Chapter 4

Structural and Functional Studies on β-bulge Mutants of Ubiquitin

4.1. Introduction

As described in **Chapter 1** ubiquitin is 76 residues long, single domain α/ β protein. Along with five strands of β-sheet, 3.5 turns of α-helix and a short 3_{10} helix, ubiquitin has two β-bulges. The β- bulge is caused by an extra residue on the bulged strand. The extra residue increases the backbone length, disrupts the hydrogen bonding pattern and causes the strand to bulge out of the sheet. Usually, β-bulges partially overlap with β-turns. The N terminal bulge consists of residues Glycine at 10^{th}, Lysine at 11^{th} and Threonine at 7^{th} positions. This bulge has been studied in detail and found to be crucial in the early folding of ubiquitin (Cox et al., 1993a). The second β-bulge is present at the C-terminal end in a parallel β-sheet and associated with a type II turn which is rarely found in proteins (Vijay-Kumar *et al*., 1987). The second β-bulge is formed by residues Glutamine, Glutamate and Serine at 2^{nd}, 64^{th} and 65^{th} positions respectively.

Glu64 is positioned as 1^{st} residue on C-terminal β-bulge and 3^{rd} on a type II turn (Vijay-Kumar *et al.*, 1987). The possibility of the presence of Glu on β-bulge is just 0.23% compared to Gly with 11.3% (**Table. 4.1**) (Chan et al., 1993). Moreover, Glu64 displayed unusual Φ and Ψ angles on the Ramachandran plot. These observations suggested Gly as the more suitable candidate for 1^{st} position on β-bulge compared to Glu. In addition, structural homologs of ubiquitin RUB1 and NEDD8 also showed the presence of Gly residue at the 1^{st} position (Jentsch and Pyrowolakis, 2000).

The next residue in the sequence is Ser65, which is present at the 2^{nd} position of β-bulge and last on Type II turn (Vijay-Kumar *et al.*, 1987). The frequency of occurrence of Ser65 at second position 1.5% compared to Asp with 2.4% (**Table. 4.1**) (Chan et al., 1993). Moreover, Asp was also found to be present at 2^{nd} position on the structural homologs of ubiquitin namely,

SMT3, SUMO-1, SUMO-2, SUMO-3 and NEDD8 (Jentsch and Pyrowolakis, 2000).

The 3rd residue on β-bulge which is called X-position is held by Gln2, present on the N-terminus of β-sheet. Gln2 residue forms hydrogen bonds with Glu64 and Ser65 making them bulge out (Vijay-Kumar *et al.*, 1987). The frequency of occurrence of Gln at X-position of β-bulge is 0.34% compared to ASN with 5.4%, which is much higher (**Table. 4.1**) (Chan et al., 1993). The presence of Asn at X-position was also found in ubiquitin's structural homologs, SMT3, SUMO-2 and SUMO-3 (Jentsch and Pyrowolakis, 2000).

Table 4.1. **Percentage frequencies of occurrence of amino acids at first, second and X-position of β-bulge (Chan et al., 1993).**

Position on β-bulge	**Amino acid present on β-bulge**			**Amino acid replacement**		
	Amino Acid (i)	**Faai/ Bulge**	**Naai**	**Amino Acid (i)**	**Faai/ Bulge**	**Naai**
First position	Glu	0.23	1	Gly	11.30	84
Second position	Ser	1.5	8	Asp	2.4	10
X-position	Gln	0.34	1	Asn	5.4	18

Faai/Bulge: percentage frequency of occurrence of amino acid i in a β-bulge.

Naai: Total number of amino acid i found in a β-bulge.

From the above observations, it became interesting to know why nature selected and conserved less preferred amino acids for β-bulge. To understand this anomaly, our laboratory generated Glu64 to Gly (E64G), Ser65 to Asp (S65D) and Gln2 to Asn (Q2N) mutants of yeast ubiquitin (Fig.4.4) (Mishra et al., 2009, 2011). The structural characterization of the individual mutants was carried out by CD and Fluorescence spectroscopic techniques.

Observations based on structural studies of E64G suggested a slight change in structure by a 4-5% decrease in helicity and more stability under guanidine hydrochloride denaturation condition (Mishra et al., 2009). Mutation S65D also resulted in minor changes in the structure like change in the position of the hydrophobic region but the stability of protein remained unchanged indicating the mutation was well accommodated within the structure of the protein. Q2N mutation also did not have any effect on the secondary structure of protein although the stability of protein decreased marginally (Mishra et al., 2011). Results were in support of the hypothesis as residues naturally present inside the bulge were replaced with residues that have a greater preference for the same secondary structure improved stability of the protein.

Once the structural assessment of β-bulge mutants Q2N, E64G and S65D were done, it was found to be stable and carried similar secondary structural characters as that of wild type ubiquitin, our lab decided to check for the conservation of functionality of the mutants.

In yeast, ubiquitin comprises a family of four genes *UBI1, UBI2, UBI3* and *UBI4*. Out of these four genes *UBI1, UBI2* and *UBI3* express single ubiquitin gene fused with ribosomal subunit protein while *UBI4* expresses polymer of ubiquitin having five repeats of ubiquitin fused head to tail. All four genes are expressed during the exponential phase, whereas expression of *UBI1* and *UBI2* genes are found to be suppressed during the stationary phase.

