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Histone-DNA assemblies in archaea : shaping the genome on the edge of life

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CHAPTER 5

**DNA binding by histones from *Ca.*
*Nanohaloarchaeota***

Abstract

Members of the archaeal phylum *Candidatus* Nanohaloarchaeota have recently been found in hypersaline lakes and salt crusts. For functionality, the macromolecules of these organisms, including histones, must be adapted to high cytoplasmic salt concentrations. Based on our sequence analysis and classification, three histone types have been identified in *Candidatus* Nanohaloarchaeota. Histones of type A are predicted to form tetramers and hypernucleosomes, whereas histones of type B can probably form no structure larger than a dimer, and may have a lower binding affinity than histones of type A. Prediction of characteristics of type C histones was less conclusive. Here, we investigate two histones encoded by *Nanosalina* sp. J07AB43. *Nanosalina* HA and HB, type A and type B histones, respectively, were recombinantly expressed and their interactions with DNA were characterized using microscale thermophoresis and tethered particle motion. We find that at 75 mM KCl, HA binds and probably compacts DNA, whereas HB does not. At 1.5 M KCl, no direct binding is observed, but both proteins do affect fluorescence when added, suggesting binding to DNA. HA does this at lower concentrations than HB, indicating that HA indeed is able to better bind DNA than its type B counterpart.

Introduction

Archaea, the domain of prokaryotic organisms from which eukaryotes have emerged, have recently seen a sharp increase in the availability of genetic information. Whereas initially archaea were found in extreme environments such as hydrothermal vents and hot springs, scrutiny of the archaeal domain revealed that they exist in nearly every habitat on earth. The archaeal tree of life dramatically changed shape over the last ten years as a result of the discovery of novel archaeal superphyla, phyla and classes (47). One of these more recent additions is the candidate phylum Nanohaloarchaeota (329). After its discovery at Lake Tyrell, Australia, it was initially classified as a new euryarchaeal class, but was later categorized as a phylum part of the DPANN superphylum (39). Three classes of *Candidatus* (*Ca.*) Nanohaloarchaeota have been identified, Haloredivivus, Nanosalina and Nanosalinarum, all resulting from metagenomic sequencing of samples from hypersaline lakes (329, 330). Additionally, fifteen unclassified genomes of *Ca. Nanohaloarchaea* were discovered on salt crusts in the Chilean Atacama desert (331, 332). None of the *Ca. Nanohaloarchaeota* have been reported to have grown in culture. The cells of *Ca. Nanohaloarchaeota* are small with an estimated cell size of 0.6 μm (329). Their genomes are also small, ranging from 0.40 to 1.48 Mbp. Most of these genomes have a GC content of 40-50%, which is low compared to other archaeal genomes that were found in the same habitat, but similar to Methanobacteria and Nanoarchaeota (329).

The proteins predicted to exist in archaeon SG9 (genome length 1.1 Mbp) have a median theoretical isoelectric point (pI) of 4.7. This would place archaeon SG9 in the first percentile of average pIs of more than 5000 proteomes from throughout the tree of life (333). The average pIs of some halophilic archaea from the euryarchaeal class Halobacteria are comparable to those of Nanohaloarchaeota. The median pI of the average of all archaeal proteomes is 6.4, with half of all archaeal proteomes having an average pI between 5.8 and 7.1 (331, 333). Scrutiny of the proteins encoded by the genome of *Nanosalina* sp. J07AB43 also indicated pIs between 4 and 6, similar to archaeon SG9. Living in conditions with high salinity is challenging, as high salt concentrations in the environment lead to an increase of the osmotic pressure inside the cell. A low average pI of the proteome is indicative of the 'salt-in' osmotic strategy, which microorganisms inhabiting hypersaline environments use to balance the osmotic pressure of their cellular contents with the pressure of the environment (334). In this strategy, ions, mainly Cl^- and K^+ , are accumulated inside the cell, which means that the proteome needs to be adapted for functionality at very high intracellular salt concentrations (335, 336). For species of Halobacteria, intracellular KCl concentrations up to 7 M have

