larger than those formed by Hfq:K56A. This supports the anisotropy and semi-native gel results showing that these mutations perturb the quaternary structure of Hfq. However, the DLS results suggested the protein adopts a more extended structure ($R \approx 75$ -100 nm, 125 Å and 25 Å, respectively) than predicted from crystal structures of the hexamer core or from SAXS data on the WT protein ($R_g = 68$ -75 Å; Fig. S3). This hydrodynamic behavior has been attributed to extension of the hydrophilic C-terminal region away from the Sm core.²³

Steady state FRET measurement

The observation of FRET between Cy3- and Cy5-labeled subunits in the hexamer band (Fig. 1b) suggested that hexamers can exchange subunits. To obtain further evidence for subunit exchange in solution, we used fluorophore-labeled protein as before. First, the increase in FRET efficiency (E_{FRET}) was measured as 300 nM Cy3-Hfq in HB buffer was titrated with increasing concentrations of Cy5-Hfq (Fig. 3). To ensure that most of Hfq was in hexamers, the total Hfq concentration was maintained between 0.3 μ M and 2.5 μ M. The FRET efficiency increased almost linearly against the Cy5-Hfq: Cy3-Hfq ratio (black line, Fig. 3a), indicating that subunits either exchange between hexamers or associate to form new hexamers.

To avoid hexamer-hexamer interactions, which could also contribute to E_{FRET} , we repeated the titration in the presence of 500 nM A₁₈ RNA, which binds Hfq tightly (3 nM). Very similar results were obtained in the presence of A₁₈ RNA, except that the transition saturated at a lower maximum E_{FRET} as expected (Fig. 3a, red line). As previous studies suggested that a high RNA:protein ratio disfavors multimers^{16; 20}, these results indicated that the FRET signal most likely arises from interactions between monomers (due to exchange or de novo assembly), rather than the association of pre-formed hexamers.

We next measured the dynamics of Hfq subunit interactions using stopped-flow FRET. When Cy3-Hfq and Cy5-Hfq were mixed in equal proportion, the FRET intensity increased rapidly, at rates of 6 to 18 s⁻¹ (Fig. 3b). The kinetics were well fit by a single exponential decay model, and the apparent rate of exchange increased almost linearly with protein concentration between 0.5 to 2.5 μ M Hfq (Fig. 3b, inset). These results show that Hfq monomers can rapidly interact even in protein concentrations expected to favor hexamers, and are consistent with a dynamic equilibrium between monomers and hexamers in vitro.

Effect of Hfq oligomerization on RNA annealing

The results in Fig. 1 show that Hfq transforms from hexamers to high molecular weight multimers above 1 μ M protein. To test whether monomers, hexamers and multimers are equally active in RNA annealing, we measured the rate of base pairing between an RNA molecular beacon (rMBD16) and DsrA sRNA at 30 °C (Fig. 4a). We previously showed that Hfq stabilizes the complex between the beacon and its complementary target near the Hfq binding site in DsrA, and that 300 nM Hfq increases the rate of annealing by 3-4 fold¹⁷.

As expected, the rate of RNA annealing did not change in presence of 10 nM Hfq (red line; Fig. 4b), which is below the 138 nM dissociation constant of Hfq for DsrA^{16; 25} and below the subunit concentration needed to assemble hexamers (Fig. 1d). When the Hfq concentration was increased to 1 μ M, the rate of RNA annealing increased 40-fold (green line) compared to the no Hfq control (black line; Fig. 4b). When the Hfq concentration was increased to 3 μ M (blue-green) or 10 μ M (red), however, the rate of RNA annealing was again close to the background rate in no Hfq (Fig. 4b). Therefore, the activity of Hfq is greatest between 0.5 and 1 μ M subunits, when most of the protein is in the hexamer form.

By contrast, the activity is very low when a high concentration of free Hfq ($\geq 3 \mu\text{M}$) favors multi-hexamer complexes. Similar results were obtained with a 16-mer RNA derived from DsrA (Fig. S4). These results are consistent with earlier observations that Hfq activity passes through an optimum and that high concentrations of Hfq inhibit the annealing of DsrA sRNA and *rpoS* mRNA¹⁶.