Under stress conditions, *UBI4* is strongly expressed as it contains a heat shock box upstream of the coding region. The *UBI4* gene is essential to produce ubiquitin moieties under various stress conditions like heat stress, nutritional stress, UV stress and antibiotic stress to the cell (Finley *et al.*, 1987, 1994; Ozkaynak *et al.*, 1987). If *UBI4* is deleted from cells, they would function normally under normal conditions but they would be highly sensitive under various stresses. As cells will be exposed to certain stress conditions, a pool of misfolded and denatured protein aggregate would form inside the cell. To combat this condition, cells need to increase the rate of ubiquitination to get rid of unwanted aggregates and help cell survival.

Yeast SUB60 cells gifted by Prof. Daniel Finley consisted of intact cellular machinery but lacked the *UBI4* gene (Finley *et al.*, 1987). So it became an ideal system to check if mutated ubiquitins are functioning efficiently to ensure cell survival under stressful conditions. In earlier work, our laboratory addressed the question of functionality of the mutant ubiquitins Q2N, E64G and S65D by expressing them in SUB60 cells and checked for stress complementation (Mishra et al., 2009, 2011).

The β- bulge mutants of ubiquitin Q2N, E64G and S65D found to be extending the half-life of ubiquitin-Pro-Protein fusions, where proline is the N-terminal residue of the protein (Ub-Pro-β-galactosidase). ubiquitin-Pro-Protein fusions are degraded by ubiquitin fusion degradation (UFD) pathway and these mutations inhibit the degradation of proteins by the UFD pathway. In the presence of translational inhibitor cycloheximide, the survival of cells decreased in mutant ubiquitin containing cells compared to cells expressing wild-type ubiquitin. however, cells with β-bulge mutants complemented heat stress and survived equally well as wild-type ubiquitin containing cells (Mishra et al., 2009, 2011).

As discussed above, in yeast cells ubiquitin is expressed from four genes UBI1, UBI2, UBI3 and UBI4. The first three are house keeping. UBI4

is a polyubiquitin gene, the expression of which is induced under stress conditions. Previously all the *in vivo* studies with mutant forms of ubiquitin were carried out in the UBI4 deletion mutant, as it is a standard method. *In vitro* studies provide direct and unequivocal evidence on actual interactions between enzymes of the ubiquitination pathway and mutant forms of ubiquitin. Hence, activation of mutant forms of ubiquitin by the E1 enzyme was addressed in the present study.

The intracellular pH of yeasts is different from that of other eukaryotic cells. Previously, some work was done on the effect of pH on β-bulge mutants of ubiquitin CD spectra were recorded at pH values of 2.2, 5.0, 7.4 and 10 (Pradeep Mishra's Thesis). As a continuation of this work on pH CD spectra was recorded at regular intervals 2 pH units and the results were analyzed.

4.2. Plan of Work

4.2.1. Assay of Ubiquitin Activation by E1

Ubiquitin β-bulge mutants Q2N, E64G and S65D present in vector pKK223-3 were purified by size exclusion chromatography. pKK223-3 containing the gene for UbF45W was received as a gift by Prof. Mark Searle's laboratory (School of Chemistry, Nottingham, United Kingdom). Ubiquitin activation assay with E1 enzyme and ubiquitin mutants was done as described in **Fig. 4.1**.

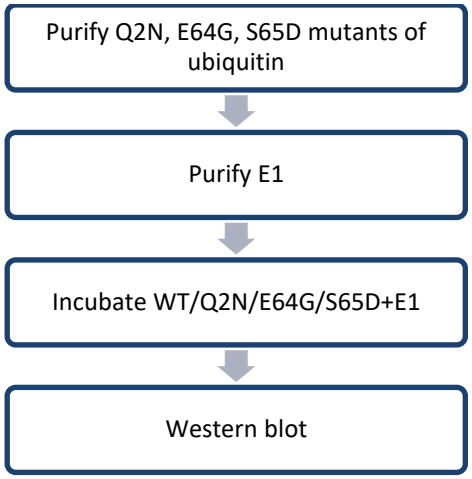


Fig. 4.1. Schematic diagram of work plan A) Purification β-bulge mutants Q2N, E64G and S65D, B) Purification ubiquitin activating enzyme E1, C) Incubation of mutant ubiquitin and E1 proteins, D) western blot analysis.

4.2.2. Testing the Effect of Different pHs on β-bulge Mutants of Ubiquitin

Purify ubiquitin wildtype and β-bulge mutants Q2N, E64G, and S65D proteins. Treat these proteins with a range of different pH buffers. Now, record far-UV CD spectra and check for structural intactness.

4.3. Materials and Methods

4.3.1. Strains, Media and Plasmid

E. coli bacterial strain BL21 DE3 (BF^- ompT gal dcm lon hsdSB(rB^- mB^-) λ(DE3 [lacI lacUV5-T7p07 ind1 sam7 nin5]) $[malB^+]_{K\text{-}12}(\lambda^S)$ was used to express proteins for the purpose of purification. The media used to grow *E. coli* was Luria broth and Luria agar (Hi-media). Ubiquitin mutants were

encoded in the pKK223-3 vector. The selection marker for vector pKK223-3 containing β-bulge mutants is ampicillin (100μg/ml). Kanamycin was used for pETE1 plasmid at the concentration of 100μg/ml.