been recorded (335). For many proteins that are expressed by organisms that use the salt-in strategy, a high intracellular salt concentration contributes to protein folding. Exposure to low salt environments can therefore lead to denaturation. Only some halobacterial archaea, as well as some bacteria, use the salt-in strategy for haloadaptation. Most other salt loving or salt tolerant organisms exclude rather than let in salt ions, using high concentrations of sugars, alcohols, glycerol or other organic compounds without net charge to counterbalance osmotic pressure (337). This places members of *Ca. Nanohaloarchaeota* in a small subgroup of extremophiles.

Genomes of *Ca. Nanohaloarchaeota* encode the nucleoid-associated protein Alba, as well as histones. Alba-encoding genes were identified on approximately half of the annotated nanohaloarchaeal genomes, whereas genes coding for histones can be found in all annotated genomes. Both Alba and histones have, like most other proteins in this phylum, exceptionally low theoretical pIs, on average 4.2 and 5.3, respectively. All nanohaloarchaeal Alba proteins contain the residue Lys16, which is subject to acetylation or methylation (119, 120). Post-translational modification alters Alba's binding affinity to DNA, and is therefore important for modulation of its activity. Most Alba proteins encoded by *Ca. Nanohaloarchaeota* are of type Alba1, and only *Haloredivivus* sp. G17 encodes a protein of type Alba2 (338). Histones encoded by the genomes of *Ca. Nanohaloarchaeota* are predicted to contain a single histone fold, unlike the 'tandem histones' from Halobacteria, which are found in the same habitat (185, 339). They likely form dimers in solution, like other archaeal histones (160, 193). Nanohaloarchaeal histones have been categorized in three types (*see also* CHAPTER 2). Although histones of all nanohaloarchaeal types are predicted to bind DNA, histones of type A are predicted to be able to tetramerize and assemble into hypernucleosomes, which strongly compact DNA (197, 339). Histones of type B lack the residues providing interactions necessary for tetramerization, which likely makes them unable to form structures larger than dimers (FIGURE 5.1). Especially the absence of H49 likely makes tetramerization impossible. Furthermore, they lack AGA at position 15-17, situated at the N-terminal loop region. Bulkier amino acid residues at this

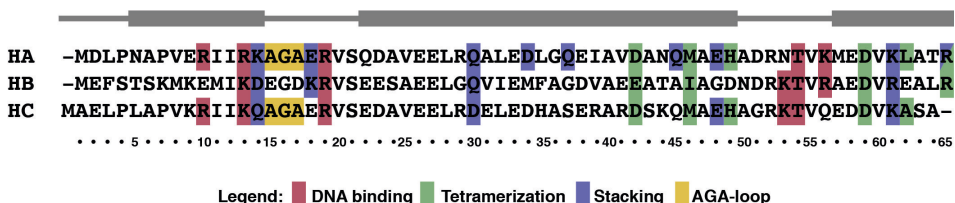


Figure 5.1: Alignment of histones HA, HB and HC encoded in the genome of *Nanosalina* sp. J07AB43. Grey bars represent α -helices.

position generally cause steric hindrance between histone dimers when multimerizing, which means that substitution of AGA for amino acids with larger side chains prevents hypernucleosome formation. Also, histones of type B have 5 sites of potential DNA-binding interactions, whereas histones of type A have 6. This may result in weaker DNA binding of type B histones compared to histones of type A. Histones of type C may be able to tetramerize, although their tetramers are likely less stable than those of type A, due to a lower number of tetramerization interactions. It is unknown if histones of type C form hypernucleosomes, but if they do, these hypernucleosomes are likely less stable than hypernucleosomes composed of histones of type A, as a result of less stacking interactions (*see also CHAPTER 2*).

