# CHAPTER 4

# CONSTRUCTION, EXPRESSION, PURIFICATION OF UbQ2N AND ITS STRUCTURALANALYSIS

## 4.1. INTRODUCTION

Glutamine, the second residue of ubiquitin present at the N-terminal end in β-sheet, forms hydrogen bonds with the Glu64 and Ser65 which are bulged out. Hence, Gln2 forms the third (X-position) residue in the β-bulge. Being a part of the parallel β-bulge, its interaction with Glu and Ser is important in the maintenance of the bulge. Gln2 shows total conservation in all ubiquitins irrespective of species. With the purpose of understanding the importance of Gln2 in β-bulge and in overall structure of the protein site directed mutant was constructed replacing Gln2 with Asn.

Percentage frequency of occurrence of Gln2 at 0.34 in β-bulge structure is much lower than Asn (Chan et al., 1993). The percentage frequency of Asn is the highest at 5.4 and hence becomes first choice for the replacement of Gln2 (Figure 4.1).

| Amino acid | I | F | V | L | W | M | A | G | C | Y | P | T | S | H | E | N | Q | D | K | R |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Faai/bulge | 0.53 | 0.17 | 0.76 | 0.8 | 0.57 | 0.00 | 0.81 | 1.0 | 2.3 | 0.20 | 0.00 | 0.9 | 1.6 | 2.6 | 0.68 | 5.4 | 0.34 | 3.5 | 0.17 | 0.25 |
| Naai | 5 | 1 | 10 | 8 | 1 | 0 | 6 | 8 | 6 | 1 | 0 | 7 | 11 | 6 | 3 | 18 | 1 | 15 | 1 | 1 |

| |
|---|
| **Faai/bulge - % frequency of occurrence of amino acid i in a bulge.** |
| **Naai - Total number of amino acid i found in a bulge.** |

**Figure 4.1. Percentage frequencies of amino acids in the X-position of β-bulge. (Chan et al., 1993)**

Asn is also found at the X-position in the structural homologs of ubiquitin (Jentsh et al., 2000), namely SMT3, SUMO-2 and SUMO-3 (figure 4.2). Thus, in order to understand the importance of Gln2 it was substituted by Asn.


**Figure 4.2. Sequence comparison of ubiquitin with its homologs. (Jentsh et al., 2000)**

## 4.2. MATERIALS AND METHODS

### 4.2.1. Construction and expression of UbQ2N-pKK 223-3

Plasmid pKK 223-3 carrying fluorescent mutant form of ubiquitin gene with mutation for F45W present between *Eco* RI and *Hind* III restriction sites was used to introduce Q2N mutation. Mutation was introduced using primer based mutagenesis technique of in vitro site directed mutagenesis protocol. Ubiquitin gene was PCR amplified using forward primer of 5′ACAGAATTCATGAACATCTTCGTCAAG3′ having the mutation at the second codon just after the start site and reverse primer of 5′GCCAAGCTT CGCTCAACC3′. The amplified product of 248 bp was cloned in the vector pKK 223-3 in the *Eco* RI and *Hind* III restriction sites. Introduction of mutation was confirmed by loss of *Bgl* II site initially and by DNA sequencing subsequently. The sequence data confirmed introduction of mutation. Expression of ubiquitin was confirmed on 15% PAGE after two hours of induction with 0.5mM IPTG.

### 4.2.2. Purification of UbQ2N protein

Protein purification and protein estimation were done as mentioned in Materials and Methods section (2.2.3.) of Chapter 2.

### 4.2.3. Circular dichroism and fluorescence spectroscopy

CD spectra were recorded using a Jasco J-715 spectropolarimeter. For far uv CD spectra from 200 to 250nm, cells of 1mm path length were used. Spectra were recorded at 2nm resolution. The data pitch was 0.2nm. Scan speed was 50nm/sec. To reduce the noise five spectra were accumulated. Protein solutions were prepared in 10mM Tris HCl, pH7.4 and concentration of the protein was 0.2mg/ml (23.4μM). The near uv CD spectra of the proteins were recorded between 250 and 320nm. Path length was 1cm and protein concentration was 1mg/ml (117μM). Spectra for far and near uv were also recorded at buffer of pH2.2 (Gly-HCl), pH5 (sodium acetate buffer) and pH10 (Gly-NaOH) with 10mM concentration final. Proper blanks were prepared for all samples and the spectra of the samples were blank corrected.