4.3.2. Purification of Ubiquitin F45W and β-bulge Mutants Q2N, E64G and S65D

For this experiment, we used ubiquitin mutation F45W as a wild-type ubiquitin. This mutation does not have any effect on the structure of the protein and has the advantage of having tryptophan for fluorescence analysis. All the β-bulge mutants were constructed in the F45W variant of ubiquitin. Ubiquitin F45W and β-bulge mutants Q2N, E64G and S65D present in pKK223-3 plasmid construct were expressed in *E. coli* strain BL21 (DE3) under the control of IPTG inducible p*tac* promoter. Cells were grown at 37°C to the mid-log stage and harvested after overnight induction (1mM IPTG). Cells were then re-suspended in lysis buffer pH7.8 containing 5% glycerol, 50mM Tris and 2mM EDTA. Now cell lysis was carried out by sonication under conditions, 40% amplitude for 4-5 minutes with an interval of 2 seconds pulse on and off. Lysed cells were centrifuged at 12k RPM for 10 min at 4°C. The cell lysate was treated with 1mM protease inhibitor cocktail. Now, the supernatant containing the remaining soluble cellular proteins was collected and treated with 0.2% polyethyleneimine (PEI). This step precipitates DNA content which then removed by centrifugation at 12k RPM for 10 minutes. As ubiquitin is a heat-stable protein, the supernatant collected from the above step is heat treated at 80°C for 10 minutes. The coagulated proteins were removed by centrifugation at 12k RPM for 30 minutes. Ubiquitin is further precipitated out of the supernatant at 40% ammonium sulfate. Again the precipitates were removed by centrifugation at 12k RPM for 30 minutes. To remaining supernatant 80% ammonium sulfate was added and centrifuged for 30 minutes at 12k RPM. Now, the pellet was collected and resuspended in 20mM tris

buffer (pH 8). After dialysis ubiquitin containing fraction was purified on Sephadex G-50 column. The protein was concentrated by precipitation and dialysis.

4.3.3. Purification of E1 Protein

The protocol followed for the purification of the E1 enzyme from the pETE1 vector was as described below. *E. coli* BL21 DE3 Cells transformed pETE1 was grown at 37°C in Luria broth (LB) containing antibiotic kanamycin (100µg/ml) to the mid-log stage. Cells were induced by 1mM IPTG overnight and harvested. Now, cells were resuspended in lysis buffer containing 50mM Tris, 2mM EDTA, 5% glycerol, pH7.8. Cell lysis was done by sonication under 40% amplitude for 4-5 minutes with an interval of 2 seconds pulse on and off. The cell lysate was then centrifuged at 12000rpm for 10 min at 4°C. A protease inhibitor cocktail was added to inhibit proteases. Following centrifugation, the supernatant containing the remaining soluble cellular proteins was collected. The collected supernatant was treated by Ni-NTA chromatography. Samples were run 12% SDS PAGE.

4.3.4. Method for Assay of Ubiquitin Activation by E1

The ubiquitination assay kit from Abcam (ab139467) was used for the detection of binding of E1 with ubiquitin variants. Further, western blot analysis was done by the anti-ubiquitin antibody for the detection of the presence of ubiquitin. protocol for the assay is as described in **Table 4.2.**

Table 4.2. Components of Ubiquitin Activation by E1 Assay

Sr. No.	Component	Volume (50 µl)
1	dH_2O	26.5
2	10X Ubiquitination Buffer	5
3	IPP (100 U/mL)	10
4	DTT (50 mM)	1
5	Mg-ATP (0.1 M)	2.5
6	20X E1 (2µM)	2.5
7	20X Ub (50µM)	2.5

All the components were mixed as described in **Table 4.2** and were incubated for 4 hours at 37°C. Samples were run on 12% SDS-PAGE and preceded for western blot using an anti-ubiquitin antibody.

4.3.5. Method for Western blotting

The nitrocellulose membrane was used for the transfer of protein bands. The membrane was activated by 20% methanol by incubating for 1 minute. It was rinsed by transfer buffer (25 mM Tris, 192 mM glycine in 20% methanol, pH 8.3) before the preparation of the gel-membrane stack. The transfer was carried out for 120 minutes at 100V. Once the transfer was done, the membrane was blocked by 5% BSA and incubated for 1 hour at 4°C. The

primary antibody used here was the anti-ubiquitin antibody. It was added to the blot and was incubated overnight at 4°C. Blot was washed by PBST buffer (Phosphate buffer with 0.1% Tween-20) 5 times for 10 minutes. Now, secondary antibody HRP conjugated Anti-Mouse IgG was added to the membrane and kept for 1-hour incubation at 4°C. Again membrane was washed by PBST 5 times, for 10 minutes every time. The membrane was developed by ECL (Enhanced Chemiluminescence) kit.

4.3.6. Method to Check the Effect of Different pH on Ubiquitin (F45W) and β-bulge Mutants Q2N, E64G and S65D

Proteins were prepared in 20 mM Tris-Cl (pH 8.0) buffer and the concentration of proteins used in this experiment was 25 μM. Purified ubiquitin protein F45W and β-bulge mutants Q2N, E64G and S65D were incubated with various buffers. The range of pH buffers taken was from pH-2.0 to pH-8.0. After which far-UV-CD spectra were recorded to check the structural integrity of the protein. Buffer composition is given below in **Table 4.3**.

Table 4.3. Buffer composition used to check the effect of different pHs on ubiquitin β-bulge mutants. (Buffers were prepared and adjusted to required pH).