Here, we investigate DNA binding and compaction by histones from the genome *Nanosalina* sp. J07AB43, HA and HB. These histones belong to nanohaloarchaeal histone types A and B, respectively. Histone types A and B are hypothesized to exhibit clear differences in terms of multimerization, which can be empirically tested by *in vitro* experiments. We use microscale thermophoresis (MST), which measures difference in the sum of molecular size, charge and hydration shell, and tethered particle motion (TPM), which gives a read-out of DNA organization, to qualitatively probe compaction of DNA by these histones, permitting assessment of the protein's ability to form tetramers or hypernucleosomes.

Results and discussion

In order to investigate interaction between *Nanosalina* sp. J07AB43 histones HA and HB, we expressed and purified recombinant HA and HB. To determine whether HA or HB binding affects DNA conformation, we used TPM. We tethered polystyrene beads to a glass surface using 685 bp long digoxigenylated and biotinylated nonspecific DNA substrates. The root mean square displacement of the tethered beads gives a quantitative readout of changes in DNA conformation. DNA tethers were incubated with HA or HB at 75 mM KCl, a concentration at which other histones have been reported to bind DNA (*see also* CHAPTERS 4 and 6). Although low pH is physiologically more relevant considering the mode of life of members of *Ca. Nanohaloarchaeota*, in TPM, buffers with pH below 6.0 cannot be used following routine protocols: lower pH causes sticking of beads to the surface. Therefore, a pH of 6.0 was chosen for TPM experiments. Addition of up to 1000 nM HA or HB did not affect the root mean square displacement (RMS) of DNA (FIGURE 5.2). This suggests that in these conditions, HA and HB do not change DNA conformation.

We further investigated HA and HB interacting with DNA using microscale thermophoresis, which also responds to proteins binding to DNA without DNA

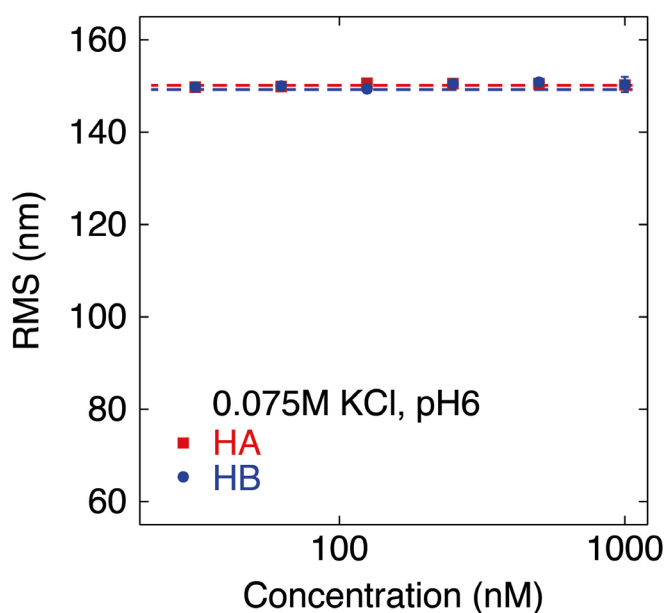


Figure 5.2: Tethered particle motion experiments show that HA and HB do not deform DNA at pH6 and 75 mM KCl. Error bars represent the standard deviation of the different experiments, and are mostly hidden behind the data points. Dashed lines are to guide the eye.

deformation. We used linear, Cy5-labeled, nonspecific DNA with a length of 78 bp and a GC content of 50%, close to the average GC content of nanohaloarchaeal genomes. The length of the DNA substrate is sufficient to harbor a histone tetramer, but is too short to accommodate larger histone assemblies (340, 341). To mimic physiological pH values, we incubated *Nanosalina* histones with the DNA substrate in a solution containing 75 mM KCl at pH 4.0. Next, we measured thermophoresis at histone concentrations between 0 and 1000 nM (FIGURE 5.3A and B). We quantify thermophoresis by taking the ratio of two regimes of the