Fluorescence spectra were recorded using Hitachi F-4010 fluorescence spectrophotometer. Slit width was 5nm. Protein concentration was 0.2mg/ml (23.4μM).

Samples were excited at 295nm to record the intrinsic fluorescence due to Trp. Emission was recorded between 320-440nm.

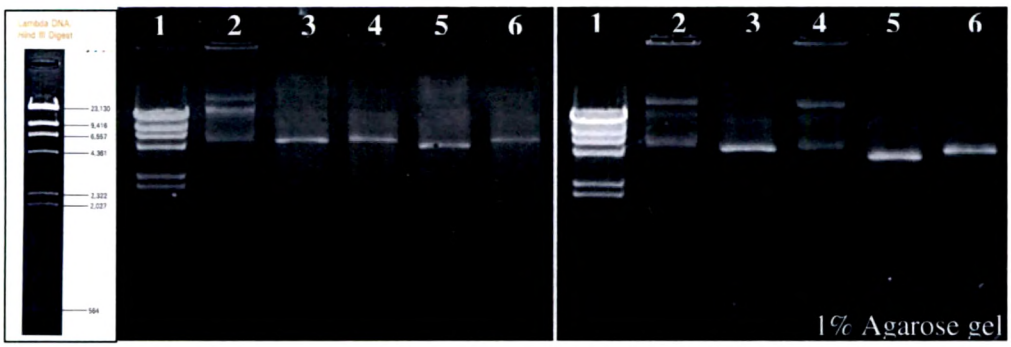
Bis-ANS was used as an extrinsic fluorophore at a concentration of 10μM. Bis ANS was excited at 390nm and emission was recorded between 450-600nm. Slit width was 5nm. Protein concentration was 0.2mg/ml (23.4μM). Protein solutions were prepared in Tris HCl buffer, pH 7.4. The experiment was repeated with 50μM ANS. Other parameters were not changed.

## 4.3. RESULTS

### 4.3.1. Construction, expression and purification of UbQ2N from pKK 223-3

Construction of pKK 223-3-UbQ2N was done by primer based mutagenesis technique of in vitro site directed mutagenesis protocol. The ubiquitin gene thus constructed is a double mutant at 45$^{th}$ and 2$^{nd}$ position. Q2N replacement was screened by loss of *Bgl* II site (Figure 4.3.), which was destroyed after the mutation is introduced (Figure 2.5). UbQ2N protein was expressed by using pKK 223-3-UbQ2N vector and purification was done by same method as used for UbWt and the purity was checked on 15% SDS-PAGE.

**Restriction analysis of pKK223-3/UbQ2N**




**(a)** **(b)**

**Figure 4.3. Restriction digestion patterns of (a) pKK223-3-UbF45W and (b) pKK223-3-UbQ2N. Lane 1. Contains λ *Hin*d III digest as marker. Lane 2. Contains undigested plasmid of pKK223-3-UbF45W (a) and pKK223-3-UbQ2N (b). Lane 3. Contains *Eco* RI digest. *Eco* RI has a unique restriction site. Lane 4. Contains *Bgl* II digest and gives a single band of 4791bp fragment with (a) pKK223-3-UbF45W and (b) the site is lost after cloning of UbQ2N resulting in no digestion of vector backbone. Lane 5. Contains *Sal* I digest. *Sal* I has two sites in (a) and releases a 728bp fragment. Lane 6. Contains *Hind* III digest. *Hind* III also has a unique restriction site. *Eco R*I and *Hind* III sites were used for cloning UbQ2N gene in pKK223-3.**

### 4.3.2. Sequence analysis of UbQ2N gene in pKK223-3

Introduction of the mutation was also confirmed by sequencing of the UbQ2N gene.