Sr. No.	pH value	Buffer Composition
1	2.0	0.1M Potassium Chloride + 0.1M Hydrochloric Acid
2	4.0	0.1M Acetic Acid + 0.1M Sodium Acetate
3	6.0	0.1M Sodium Phosphate Monobasic + 0.1M Sodium Phosphate Dibasic
4	8.0	0.1M Tris + 0.1M Hydrochloric Acid

Jasco J-715 CD spectrophotometer was used to record the Far UV CD spectra of all proteins. The range for Far-UV-CD spectra was 200 to 250 nm. The path length was 1 mm. All the spectra were blank corrected. The spectra were accumulated for five times to improve the signal-to-noise ratio. Scan speed was 50 nm/ sec.

4.4. Results

4.4.1. Purification of Ubiquitin F45W and β-bulge Mutants Q2N, E64G and S65D

F45W ubiquitin and its mutant forms were purified by the method described earlier. These purified proteins were analyzed on 15% SDS-PAGE (**Fig. 4.2**). The purity of F45W ubiquitin and its mutant forms was confirmed.

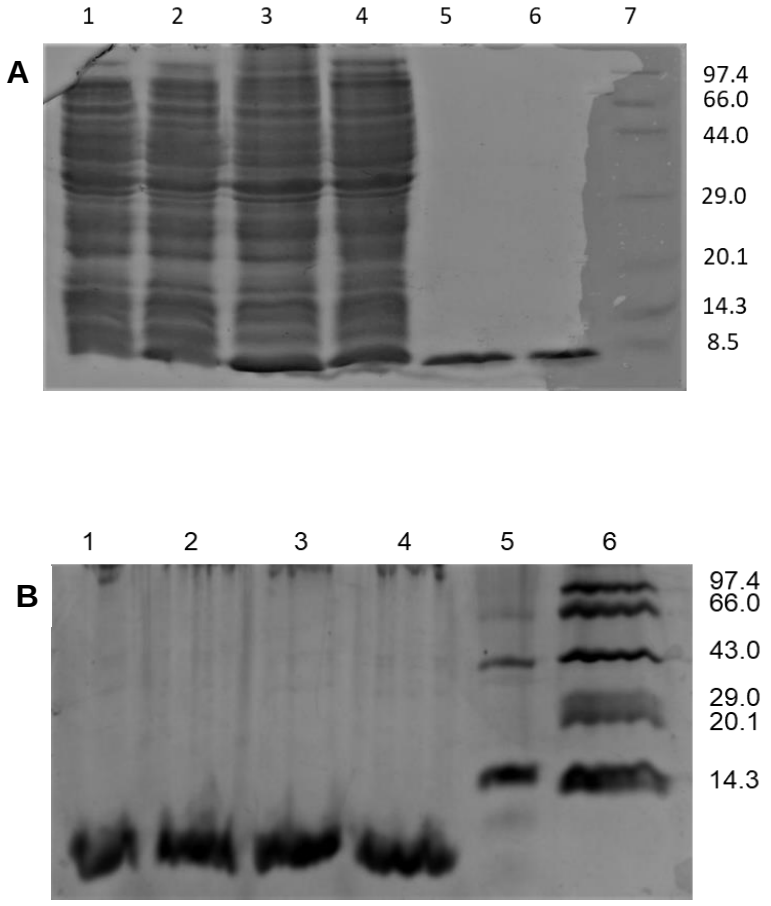


Fig. 4. 2. **Expression and purification of ubiquitin wild type and β-bulge mutants.** A) Lane 1 contains BL21 DE3 cells, Lane 2 is BL21 DE3 cells transformed with F45W Ubiquitin in the absence of inducer, Lane 3 is cells transformed with F45W Ubiquitin with 1mM IPTG inducer, Lane 4 is BL21 DE3 cell lysate expressing F45W ubiquitin protein, Lane 5 is purified ubiquitin F45W protein (8kDa), Lane 6 is purified Q2N protein (8kDa), Lane 7 is protein molecular weight marker.

B) Lane 1 contains purified ubiquitin F45W protein (8kDa) as a marker, Lane 2 is purified Q2N protein (8kDa), Lane 3 is purified E64G protein (8kDa), Lane 4 is purified S65D protein (8kDa), Lane 5 is protein molecular weight marker, Lane 6 is protein molecular weight marker.

The purified β-bulge mutant proteins were concentrated by ammonium sulfate precipitation and the precipitated product was redissolved in buffer dialyzed to get a purified product to conduct the experiments.

4.4.2. Purification of Ubiquitin Activating Enzyme E1

UBA1 gene of Ubiquitin activating enzyme E1 cloned in pET28a was received as a gift. The gene UBA1 in the pET28a vector has a 6x-His-tag as N-terminal fusion. The protein was expressed under the following conditions. Mid-log cultures of cells were added with 1 mM IPTG to induce the protein overnight. The next day the cells were harvested and loaded on 12% SDS PAGE to check purification (**Fig. 4.3**).