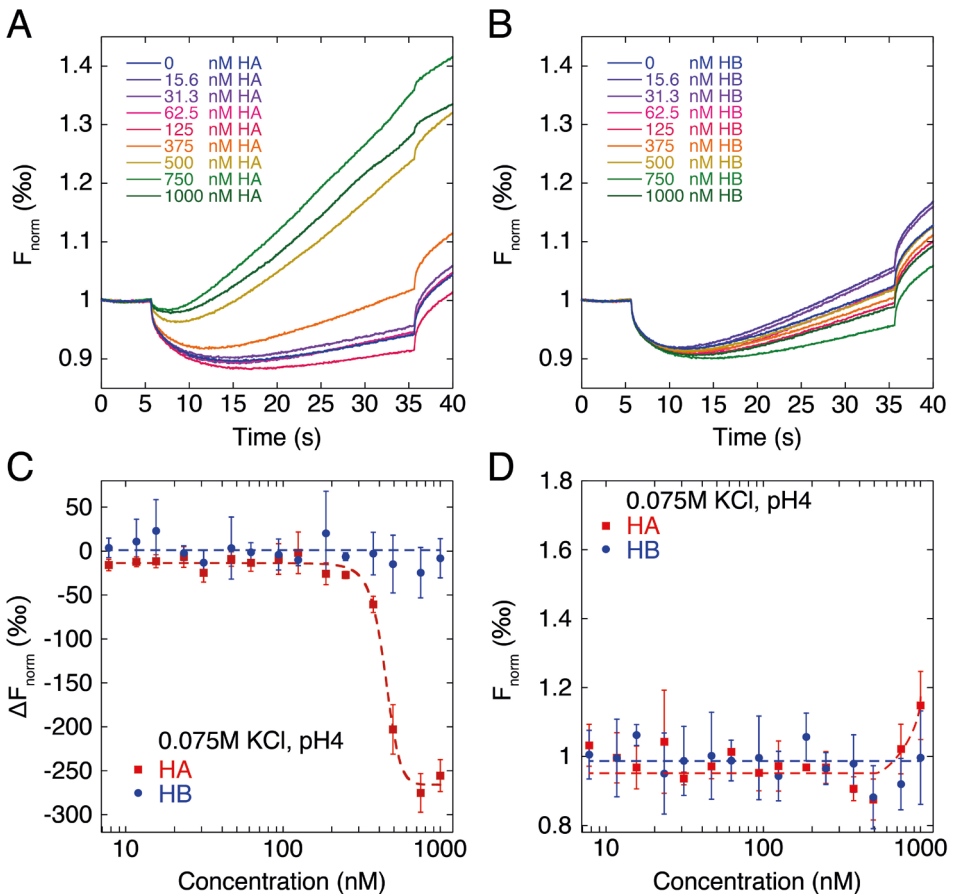


Figure 5.3: Microscale thermophoresis experiments show reduction in thermophoresis by HA, but not by HB at pH4 and 75 mM KCl. Representative normalized time traces of thermophoresis experiments on 78 bp nonspecific DNA substrates for *Nanosalina* histones HA (A) and HB (B). Normalized fluorescence (F_{norm}) is expressed as function of time. C) Thermophoresis of *Nanosalina* histones HA and HB on 78 bp nonspecific DNA substrates at pH4, expressed as the difference in normalized fluorescence compared to the value at 0 nM (ΔF_{norm}) as function of histone concentration. Error bars represent standard deviation of the experiments. D) Initial normalized fluorescence (F_{norm}) of *Nanosalina* histones HA and HB on 78 bp nonspecific DNA substrates, as function of concentration. Error bars represent standard deviation of the experiments.