Conclusion

Many studies have established that Hfq forms ring shaped homo-hexamer that chaperones RNA annealing and strand exchange reactions^{1; 26}. Here, we show that Hfq hexamers are in dynamic equilibrium with monomer subunits and with multi-hexamer complexes in vitro. The hexamer form of Hfq is active in RNA annealing assays, consistent with previous studies^{27; 28; 29}. Therefore, Hfq's activity as a chaperone depends on total protein concentration, with the transition from monomer to hexamer occurring around $1 \mu\text{M}$ protein. Because Hfq binds RNA substrates such as A₁₈ at concentrations below that needed for hexamer assembly, under these conditions RNA binding must be coupled to hexamer assembly, explaining the cooperative binding observed previously¹⁶.

Our results resolve conflicting data on the stoichiometry of Hfq binding to RNA, which has been variously reported as 6:1^{11; 20} or 12:1^{16; 30}. When the Hfq concentration is high relative to the RNA, we consistently find that it forms a 12:1 complex in solution¹⁷. These 12:1 complexes form in the same concentration range as the protein multimers, and are thus best explained by interactions between hexamers. The weak association of Hfq hexamers ($\sim 5 \mu\text{M}$) would make these interactions hard to observe in gel mobility shift assays. When the RNA concentration is high, as in ITC or ultracentrifugation experiments, the 6:1 complex is favored because Hfq interacts more strongly with RNA than with itself. The loss of annealing activity above $3 \mu\text{M}$ protein (Fig. 4) is consistent with the finding that multi-hexamer complexes inhibit RNA annealing¹⁶.

The concentration of Hfq in *E. coli* cells is approximately $15 \mu\text{M}$ subunits¹⁸, which is sufficient to form hexamers and multimers in our experiments. Thus, we anticipate that these forms of Hfq are in dynamic equilibrium in vivo, and that mutations or other factors that shift the equilibrium between quaternary structures can alter Hfq's biological activity. As the intracellular RNA concentration is high, Hfq presumably spends most of the time bound to RNA or to the DNA nucleoid, which is expected stabilize hexamers and disfavor multimers. Once assembled and bound to RNA, Hfq hexamers could transfer from one binding site to another without disassembly³¹. Further work is needed to know how RNA binding changes the quaternary structure and subunit dynamics of Hfq and how this affects its biological function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Vogel J, Luisi BF. Hfq and its constellation of RNA. Nat Rev Microbiol. 2011; 9:578–589. [PubMed: 21760622]