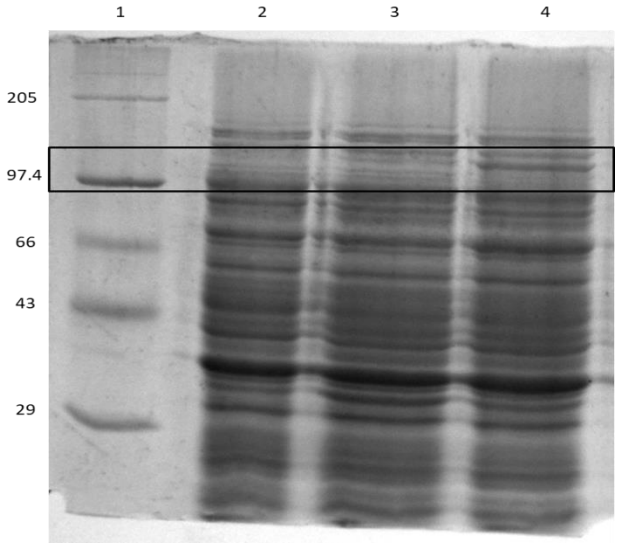


Fig. 4.3. **Expression of E1 protein.** Lane 1 contains protein molecular weight marker, Lane 2 contains BL21 DE3 cells, Lane 3 consists of BL21 DE3 cells with transformed pETE1 plasmid without inducer, Lane 4 has BL21 DE3 cells with transformed pETE1 plasmid with 1mM IPTG inducer which shows E1 expression at ~110kDa.

The expression of E1 we obtained was very low. To improve the quantity of expression of E1 protein various parameters were tried. The concentration of Inducer IPTG was varied from 0.4mM to 2mM and cells were incubated for various time durations ranging from 2 hours to overnight.

Purification of E1 by affinity chromatography was attempted with cultures showing mild expression. However, the protein levels in the scaled-up experiment were so low that the band could not be observed at all in SDS-PAGE. In spite of trying with different buffers during lysis like 20mM Tris, PBS buffer, PBS buffer with denaturing agent urea, the protein could not be observed in the lysate. As E1 could not be purified even after repetitive attempts under various conditions, the Abcam kit had to be used for other experiments.

4.4.3. Assay of Ubiquitin Activation by E1

To check ubiquitin activation by E1, an assay was performed where ubiquitin and its mutants were incubated with E1. Free ubiquitin was used as a control, where E1 was not added which could be seen in lane 1 (**Fig 4.4)**. Lane 2 was another negative control where ubiquitin was not added to the reaction, only E1 enzyme was added which did not show reaction with anti-ubiquitin antibody, hence no bands could be seen. Lane 3-7 was reaction mixtures of ubiquitin and its β-bulge mutants incubated with E1.

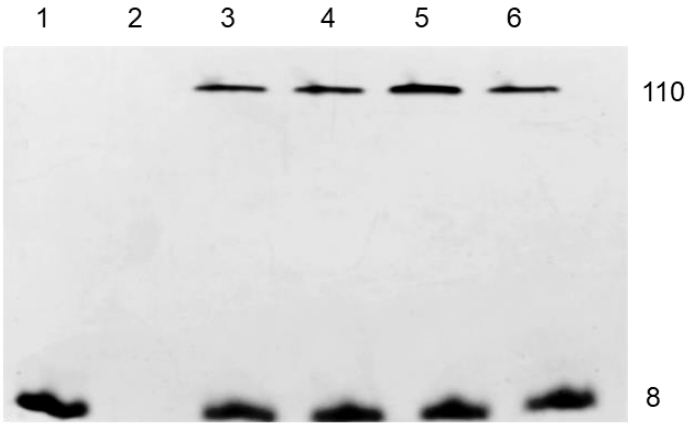


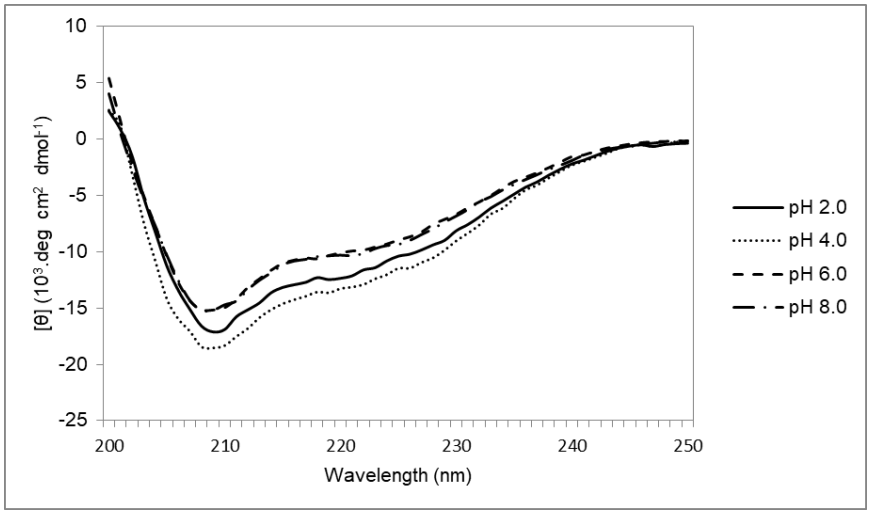
Fig. 4. 4. **Western blot analysis of ubiquitin activation by E1.** Lane 1 is free ubiquitin acting as a control, Lane 2 is Free E1 acting as negative control, Lane 3 is wild type ubiquitin F45W incubated with E1, Lane 4 is mutant ubiquitin Q2N incubated with E1, Lane 5 is mutant ubiquitin E64G incubated with E1, Lane 6 is mutant ubiquitin S65D incubated with E1.

Western blot was developed using an anti-ubiquitin antibody which showed a band at around 110kDa which indicated the presence of Ub~E1 complex and free ubiquitin showed the second band at ~8kDa. This result indicated all β-bulge mutants are capable of reacting with E1 upon activation. Hence, the intensity of the bands of all three ubiquitin mutants Q2N, E64G and S65D were comparable to that of wild type ubiquitin. Therefore they are activated by E1almost at the same level as wild type ubiquitin and showed binding to its active site.