thermophoresis time traces, by which we compare fluorescence before heating and fluorescence just before the end of heating (*see also MATERIALS & METHODS*). The ratio gives a read-out of how fast the DNA substrates diffuse out of the heated spot, which in turn indicates the change in size, charge and hydration shell when compared to a situation where only labeled DNA is present. Thermophoresis experiments showed that at concentrations of HA >250 nM, the difference in normalized fluorescence (ΔF_{norm}) decreases (**FIGURE 5.3C**). This means that the combination of change in molecular assembly, charge and/or hydration shell changes as a result of histone binding and possibly DNA deformation (342). The response to HA is similar to the results of MST experiments on HMfA and HMfB, although that study also showed that ΔF_{norm} increases at protein concentrations >1000 nM (341). Due to limited availability of protein, we were not able to reach these concentrations. In contrast to the response to thermophoresis caused by HA, ΔF_{norm} did not change in the presence of HB, which suggests that HB does not bind and compact the DNA substrate. Since the footprint of archaeal histones is 30 bp per dimer, the 78 bp substrate can accommodate a tetramer. Based on structural and sequential analyses, we predicted that HA is able to bind DNA as tetramers, whereas HB also binds DNA but probably cannot form tetramers. The results of the experiments presented here are in line with this hypothesis. In addition to thermophoresis, protein binding to DNA can also have an effect on the initial fluorescence before heating of the sample. Therefore we normalized and plotted initial fluorescence as function of concentration. The initial fluorescence did not significantly change in response to protein concentration (**FIGURE 5.3D**).

Based on the notion that the average pI of nanoarchaeal proteins is very low, we expect that members of *Ca. Nanohaloarchaeota* use the salt-in strategy for regulation of osmotic pressure. Reduction of salt concentrations to below 0.5 M (as used in the experiments reported above) leads to a shortage of cations shielding the protein surface, resulting in repulsive forces that weaken the conformation of some proteins (337). As it may lead to more stable histones, it is thus relevant to measure nanoarchaeal histones at high salt concentrations. However, when we carried out HA protein titrations using MST at 1.5 M of KCl, we no longer detect an effect of HA on thermophoresis (**FIGURE 5.4A-C**). This suggests that HA is no longer binding and compacting the DNA substrate, or binds with low affinity. A similar behavior was observed for HB. However, when we look at initial fluorescence, we observe a positive correlation between histone concentration and fluorescence for both HA and HB (**FIGURE 5.4D**). Fluorescence increases starting at a concentration of 125 nM for HA, whereas for HB, we observe an increase in fluorescence from 375 nM and onwards. One possible explanation for the increase in fluorescence signal is protein induced fluorescence enhancement (PIFE). This effect entails that fluorophore intensity increases upon binding of

a protein in its vicinity, more precisely within 0 and 3 nm of the dye (343, 344). Cy5, the dye attached to our DNA substrates, has been shown to exhibit PIFE (343). This means that if PIFE is a proxy of histone binding to DNA, initial fluorescence data does not correspond with thermophoresis data. It should be noted that fluorescence is proportional to the viscosity of the experimental buffer and that protein concentration contributes to viscosity (345, 346). However, we do not observe a proportional relation between protein concentration and initial fluorescence. Also, HA and HB are very similar in terms of size, charge and structure, which would suggest that their contribution to buffer viscosity is roughly equal. As our data indicate that HA and HB affect initial fluorescence at different con-