2. Beisel CL, Storz G. Base pairing small RNAs and their roles in global regulatory networks. *FEMS Microbiol Rev.* 2010; 34:866–882. [PubMed: 20662934]
3. Gottesman S, McCullen CA, Guillier M, Vanderpool CK, Majdalani N, Benhammou J, Thompson KM, FitzGerald PC, Sowa NA, FitzGerald DJ. Small RNA regulators and the bacterial response to stress. *Cold Spring Harbor Symp Quant Biol.* 2006; 71:1–11. [PubMed: 17381274]
4. Kaberdin VR, Blasi U. Translation initiation and the fate of bacterial mRNAs. *FEMS Microbiol Rev.* 2006; 30:967–979. [PubMed: 16989654]
5. Aiba H. Mechanism of RNA silencing by Hfq-binding small RNAs. *Curr Opin Microbiol.* 2007; 10:134–139. [PubMed: 17383928]
6. Caron MP, Lafontaine DA, Masse E. Small RNA-mediated regulation at the level of transcript stability. *RNA Biol.* 2010; 7:140–144. [PubMed: 20220305]
7. Sun X, Zhulin I, Wartell RM. Predicted structure and phyletic distribution of the RNA-binding protein Hfq. *Nucleic Acids Res.* 2002; 30:3662–3671. [PubMed: 12202750]
8. Schumacher MA, Pearson RF, Moller T, Valentin-Hansen P, Brennan RG. Structures of the pleiotropic translational regulator Hfq and an Hfq-RNA complex: a bacterial Sm-like protein. *EMBO J.* 2002; 21:3546–3556. [PubMed: 12093755]
9. Link TM, Valentin-Hansen P, Brennan RG. Structure of *Escherichia coli* Hfq bound to polyribadenylate RNA. *Proc Natl Acad Sci USA.* 2009; 106:19292–19297. [PubMed: 19889981]
10. Sauer E, Weichenrieder O. Structural basis for RNA 3'-end recognition by Hfq. *Proc Natl Acad Sci USA.* 2011; 108:13065–13070. [PubMed: 21737752]
11. Mikulecky PJ, Kaw MK, Brescia CC, Takach JC, Sledjeski DD, Feig AL. *Escherichia coli* Hfq has distinct interaction surfaces for DsrA, rpoS and poly(A) RNAs. *Nat Struct Mol Biol.* 2004; 11:1206–1214. [PubMed: 15531892]
12. Beich-Frandsen M, Vecerek B, Sjoblom B, Blasi U, Djjinovic-Carugo K. Structural analysis of full-length Hfq from *Escherichia coli*. *Acta Crystallogr Sect F Struct Biol Cryst Commun.* 2011; 67:536–540.
13. de Haseth PL, Uhlenbeck OC. Interaction of *Escherichia coli* host factor protein with oligoriboadenylates. *Biochemistry.* 1980; 19:6138–6146. [PubMed: 6162476]
14. Mura C, Kozhukhovskiy A, Gingery M, Phillips M, Eisenberg D. The oligomerization and ligand-binding properties of Sm-like archaeal proteins (SmAPs). *Protein Sci.* 2003; 12:832–847. [PubMed: 12649441]
15. Arluison V, Mura C, Guzman MR, Liquier J, Pellegrini O, Gingery M, Regnier P, Marco S. Three-dimensional structures of fibrillar Sm proteins: Hfq and other Sm-like proteins. *J Mol Biol.* 2006; 356:86–96. [PubMed: 16337963]
16. Lease RA, Woodson SA. Cycling of the Sm-like protein Hfq on the DsrA small regulatory RNA. *J Mol Biol.* 2004; 344:1211–1223. [PubMed: 15561140]
17. Hopkins JF, Panja S, McNeil SA, Woodson SA. Effect of salt and RNA structure on annealing and strand displacement by Hfq. *Nucleic Acids Res.* 2009; 37:6205–6213. [PubMed: 19671524]
18. Kajitani M, Kato A, Wada A, Inokuchi Y, Ishihama A. Regulation of the *Escherichia coli* hfq gene encoding the host factor for phage Q beta. *J Bacteriol.* 1994; 176:531–534. [PubMed: 8288550]
19. Banik U, Mandal NC, Bhattacharyya B, Roy S. A fluorescence anisotropy study of tetramer-dimer equilibrium of lambda repressor and its implication for function. *J Biol Chem.* 1993; 268:3938–3943. [PubMed: 8440687]
20. Updegrove TB, Correia JJ, Chen Y, Terry C, Wartell RM. The stoichiometry of the *Escherichia coli* Hfq protein bound to RNA. *RNA.* 2011; 17:489–500. [PubMed: 21205841]
21. Arluison V, Folichon M, Marco S, Derreumaux P, Pellegrini O, Seguin J, Hajnsdorf E, Regnier P. The C-terminal domain of *Escherichia coli* Hfq increases the stability of the hexamer. *Eur J Biochem.* 2004; 271:1258–1265. [PubMed: 15030475]
22. Moskaleva O, Melnik B, Gabdulkhakov A, Garber M, Nikonov S, Stolboushkina E, Nikulin A. The structures of mutant forms of Hfq from *Pseudomonas aeruginosa* reveal the importance of the conserved His57 for the protein hexamer organization. *Acta Crystallogr Sect F Struct Biol Cryst Commun.* 2010; 66:760–764.
23. Beich-Frandsen M, Vecerek B, Konarev PV, Sjoblom B, Klobner K, Hammerle H, Rajkowsch L, Miles AJ, Kontaxis G, Wallace BA, Svergun DI, Konrat R, Blasi U, Djjinovic-Carugo K.