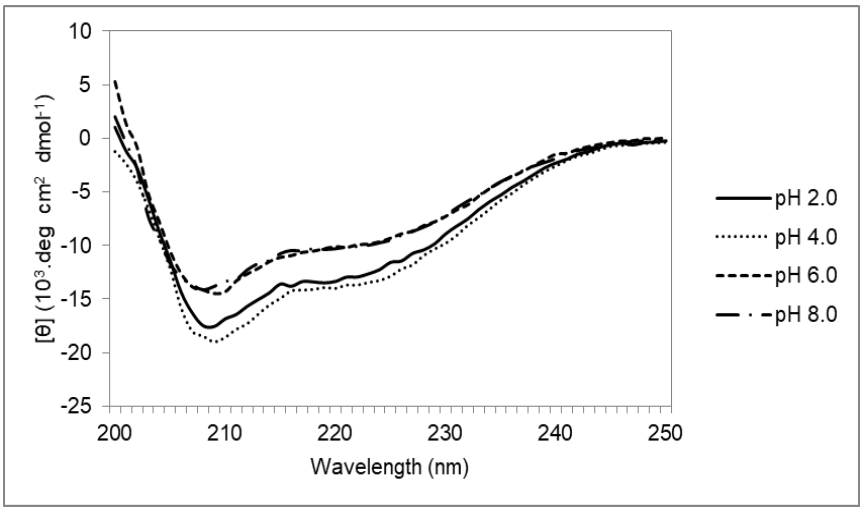
4.4.4. Effect of Different pH on Ubiquitin (F45W) and β-bulge Mutants Q2N, E64G and S65D

Far-UV-CD spectra were recorded to check the effect of mutations on the stability of the protein. Results are plotted as a graph and compared with wild-type ubiquitin protein.