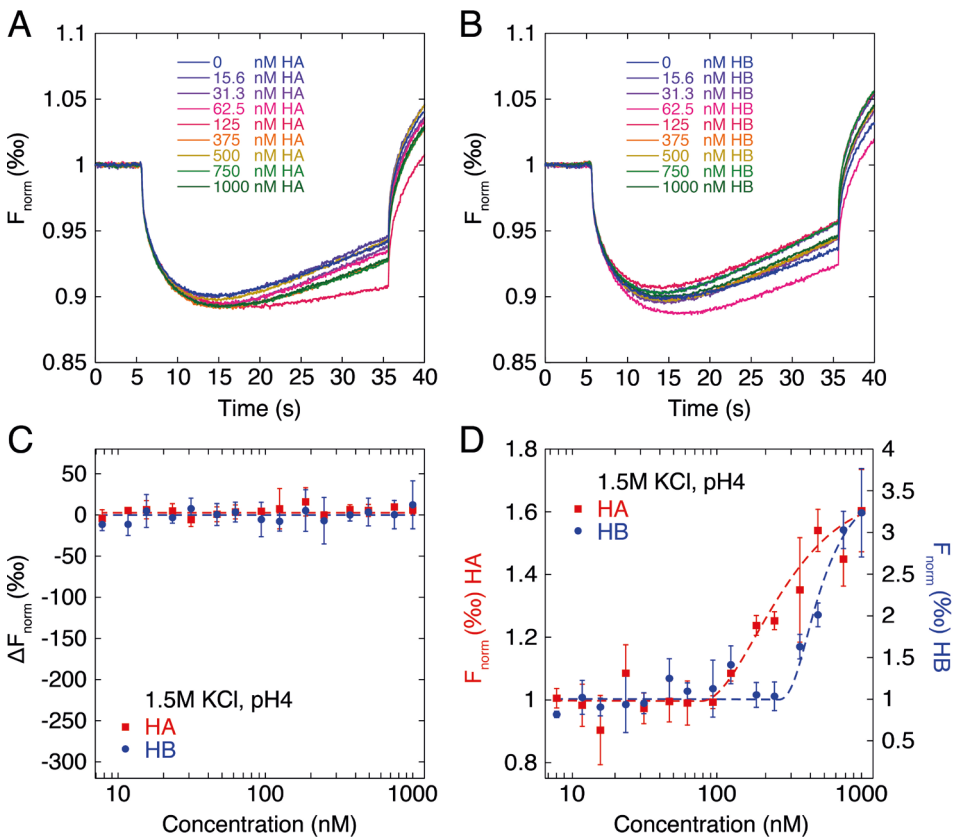


Figure 5.4: Microscale thermophoresis experiments indicate binding of DNA by HA and HB at pH4 and 1.5 M KCl. Representative normalized time traces of thermophoresis experiments on 78 bp nonspecific DNA substrates for Nanosalina histones HA (A) and HB (B). Normalized fluorescence (F_{norm}) is expressed as function of time. C) Thermophoresis of Nanosalina histones HA and HB on 78 bp nonspecific DNA substrates, expressed as the difference in normalized fluorescence compared to the value at 0 nM (ΔF_{norm}) as function of histone concentration. Error bars represent standard deviation of the experiments. D) Initial normalized fluorescence (F_{norm}) of Nanosalina histones HA and HB on 78 bp nonspecific DNA substrates, as function of concentration. Error bars represent standard deviation of the experiments.

centrations (**FIGURE 5.4D**), a viscosity-induced increase in fluorescence unlikely. The discrepancy between thermophoresis and initial fluorescence experiments may be explained by binding dynamics of HA and HB. High dissociation rates may result in a PIFE effect but may not be visible as an effect on thermophoresis. We did not observe an increase in initial fluorescence as function of protein concentration for HA and HB at 75 mM KCl. Possibly, higher protein concentrations would result in an effect on initial fluorescence, as the initial fluorescence seems to slightly increase at the highest concentrations of the titration used in this study. This may indicate a different form of binding at 1.5 M KCl compared to 75 mM. The observation of DNA binding at high salt concentrations might be indicative of the involvement of hydrophobic interactions. It should be noted that initial fluorescence cannot be quantitatively compared between conditions with different KCl concentrations, as fluorescence is inversely related to KCl concentration (**FIGURE S5.1**). We did not use concentrations of 1.5M KCl in TPM, because high salt concentrations are not compatible with our routine protocols. In order to establish whether HA and HB form multimers, we recommend further optimization to establish conditions at which histone-DNA complexes are stable. Establishing such conditions could also allow us to quantify DNA deformation by HA and HB. These proposed experiments may provide further insight into the function of multimerizing and non-multimerizing histones.

