Engineering spectroscopic reporters of structure and DNA-binding function in the histone-like protein, HU, through Phe-to-Trp substitutions.

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A dissertation submitted for the partial fulfillment of

BS-MS dual degree in Science

Department of Biological Sciences

Indian Institute of Science Education and Research (IISER)

Mohali

April 2014

Certificate of Examination

This is to certify that the dissertation titled "**Engineering spectroscopic reporters of structure and DNA-binding function in the histone-like protein, HU, through Phe-to-Trp substitutions.**" submitted by **Abhishek Anand** (MS09003) for the partial fulfillment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Prof. Purnananda Guptasarma (Supervisor)

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Dated: April 25, 2014

Declaration

The work presented in this dissertation has been carried out by me under the supervision of Prof. Purnananda Guptasarma at the Department of Biological Sciences, Indian Institute of Science Education and Research (IISER) Mohali.

This work has not been submitted in part or full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bona fide record of original work done by me and all sources listed within have been detailed in the bibliography.

Abhishek Anand

(Candidate)

Dated: April 25, 2014

In my capacity as supervisor of the candidate's thesis work, I certify that the above statements by the candidate are true to the best of my knowledge.

Prof. Purnananda Guptasarma
(Supervisor)

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Engineering spectroscopic reporters of structure and DNA-binding function in the histone-like protein, HU, through Phe-to-Trp substitutions.

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Abstract: Three phenylalanine residues in Histone like protein HUA of *E. coli* were mutated to tryptophan. Systematically, the experiments were conducted to monitor the effect of these mutations on the structure, stability and function of these mutant proteins. Of three, one mutant F51W (Mut 2) had a very high aggregation tendency, the other 2 mutants F47W (Mut 1) and F79W (Mut 3) also showed aggregation but had dimeric population also. Both the mutants show significant DNA binding. No significant change in structure of protein was observed on DNA binding, as observed by Far-UV CD spectra and trp fluorescence. Tryptophan anisotropy showed increase in anisotropy, more in case of Mut 1. Protein DNA complexes were irradiated with UV light to look for changes in DNA binding and size upon generation of tryptophan oxidation by-products.

Chapter 1

1. Introduction

1.1 Basic theory:

Histones are highly basic proteins, found in eukaryotes that help in compaction of DNA into structural units called nucleosomes. Different families of histones come together to form an octamer around which the DNA winds. Histones also undergo post translational modifications and hence play a role in gene regulation[1]. Prokaryotes lack histones, but use other proteins which due to same functional characteristics as histones, are termed as histone-like proteins. These proteins primarily include IHF (integration host factor), CbpA (curved DNA-binding protein A), CbpB (curved DNA-binding protein B), Fis (factor for inversion stimulation), DnaA (DNA-binding protein A), Dps (DNA-binding protein from starved cells), Lrp (leucine-responsive regulatory protein), H-NS (histone-like nucleoid structuring protein), Hfq (host factor for phage Qb), H-NS (histone-like nucleoid structuring protein), IciA (inhibitor of chromosome initiation A), and StpA (suppressor of td2 phenotype A) and HU (heat unstable nucleoid protein) [2]. Of these, HU is the most abundant and widely studied.

HU is a 90-99 amino acid alkaline, dimeric histone-like protein [3] capable of wrapping DNA. It belongs to DNAB II family of DNA binding proteins. Unlike other proteins of the family, HU is ubiquitous in all eubacteria, with more than one homologue in some species[4] (e.g. *Escherichia coli, Serratia marcescens*), where it exists both as homo and heterodimers of the two homologues. As all other histone-like proteins, it binds to the nucleoid and helps in DNA compaction. HU is present in very high concentrations i.e. 20μM/cell [5] (12,000 dimers/cell). It is also present in eukaryotes, and is localized in organelles such as chloroplast and mitochondria [6].

The structures have been solved for homodimeric HU protein from *Bacillus stearothermophilus*, *Thermotoga maritima* and *Anabaena* by using NMR and X-ray crystallographic analysis [7-10]. The amino-terminal half has two α-helices connected by a turn; the carboxy terminal half has one α-helix. The remainder of the protein has a three-stranded β-sheet structure which includes a β-ribbon extension in the middle (Fig. 1). This extension is disordered in absence of DNA[9] but in presence of DNA it becomes structured and binds to minor groove of DNA. Two monomers come together to form a compact α-helical body capped by β-sheets that extend as two β-ribbon arms.