A



B



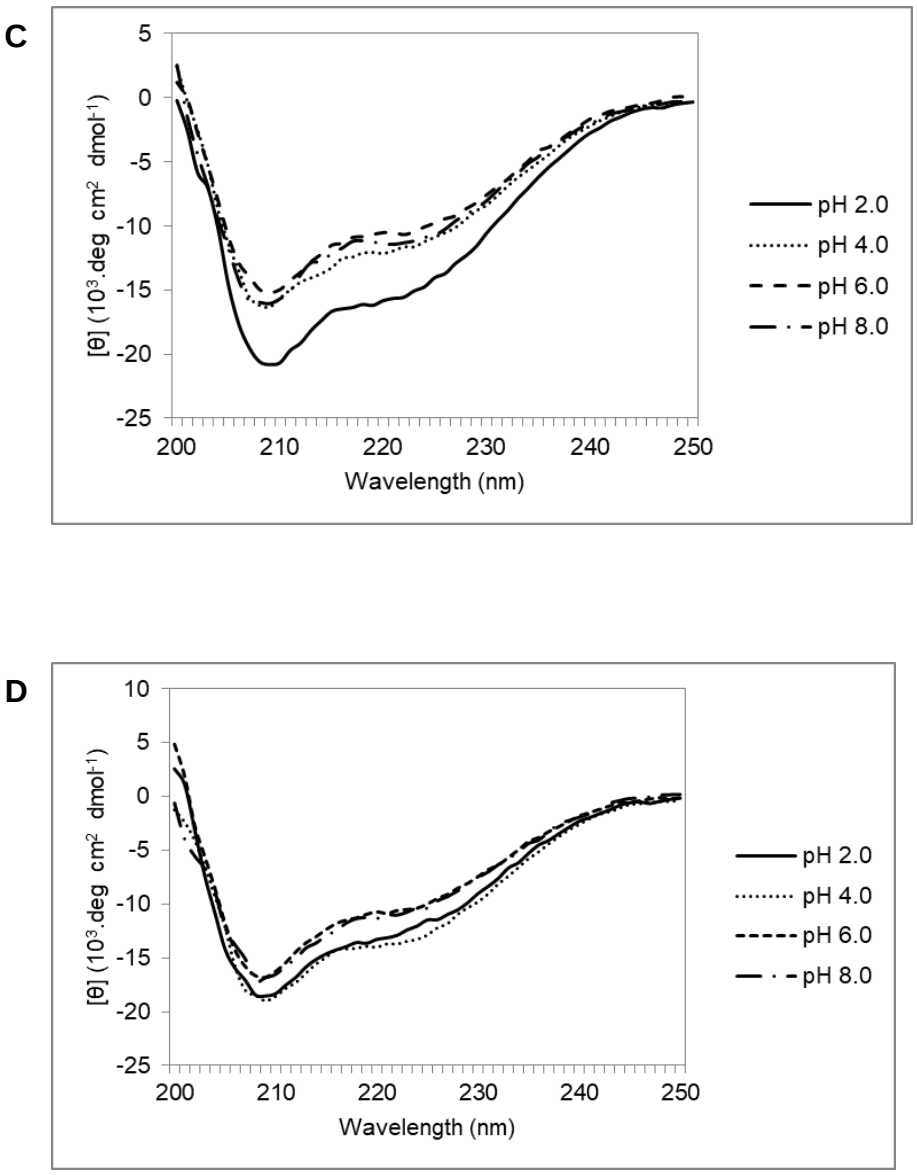


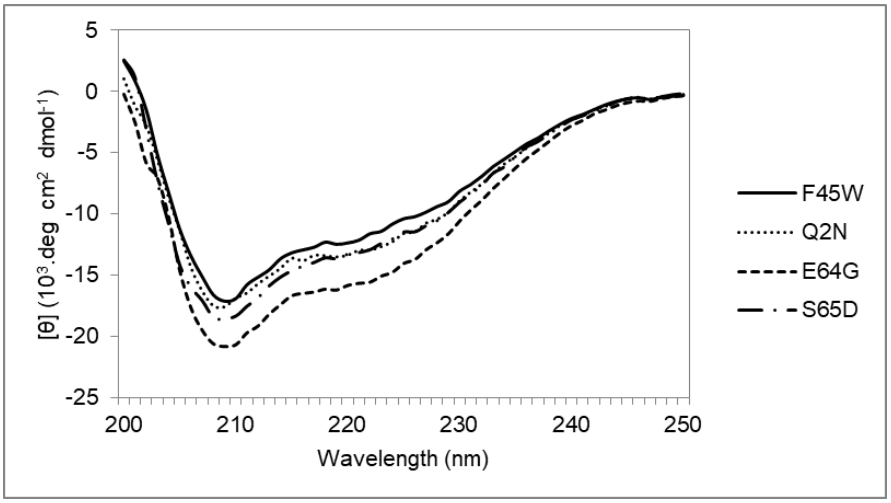
Fig. 4.5. CD spectra of Ubiquitin (F45W) and β-bulge mutants. A) Ubiquitin F45W, B) Ubiquitin Q2N, C) Ubiquitin E64G, D) Ubiquitin S65D with each mutant showing effect of different pH point.

From results (**Fig 4.5**), it can be seen there is minimal effect of pH change on wild-type ubiquitin F45W and β-bulge mutant S65D. All three mutants and wild-type ubiquitin have retained their secondary structure.

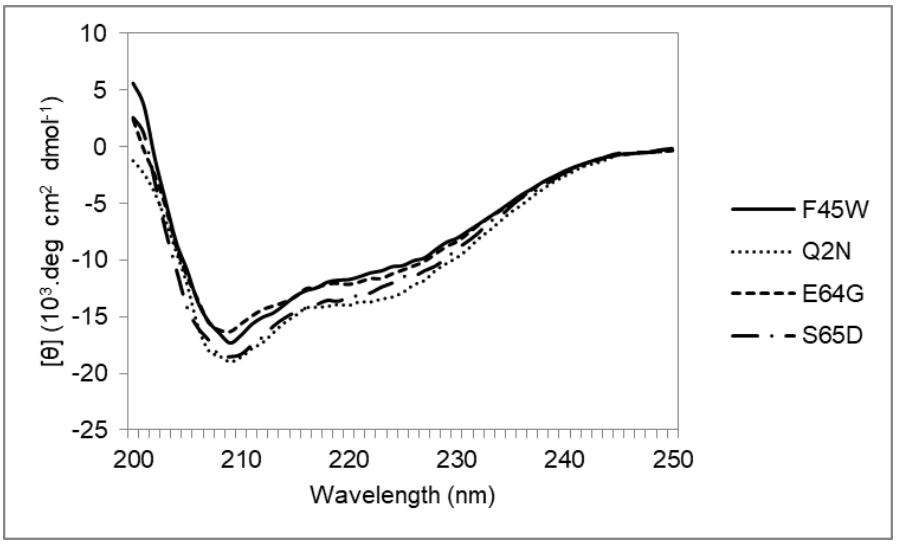
However, β-bulge mutant E64G is showing increased helicity at pH 2.0, and Q2N is also showing some change in spectra at pH 2.0 and pH 4.0. This effect might be there due to loss in β-sheet content, which resulted in random coils in turn showing higher helicity.

Later, we decided to check the specific pH point to get a better picture of the comparative effect on the structure of all ubiquitin variants.