- Structural insights into the dynamics and function of the C-terminus of the *E. coli* RNA chaperone Hfq. *Nucleic Acids Res.* 2011; 39:4900–4915. [PubMed: 21330354]
24. Wang W, Wang L, Zou Y, Zhang J, Gong Q, Wu J, Shi Y. Cooperation of *Escherichia coli* Hfq hexamers in DsrA binding. *Genes Dev.* 2011; 25:2106–2117. [PubMed: 21979921]
25. Brescia CC, Mikulecky PJ, Feig AL, Sledjeski DD. Identification of the Hfq-binding site on DsrA RNA: Hfq binds without altering DsrA secondary structure. *RNA.* 2003; 9:33–43. [PubMed: 12554874]
26. Brennan RG, Link TM. Hfq structure, function and ligand binding. *Curr Opin Microbiol.* 2007; 10:125–133. [PubMed: 17395525]
27. Franze de Fernandez MT, Hayward WS, August JT. Bacterial proteins required for replication of phage Q ribonucleic acid. Purification and properties of host factor I, a ribonucleic acid-binding protein. *J Biol Chem.* 1972; 247:824–831. [PubMed: 4550762]
28. Zhang A, Wassarman KM, Ortega J, Steven AC, Storz G. The Sm-like Hfq Protein Increases OxyS RNA Interaction with Target mRNAs. *Mol Cell.* 2002; 9:11–22. [PubMed: 11804582]
29. Arluison V, Derreumaux P, Allemand F, Folichon M, Hajnsdorf E, Regnier P. Structural Modelling of the Sm-like Protein Hfq from *Escherichia coli*. *J Mol Biol.* 2002; 320:705–712. [PubMed: 12095248]
30. Sun X, Wartell RM. *Escherichia coli* Hfq binds A18 and DsrA domain II with similar 2:1 Hfq6/RNA stoichiometry using different surface sites. *Biochemistry.* 2006; 45:4875–4887. [PubMed: 16605255]
31. Fender A, Elf J, Hampel K, Zimmermann B, Wagner EG. RNAs actively cycle on the Sm-like protein Hfq. *Genes Dev.* 2010; 24:2621–2626. [PubMed: 21123649]
32. Hopkins JF, Panja S, Woodson SA. Rapid binding and release of Hfq from ternary complexes during RNA annealing. *Nucleic Acids Res.* 2011; 39:5193–5202. [PubMed: 21378124]

Figure 1. Hfq oligomerization and hexamer stability

(a) Dye-labeled Hfq monomers can interact by assembly of new hexamers or by exchanging subunits between Hfq hexamers. *E. coli* Hfq:S65C was derivatized with Cy3- or Cy5-maleimide as previously described³² and re-purified from free dyes using a Hi-Trap Co²⁺ column. The labeling efficiency, typically 20-30%, was estimated by measuring the absorbance at 280 nm and at 550 or 560 nm. Nucleic acid contamination was very low as judged by $A_{260}/A_{280} \approx 0.8$. We previously confirmed that labeling does not impair Hfq activity.³² (b) Semi-native PAGE. Hfq subunits (1 μM) labeled with Cy3, Cy5 or a mixture (1:1) of Cy3- and Cy5-Hfq were loaded on a 15% (29:1) polyacrylamide gel in 0.5% (w/v) SDS. Left; superposition of scans using the Cy3 channel (green) and Cy5 channel (red). Right; excitation with Cy3 channel (532 nm) and detection in Cy5 channel (670 nm) to measure in-gel FRET (Typhoon 9410; GE Healthcare). (c) Population of different forms of Hfq plotted as a function of total Hfq concentration, from additional experiments as in (b). (d) Association of Hfq subunits by fluorescence anisotropy. Wild type, R16A or K56A Hfq (50 μM) was treated with a 10-fold molar excess of dansyl chloride (0.5 mM in acetone) 1 hr at room temperature in 50 mM potassium phosphate (pH 7.5). Excess dye was removed with 250 μL dextran-coated charcoal (25 mg/mL in the same buffer) at 4 °C for 30. After removing charcoal by centrifugation, the supernatant was dialyzed against the storage buffer before use. Dansyl-labeled Hfq (10 μM) was diluted with 50 mM potassium phosphate [pH 7.5], 250 mM NaCl to dissociate multimers; samples were equilibrated 3 min after each dilution. Anisotropy was measured with 340 nm excitation, 520 nm emission, 5 nm slits (Horiba Fluorolog-3; L format) The change in anisotropy was fit to a two-stage binding isotherm, $\theta = \Delta\theta_1[\text{Hfq}]^{n1}/([\text{Hfq}]^{n1} + K_1^{n1}) + \Delta\theta_2[\text{Hfq}]^{n2}/([\text{Hfq}]^{n2} + K_2^{n2}) + \theta_{\text{min}}$, in which