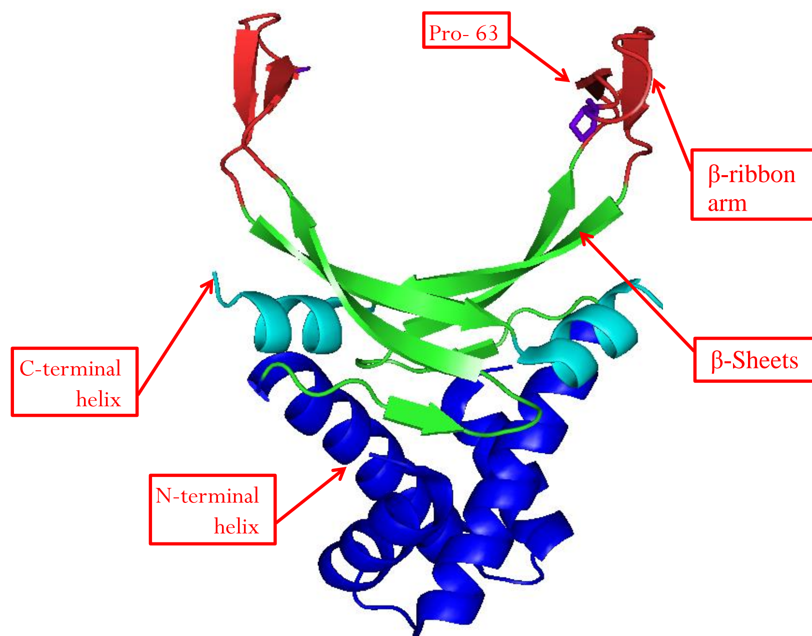

Figure 1: Structure of *Anabaena* HU showing the various structural elements.

Binding of HU to DNA is nonspecific. It binds to linear DNA, nicked as well as to bent DNA [11-13]. HU has high DNA binding affinity as reflected from binding constant which varies from 200-2500nM[14]. There is a highly conserved proline residue at position 63 at the tip of each β-ribbon arm (Fig. 1), which introduces kinks into the DNA at a spacing of 9 bp and causes stabilization of the bent DNA (by~105 to 139˚) by intercalating into the base-pair stack. Binding site length can vary from 9-42 bp, the variability comes from the fact that after binding

of β-ribbon arms to minor groove of DNA, there is an interaction between positively charged residues along the body of the protein and negatively charged groups of DNA to stabilize the interactions [15], which is variable under different conditions. This variable bending explains the more fluid structure of prokaryotic chromatin in comparison to eukaryotic.

HU also induces the formation of negative supercoiling, bends and binds DNA and helps in compaction of nucleoid, hence, plays a pleiotropic role. It is involved in various processes like DNA replication, stationary phase adaptive mutation, transcription, transposition, gene inversion, DNA repair and recombination.

Histone-like proteins, like histones are highly conserved, and there are no tryptophans in the sequence of both histone-like proteins and histones [16]. Is it that nature doesn't allow this to happen? Does the presence of tryptophan in such highly abundant DNA binding proteins have some negative effects on cells? Could such high abundance of tryptophans lead to photosensitized oxidation of DNA?

There are many factors, including UV, pollutants, metabolic end-products or presence of metal ions which are known to be responsible for formation of reactive oxygen species (ROS). These ROS lead to oxidation of tryptophan and hence formation of by-products like 5-hydroxy-tryptophan (5-HTP), oxindolylalanine (Oia), 3-hydroxyanthranilic acid (3-HAA), dioxindolylalanine, *N*-formyl-kynurenine (NFK), kynurenine (Kyn), hydroxykynurenine, and hydroxy-*N*-formyl-kynurenine[17,18]. These metabolites are known to cause DNA damage and act as carcinogens [17]

Also, because of absence of tryptophan in the sequence, it is difficult to use fluorescence spectroscopic techniques to probe structural changes and DNA binding in HU. In current study, we have used HU from mesophilic bacteria *E. coli.* In *E. coli*, HU has 2 homologues HUA and HUB, of these, we have used HU A. HU A contains three phenylalanine (Phe) residues at

positions 47, 51 and 79 (Shown in Fig 2). Residue 47 lies in the loop but residue 51 and residue 79 lie on β-strands that bind to DNA but side chains of these residues point inwards. We have made three different mutants by introducing tryptophans (Trp) (in place of phenylalanine) at the above mentioned positions. If Phe to Trp mutation in these mutants doesn't affect protein structure or its DNA binding properties, that mutant protein could serve as a substitute for wild type (WT) HU protein which can be studied using fluorescence techniques. The same mutants were used to explore the effects of incorporation of tryptophan in HU.