A



B



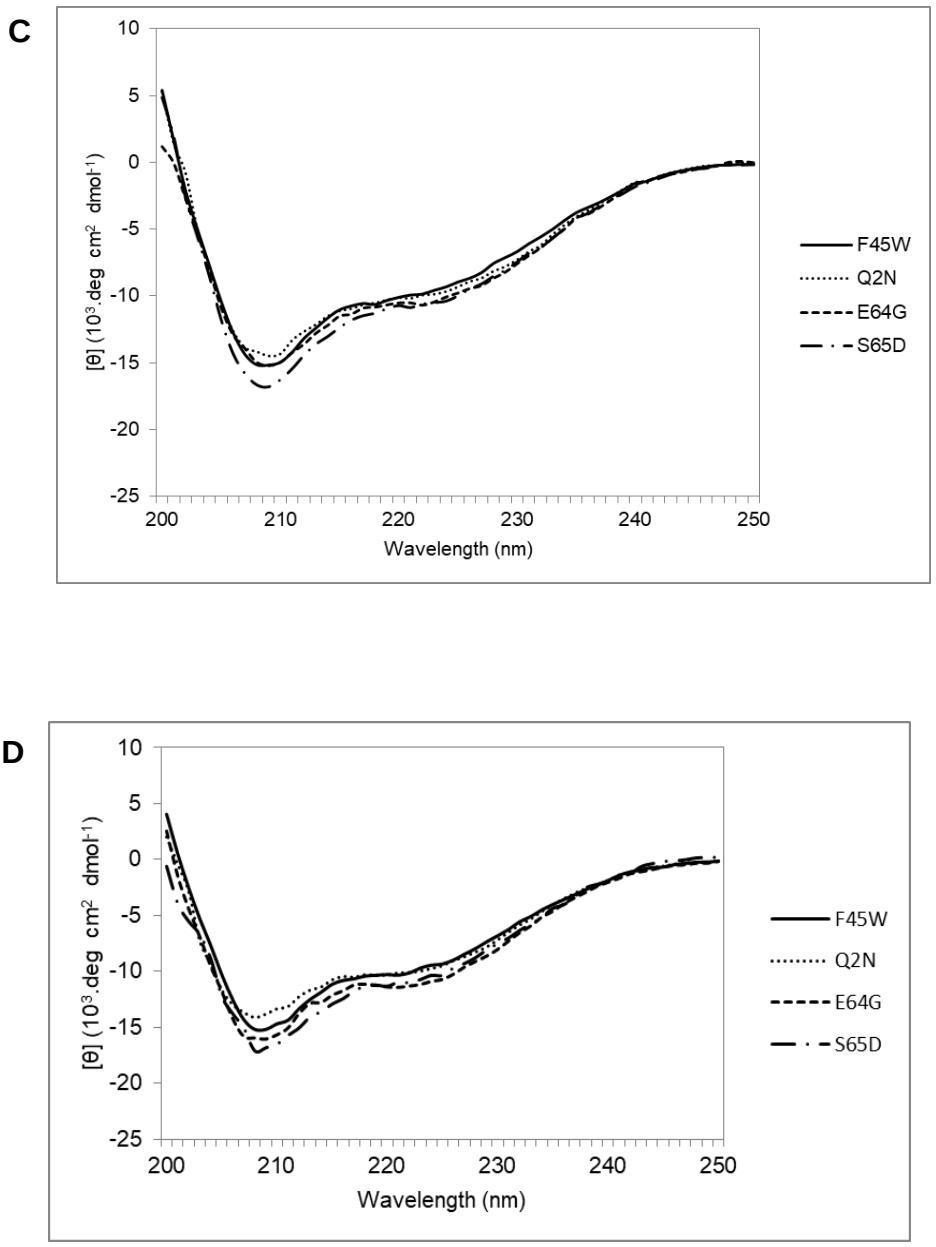


Fig. 4.6. CD spectra showing effect of different pH range on Ubiquitin (F45W) and β-bulge mutants. A) pH 2.0, B) pH 4.0, C) pH 6.0, D) pH 8.0.

As we already discussed, ubiquitin wild-type along with β-bulge mutants are retaining the structure. At pH 2.0 ubiquitin mutant E64G showed elevated helicity compared to other mutants and ubiquitin wild-type. For pH 4.0, 6.0 and 8.0 all ubiquitin variants are behaving similarly (**Fig. 4.6**). No major changes in the secondary structure could be observed.

4.5. Discussion

Despite a billion years of evolution separating humans from the yeast ubiquitin has remained more or less conserved. The reason could be the involvement of this protein in multiple pathways in the cell. Even at higher temperatures, ubiquitin has the ability to remain stable and intact. Our laboratory previously worked on the C-terminal β-bulge part of the protein. This bulge involved three residues Gln2, Glu64 and Ser65, which were not the most suitable choices for their position on β-bulge. These residues were conserved during the course of evolution by nature. Hence, it is interesting to check the effect of replacing the conserved residues with more suitable candidates on ubiquitin. This idea resulted in β-bulge mutants Q2N, E64G and S65D (Mishra et al., 2009, 2011). Structural characteristics and functionality under stresses like heat, UV, antibiotics were already checked in our laboratory and the mutants were found to be stable and carried secondary structural characteristics similar to those of wild type ubiquitin. Host cells lacking polyubiquitin gene and as a consequence showing sensitivity to various stresses, upon expressing β-bulge mutants could show resistance to thermal stress because of the functional efficacy of the mutant ubiquitins. However, the mutants showed reduced survival in the presence of antibiotic Cycloheximide (Mishra et al., 2009, 2011).

Ubiquitin activation was tested *in vitro* to quantitate the efficiency of mutant forms of ubiquitin vis-à-vis wild type ubiquitin. The experimental design included incubation of β-bulge mutants with ubiquitin activating enzyme E1 and analyze activation of ubiquitin. Even after multiple attempts, E1 protein could not be purified, as the expression level of E1 obtained was low. Various parameters were tried singularly and in different combinations such as the concentration of inducer, incubation time and temperature to amplify the expression of E1. None of the efforts could produce the desired result. Later, the assay was carried out by using the ubiquitin activation assay kit obtained from Abcam. All three β-bulge mutants Q2N, E64G and S65D showed activation by E1 and formed covalent bonds with active site cysteine of E1. This result clearly indicated despite mutation, the functionality of ubiquitin has not been hampered.

The intracellular pH of yeasts is different from that of other eukaryotic cells. Far UV CD spectra of purified wild type and mutant ubiquitin proteins were compared to understand the effect of pH on the stability of these mutants. For which we used different pH buffers from 2.0 to 8.0. At pH 2.0, the E64G mutant showed an increase in helicity. Q2N also showed comparatively more helices at pH 2.0 and pH 4.0. The secondary structure content of S65D remained unaltered and was similar to wild-type ubiquitin F45W.

Conclusively, Ubiquitin β-bulge mutants are unaffected by the effect of mutation structurally and functionally at the level of ubiquitin activation by E1.