$\Delta\theta_1$ and $\Delta\theta_2$ are the relative increase in anisotropy of the hexamers and multimers respectively, $K_1 = 0.83 \mu\text{M}$, $K_2 = 4.9$, $n_1 = 9.0$, and $n_2 = 4.2$. From the fits we obtain $\theta = 0.076$, 0.120 and 0.20 for the monomer, hexamer and multimer, respectively. Errors are approximately $\pm 20\%$. Controls in which dansyl-Hfq was diluted with unlabeled Hfq showed no appreciable change in anisotropy.

Figure 2. RNA binding site mutations disrupt the hexamer

(a) Location of amino acid substitutions in the three-dimensional structure of Hfq (PDB: 3qhs)¹²; residues are colored in one monomer for clarity. Variants of wild type (untagged) Hfq were prepared as previously described³². **(b)** Hfq (50 μ M) was analyzed by semi-native PAGE as in Figure 1, and stained with Coomassie blue.

Figure 3. FRET assay for monomer exchange

(a) Steady-state fluorescence emission upon excitation of 300 nM Cy3-Hfq (blue) at 540 nm. Titration with Cy5-Hfq at 30 °C in 1X HB buffer (10 mM Tris-HCl, pH 7.5, 50 mM NH₄Cl, 0.2 mM EDTA, 2% glycerol) decreases Cy3 emission at 565 nm and increases Cy5 emission at 665 nm. Inset: Increase in FRET efficiency (E_{FRET}) with increasing protein concentration indicates monomer exchange. The FRET efficiency was calculated from $E_{\text{FRET}} = 1 - I_{\text{D}} / (I_{\text{D}} + I_{\text{A}})$, in which I_{D} and I_{A} are the donor and acceptor intensities, respectively. Black, no RNA; red, 500 nM A₁₈ RNA. Data with 5'AU₆G RNA were indistinguishable from no RNA curve (not shown). (b) Kinetics of Hfq interactions were measured by mixing Cy3-Hfq and Cy5-Hfq, using an Applied Photophysics SX 18MV stopped flow spectrometer as described previously³². Emission intensities were recorded using a 570 (± 10) nm band pass filter. The normalized fluorescence intensity was fit to a

single exponential rate equation (red line), $\Delta F(t) = (F_t - F_0)/(F_\infty - F_0) = A (1 + \exp -kt)$. Inset, observed rate of monomer exchange versus total protein concentration. Error bars show the standard deviation between five different trials.

Figure 4. Optimum Hfq concentration for RNA annealing

(a) An RNA molecular beacon (black line) fluoresces more brightly upon binding with DsrA sRNA (blue). The loop of the beacon is complementary to the 16 nt in red; the 5' end is conjugated with fluorescein while the 3' end is modified with DABCYL¹⁷. (b) Hybridization kinetics in different Hfq concentrations. The molecular beacon (rMBD16; 10 nM) and DsrA sRNA (100 nM) were mixed in TNK (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 50 mM KCl) at 30 °C with or without Hfq as indicated. The increase in fluorescein emission (515 nm) over time was fit to a single exponential. Progress curves in 0.5 and 1 μM Hfq were normalized to equilibrium data as previously described.³²