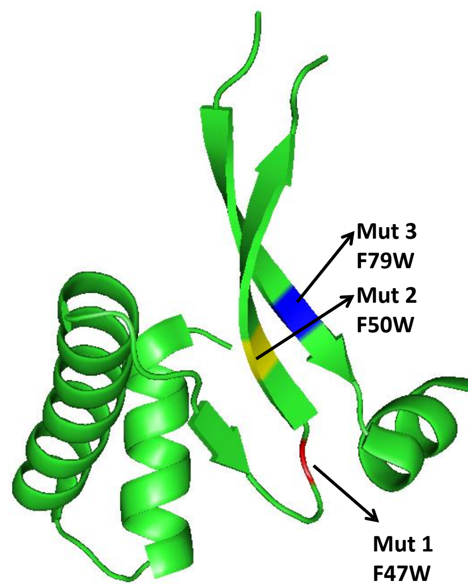

Figure 2: Crystal structure of *E. coli* HU A monomer with three Phe residues to be mutated to Trp highlighted.

1.2. Experimental Methods

1.2.1. Materials:

Primers were procured from Integrated DNA Technology (IDT). Molecular biology reagents like polymerases and ligases were procured from NEB. Restriction endonucleases were procured from fermentas. All media and buffer components were procured from Himedia. Isopropyl-β-thiogalactopyranoside (IPTG) and antibiotics (Ampicillin, Kanamycin and Tetracyclin) were obtained from Sigma. Ni-NTA Agarose/ Superflow resin was obtained from Qiagen. Superdex 75 10/300 GL column were procured from GE Healthcare Life Sciences. Dialysis tubing (molecular weight cut-off 3000 Da) was obtained from Thermo Scientific.

1.2.2. Cloning:

To mutate Phe residues to Trp, combination of SOE PCR and restriction digestion and ligation was used. Primers incorporating the mutations were designed using Gene Runner software. PCR and digestion reactions were performed as shown in Fig. 3(a) and Fig. 3(b) to obtain the spliced gene carrying the specified mutation. Spliced gene and pQE30 plasmid were digested with BamHI and HindIII restriction endonucleases and ligated. Ligation of mixtures were transformed into XL1Blue strain. Colonies obtained for each mutant were checked by colony PCR, followed by plasmid isolation. Plasmids were digested with BamHI and HindIII restriction enzymes to further confirm the presence of right size gene. Positive plasmids showing right size insert were sequenced to confirm the mutation.