Conclusion

Nanohaloarchaeal histone HA from *Nanosalina* sp. J07AB43 binds DNA at 75 mM KCl and pH4. Although we have not been able to confirm DNA compaction by HA using TPM, the strong effect of the protein on fluorescence is, in accordance with previous studies, indicative of a large conformational change. This conformational change is probably caused by wrapping around a histone tetramer. HA was predicted to better tetramerize and bind to DNA than HB, based on sequential analysis. This study indirectly confirms the prediction, although optimization of pH and KCl concentrations may reveal the conditions at which HB also binds and compacts DNA. Experiments at KCl concentrations intended to mimic physiological conditions of *Ca. Nanohaloarchaeota* did not directly show binding, but increase in initial fluorescence in MST experiments suggests that HA and HB interact with DNA. Further optimization of experimental conditions may shed more light on nanohaloarchaeal histones and their involvement in DNA compaction. Insight into the DNA-binding characteristics of nanohaloarchaeal histones may reveal how mechanisms for DNA compaction and transcription regulation were established in the archaeal domain.

Materials & Methods

Purification of *Nanosalina* HA and HB

Genes coding for *Nanosalina* sp. J07AB43 histones HA (accession number EGQ42849) and HB (accession number EGQ43804) were codon-optimized for *Escherichia coli* and synthesized at GeneArt (Thermo Fisher Scientific, MA). Genes were cloned into pET30b vectors using Gibson assembly, resulting in plasmids pRD321 expressing HA and pRD322 expressing HB. pRD321 was transformed to *E. coli* BL21 star (DE3), and pRD322 was transformed to *E. coli* BL21 Rosetta (DE3), resulting in strains NT494 and NT495. For protein overexpression 500 ml LB medium with 25 mg/ml chloramphenicol and 50 mg/ml kanamycin was inoculated with 1% v/v culture grown to saturation o/n. Cultures were grown at 37°C with shaking at 250 rpm. At $OD_{600} \approx 0.5$, expression of HA was induced with 1 mM IPTG. Cultures were grown for 1 h at 37°C with shaking at 250 rpm. Cultures were harvested by centrifugation for 30 minutes at 11,325 xg and at 4°C. Cells were frozen at -80°C and thawed the next day. DNase I, PMSF and lysozyme were added and cells were lysed using the pressure homogenizer unit 5 (Homogenising Systems Ltd, UK) at 350 MPa in 50 mM Tris-HCl pH 8.0 and 100 mM NaCl in water. Cell lysate was separated from the pellet by centrifugation for 30 min at 10,967 xg and at 4°C. Lysates were incubated at 95°C for 10 minutes, then spun down for 15 min at 10,967 xg and at 4°C. The supernatant was diluted 3x in 16.7 mM Tris-HCl pH 7.0. The treated lysates were loaded onto a 5 ml HiTrap DEAE Sepharose FF column (GE Healthcare, IL) and eluted with 150 ml 50 mM Tris-HCl pH 8 and a NaCl gradient from 0 to 250 mM (for lysates of NT494) or 0 to 1 M (for lysates of NT495) using the Äkta start protein purification system (GE Healthcare, IL) at RT. Peak fractions were analysed on Coomassie-stained SDS-PAGE gels and combined. Combined fractions were concentrated to 400 µl using Amicon ultrafiltration membranes (Merck, Germany; cut-off: 3 kDa) at 3700 rpm. The combined fractions were loaded on a Superdex 75 Increase 10/300 GL gel filtration column (GE Healthcare, IL) and eluted with a degassed solution of 50 mM Tris-HCl pH 8 and 100 mM NaCl in water, using the Äkta pure chromatography system (GE Healthcare, IL) at RT (flow rate: 0.5 ml/min). Peak fractions of 0.5 ml were collected, checked on Coomassie-stained SDS-PAGE gels and protein purity was examined using mass spectrometry. Fractions with less than ~5% protein impurities were considered pure. Coomassie-staining showed bands for HA and HB corresponding to 1x and 2x the expected molecular, indicating that a significant fraction of both proteins is present as dimer. Since the proteins are able to interact, we expect that the proteins are properly folded. Concentrations of HA and HB were determined using a Pierce BCA Protein Assay kit (Thermo Fisher

Scientific, MA) and were 100 μM and 561 μM , respectively. Protein solutions were plunge frozen and stored at -80°C .