47 F- CTGGTTGGTTGGGGTACCTTCAAAG

51 R- GGTTCACTTTCCAGGTACCGAATCC

79 F- CGTT CCGGCATGGGTTTCTG

79 R- CCAGAAACCCATGCCGGAACG

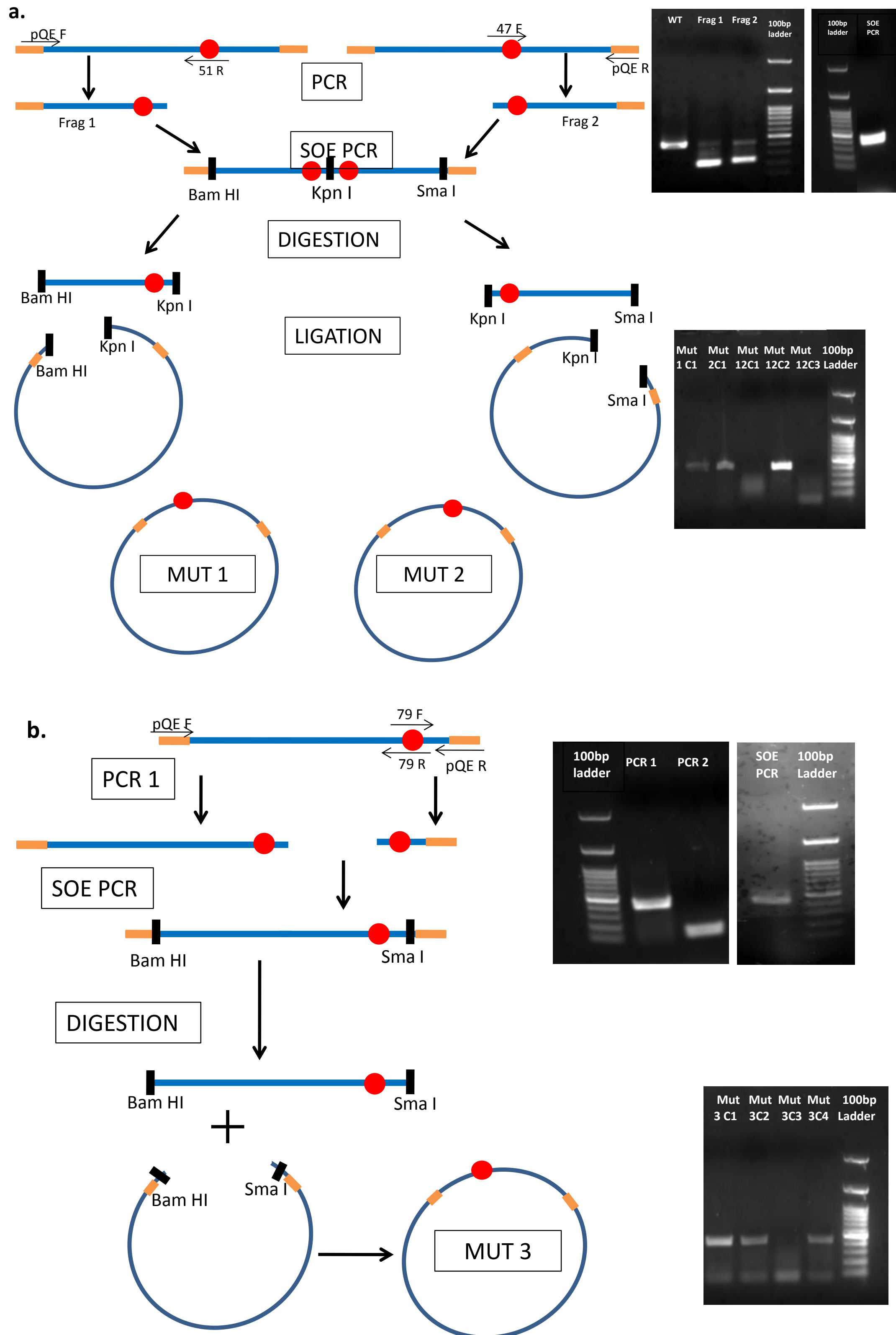

Figure 3: Schematic representation of cloning strategy used to mutate **(a)** F47 in Mutant 1, F51 in Mutant 2 and **(b)** F79 in Mutant 3.

1.2.3 Protein expression and purification:

Protein was expressed from XL1Blue strain. pQE30 contains His Tag at N-terminal of multiple cloning site such that the expressed protein contains His-Tag at N-terminal. His tagged protein was purified using Ni-NTA affinity chromatography under native conditions, followed by size exclusion chromatography using 24 ml Superdex 75 column. Proteins were dialyzed against Phosphate Buffer Saline (PBS) and all the experiments were done in the same buffer.

1.2.4. Electrophoretic Mobility shift Assay (EMSA):

For EMSA experiments, DNA template used was 4 way junction as used in [19]. 20ng of DNA was mixed with protein and loaded on 0.5% agarose gel. 4 way junction was reconstituted by mixing following 4 strands:

4WJ strand 1	CCCTATAACCCCTGCATTGAATTCCAGTCTGATAA
4WJ strand 2	GTAGTCGTGATAGGTGCAGGGGTTATAGGG
4WJ strand 3	AACAGTAGCTCTTATTCGAGCTCGCGCCCTATCACGACTA
4WJ strand 4	TTTATCAGACTGGAATTCAAGCGCGAGCTCGAATAAGAGCTACTGT

For all experiments, only DNA (4WJ) and only protein were run as controls.

1.2.5. Circular Dichorism:

Far UV-CD spectra for all the mutants was collected on Biologic CD spectrometer, using a 1 mm path length quartz cuvette. 0.2mg/ml of protein was used for all CD experiments. For each sample, three spectra were recorded (at a scan rate of 0.2nm/sec) and averaged. The final spectra were buffer subtracted, converted to Molar ellipticity (cm^2 $dmol^{-1}$) smoothened using Analysis software provided with the instrument. To monitor structural changes on DNA binding, 20μM of protein was mixed with 4μM and 8μM of DNA. Protein melting experiments

were performed on Chirascan(Applied Photophysics) system and samples were heated from 20˚C - 90˚C at a ramp rate of 1 degree/min and only the signal at 222 nm was monitored.

1.2.6. Fluorescence spectroscopy:

For fluorescence, 0.2 mg/ml of protein was used and spectra were collected on Horiba Fluoromax instrument. Samples were excited at 295nm and emission spectra were monitored in the range 300nm-400nm. Excitation and emission slit-widths used were 0.8nm and 2nm, respectively. For DNA binding experiments, 20μM of protein was mixed with DNA in ratios 5:1 and 10:1.

1.2.7. Tryptophan anisotropy:

Same concentration of DNA and protein were used as in fluorescence experiments. Excitation and emission wavelengths used were 295 nm (slit-width 2 nm) and 340 nm (slit-width 2 nm), respectively. Anisotropy value for each sample was collected 5 times and was averaged to get standard errors.

1.2.8. UV irradiation experiments:

To check if the UV irradiated oxidation of Trp has negative effects on genomic DNA and hence the cell growth, DNA samples were mixed with WT and mutant proteins were irradiated with UV light of 300 nm and slit-width of 5nm. And samples along with controls were run on 0.5% agarose gel.

Chapter 2

2. Summary and Conclusions

2.1. Results and Discussion:

2.1.1. Quaternary structure:

In native form, HU A exists as dimer and elutes at ~12 ml on Superdex 75 column, which is equivalent to 20 kDa with reference to the standard samples. To know the oligomeric status of the mutant proteins, Mut 1 and Mut 3 were also run on superdex 75 and both the mutants peak near void volume (8ml). For mutant 2 most of the protein eluted at void volume (data not shown) so all further experiments were done only with mutant 1 and mutant 3.

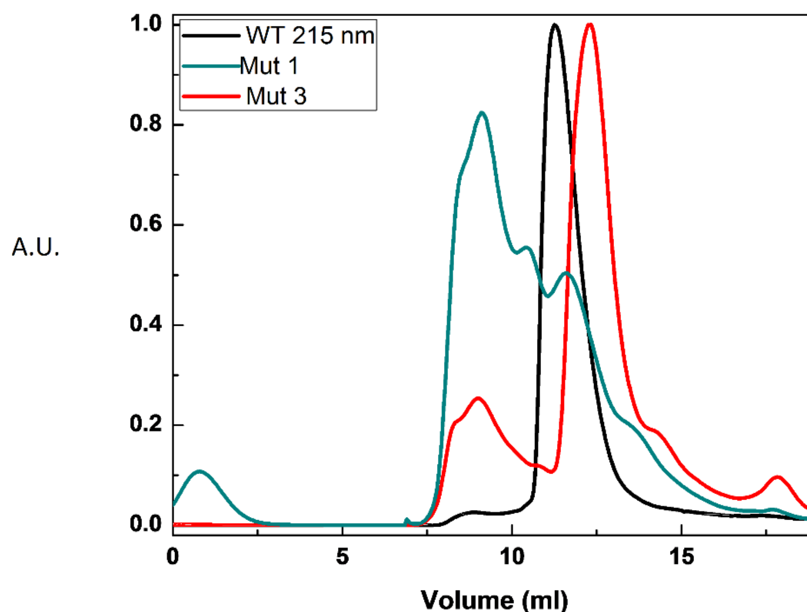

Figure 4: Superdex 75 gel filteration chromatograms of HUA WT (black), Mut 1 (Green) and Mut 3 (Red).

2.1.2. DNA binding:

DNA in wells in lanes 2-5 i.e. only protein and protein plus DNA lanes, indicates that like Wild type HU, both the mutants are bound to large sized genomic DNA in their purified form. On addition of 4WJ to the mutant proteins, there is a shift in mobility of 4WJ. This shows that both the mutant proteins can bind to DNA and are functionally active.

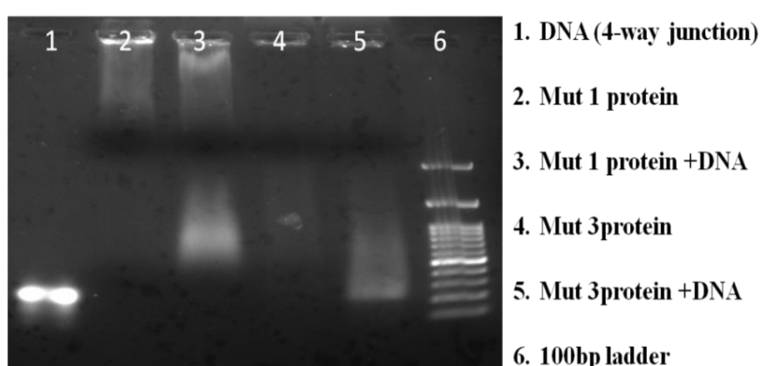

Figure 5: EMSA gel showing binding of the mutants to 4WJ.

2.1.3. Secondary structure:

CD spectra was collected for both the mutant proteins and compared to that of WT protein. Both the mutants show significant CD structure, Mut 1 being more similar to WT than Mut 3 which shows a decrease in intensity at 222nm and shift of 208nm peak to around 200nm.