DNA substrate preparation

For the MST substrate, two complementary oligos (Merck, Germany) of 78 nucleotides containing the 50% GC sequence as described in chapter 4 were mixed at 100 μM each. The oligos were annealed by heating to 95°C for 5 minutes, then decreasing the temperature in steps of 5°C , followed by 5 minutes incubation, and so on until a temperature of 20°C was reached. Substrates were subsequently diluted to 100 nM in 10 mM Tris-HCl pH 8 and 20 mM NaCl.

For the TPM DNA substrate, plasmid pRD121 was used, as described previously (see CHAPTER 4). We used PCR to generate and amplify a 685 bp linear substrate, using digoxigenin- and biotin-labeled oligonucleotides (195).

Microscale Thermophoresis

A dilution series of HA and HB starting at 2000 nM down to 15.6 nM was made using measurement buffer (50 mM acetate buffer pH 4.0 [11.3 mM sodium acetate and 38.7 mM acetic acid], KCl 75 mM or 1.5 M in water). Protein dilutions were mixed 1:1 with 100 nM Cy5-labeled MST substrate, resulting in protein concentrations from 1000 nM downwards and a constant substrate concentration of 50 nM in a total volume of 40 μl . Sample mixtures were transferred to MST capillaries (Monolith NT.115 Premium Capillaries, NanoTemper, Germany). Capillaries were measured using the Monolith NT.115 (NanoTemper, Germany) at 20% LED power and 20% MST power. Total measuring time was 40.4 s. NanoTemper Analysis software was used for data analysis of MST experiments. Time traces were normalized by alignment of the linear regime before onset of the MST laser (0-5.6 s). Thermophoresis was calculated by the ratio of normalized fluorescence between time windows 6.2-6.7 s and 34.7-36.6 s as described by Corbeski *et al.* (340). For the initial fluorescence curves, the average of initial fluorescence values at 0-62.5 nM was normalized to 1. The initial fluorescence values of the other data points are then shown as relative values to the values at 0-62.5 nM. MST experiments were carried out in triplicate.

Tethered particle motion

The tethered particle motion experiments, data analysis and representation of results were performed as previously described (322). To select single-tethered

beads, we used a standard deviation cut-off of 8% and an anisotropic ratio cut-off of 1.3. As measurement buffer we used 50 mM phosphate buffer pH 6.0 (6.8 mM dipotassium phosphate and 43.2 mM monopotassium phosphate) and 75 mM KCl in water. TPM experiments were carried out in duplicate.

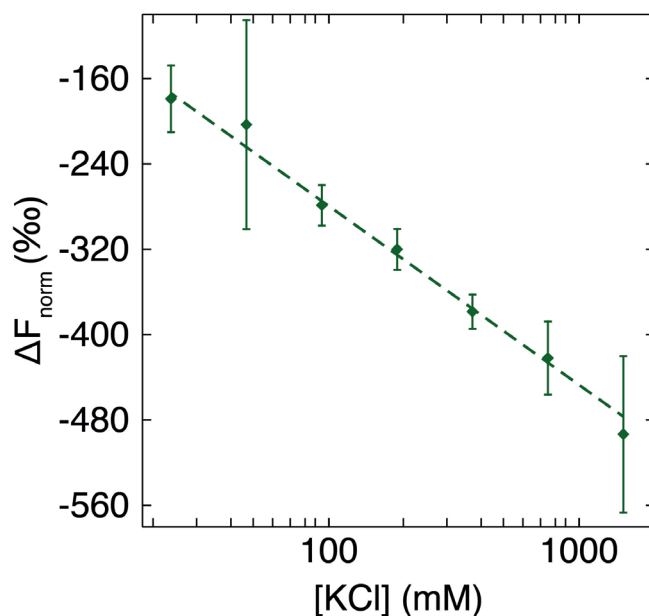
Supplemental figure

Figure S5.1: Initial fluorescence as a function of KCl concentration. Fluorescence decreases at larger KCl concentrations. Error bars represent standard deviation of the experiments. Dashed line is to guide the eye.