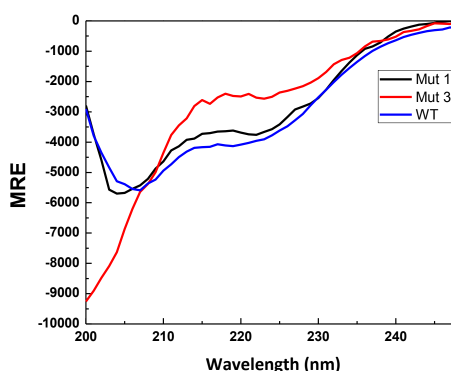

Figure 6: CD spectra showing secondary structure spectra of Mutant proteins and comparison to the WT protein.

2.1.4. Changes in structure on DNA binding:

As mentioned HU contains an unstructured region, which becomes structured only in presence of DNA, to look for such structural changes, protein was mixed with DNA in 5:1 and 10:1 molar ratios and CD spectra collected. No significant change was observed in secondary structure on binding with DNA.

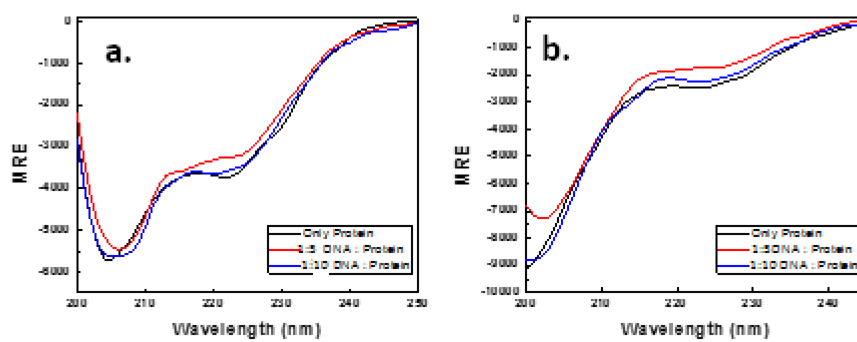

Figure 7: CD spectra of Mut 1 **(a)** and Mut 3 **(b)** in absence of DNA(black), in 5:1 Protein: DNA ratio (Red) and in 10:1 Protein: DNA ratio (Blue).

2.1.5. Chemical stability monitored using Circular Dichorism:

Protein samples were mixed with guanidine concentrations 0.1M, 0.2M, 0.5M, 1M, 1.5M, 2M, 2.5M and 3M, respectively and incubated overnight. CD spectra were collected for all the concentrations. Because of increase in HT at lower wavelengths, there is a lot of noise at lower wavelengths. 222nm value at all the guanidine concentrations were plotted for both the mutants for comparison. As observed in Fig. 6, Mut 3 has more negative MRE value at 222 nm in comparison to Mut 1, so the starting point of melting for both mutants is different but the final MRE value for both the mutants is same.

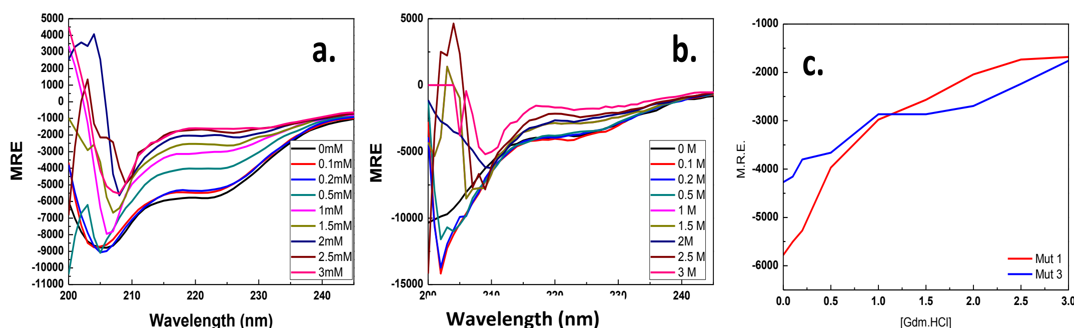

Figure 8: CD spectra of proteins incubated in different guanidine concentrations, Mut 1 **(a)** Mut 3 **(b)** and comparison of change in 222 nm value w.r.t. change in guanidine concentration **(c)**.

2.1.6. Thermal stability

0.2mg/ml of WT, Mut 1 and Mut 3 were heated inside the instrument from 20-90 degree and intensity at 222nm was monitored. Like the WT protein, none of the mutant unfolds completely.

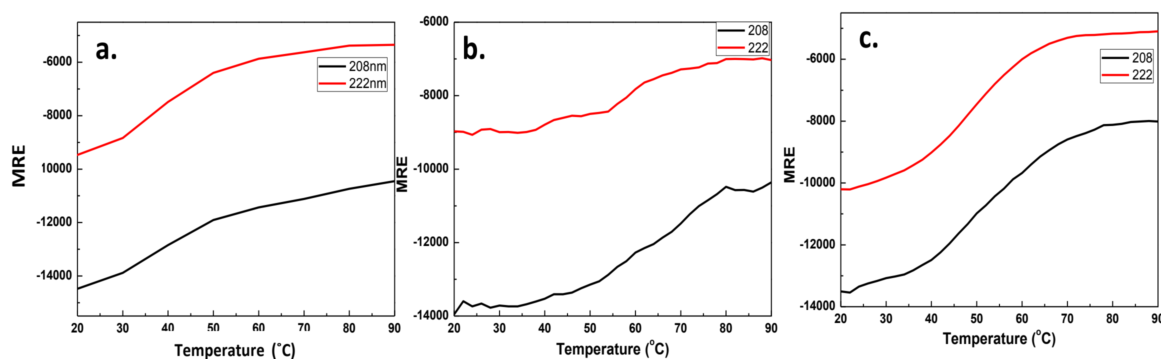

Figure 9: Thermal denaturation profiles of WT **(a)**, Mut 1 **(b)** and Mut 3 **(c)**.

2.1.7. Chemical stability monitored using tryptophan fluorescence:

The same guanidine concentrations as mentioned in CD experiments were used. Because of excitation at 295nm, raman spectra is also contributing to emission spectra. To compare both the mutants, ratio of intensity at 320nm and 360 nm was plotted.

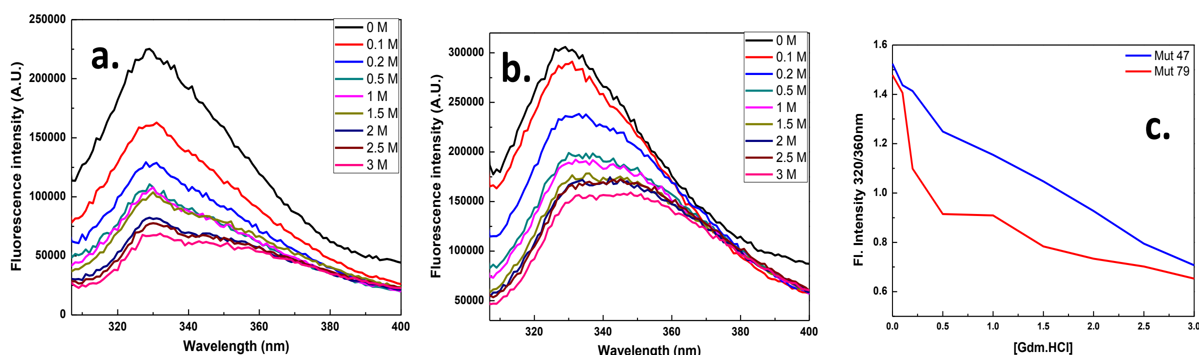

Figure 10: Fluorescence spectra of proteins incubated in different guanidine concentrations, Mut 1 **(a),** Mut 3 **(b)** and ratio of 320 and 360nm w.r.t. change in guanidine concentration **(c)**.

2.1.6. DNA binding based on changes in tryptophan fluorescence

Tryotophan fluorescence of Mutant proteins was monitored in presence in presence and in absence of DNA to look for spectral changes upon DNA binding. No significant change in spectral properties was observed.

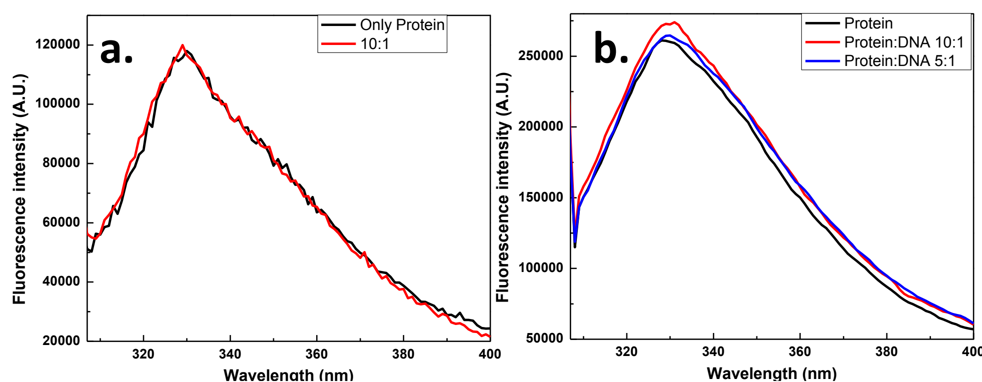

Figure 11: Fluorescence spectra of Mut 1 **(a)** and Mut 3 **(b)** in absence of DNA (Black), in 5:1 Protein: DNA ratio (Blue) and in 10:1 Protein: DNA ratio (Red).

2.1.7. DNA binding by monitoring tryptophan anisotropy:

Tryptophan anisotropy increases when the movement of protein is restricted by association with a bulky molecule. So, increase in anisotropy values is expected upon DNA binding. To avoid errors in data anisotropy value was collected multiple times and averaged.

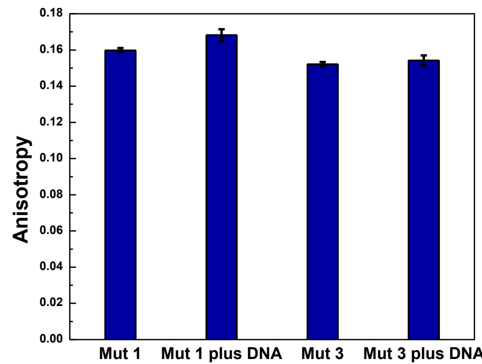

Figure 12: Change in anisotropy values on addition of DNA to Mut 1 and Mut 3 proteins.

2.1.8. Tryptophan mediated photosensitized DNA degradation?

Gel filtration, CD and EMSA results suggest that the mutant proteins are folded as well as functional. This indicates that tryptophan does not have any effect on either structure or function of protein. To explore the effect of tryptophan oxidation by-products on DNA, UV was irradiated on protein plus 4WJ mixture and run on EMSA. As seen in only protein controls, in WT protein the bound DNA (that co-elutes with protein after purification) is higher molecular weight in comparison to the bound DNA in case of mutants. Possible explanation is that low amounts of UV light have already degraded the bound DNA in mutants but not in WT protein because of lack of trp residues. This needs to be explored further but this indicates DNA damage induced by UV.

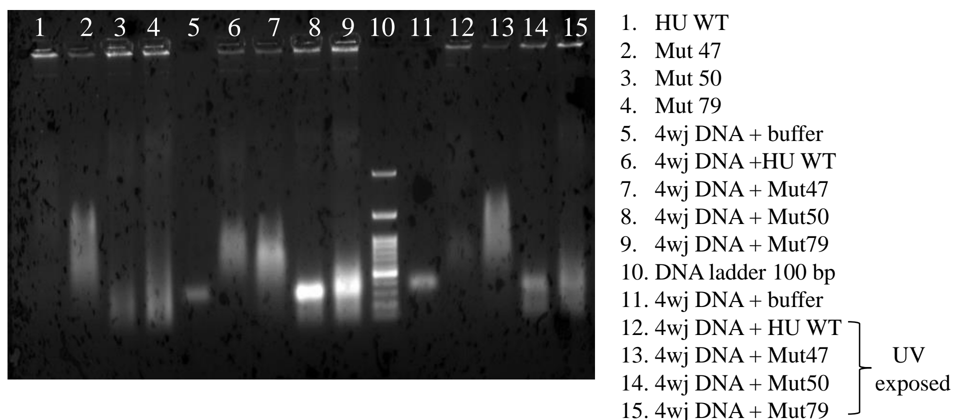

Figure 13: EMSA gel showing binding of WT and Mutant proteins to 4WJ and effect of UV exposure on DNA binding.

2.2. Conclusions and Future Perspectives:

Two mutants Mut1(Mut47) and Mut3(Mut79)were examined. Both fold similarly to wild type. Both have dimeric population but also aggregate. Both show shift in EMSA upon DNA binding. Both are purified in a form bound to large genomic DNA from *E.coli*, like wild type HU. No significant change in structure is observed upon DNA binding, through CD spectroscopy or fluorescence spectroscopy. Mut1(Mut47) shows significant change in fluorescence anisotropy of tryptophan upon DNA binding, indicating that 'W' at 47 position can be used as a spectroscopic probe of DNA binding and also Mut1(Mut47) is more like wild type in DNA binding. Introduction of tryptophan appears to be associated with reduced size of pre-bound DNA (DNA in wells appears to be getting degraded). More experiments need to be done to further explore the reason of absence of tryptophans from Histones and Histone-like proteins.

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