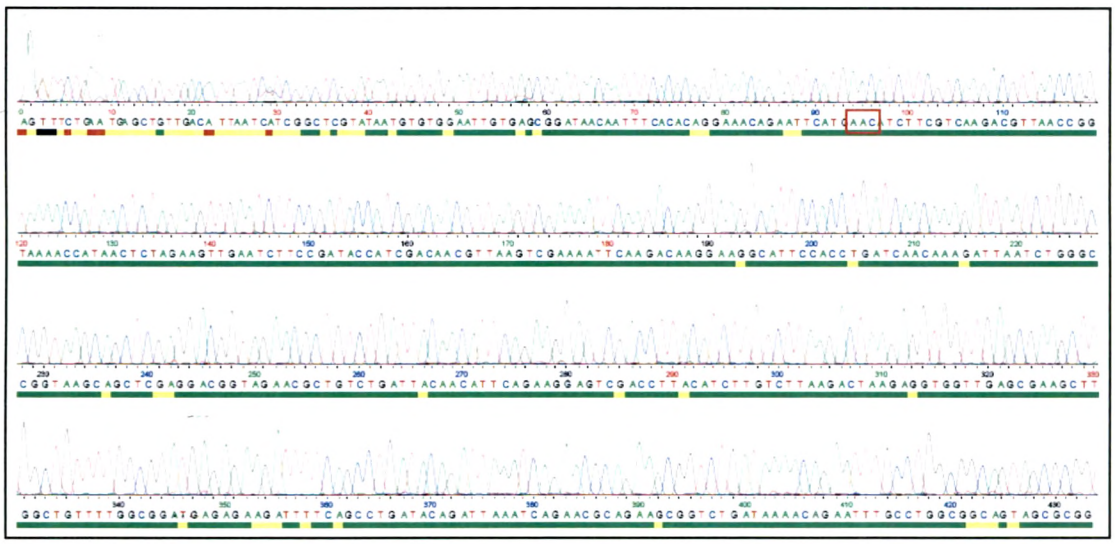
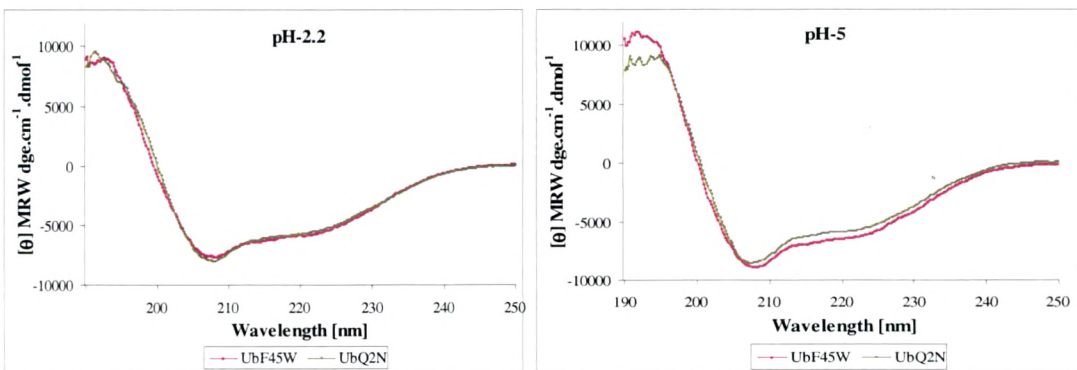


**Figure 4.4. Electrophoretogram of UbQ2N gene in pKK223-3 vector. The marked region confirms the UbQ2N substitution.**

### 4.3.3. Far and near UV CD spectra of ubiquitin variants UbF45W and UbQ2N

Far uv CD spectra of UbF45W and UbQ2N were recorded to study the changes in secondary structure due to mutations in the sequence of ubiquitin. The UbF45W spectrum was used as a reference in far and near uv CD spectra. UbQ2N shows no change in secondary structure with respect to UbF45W (Figure 4.5.).



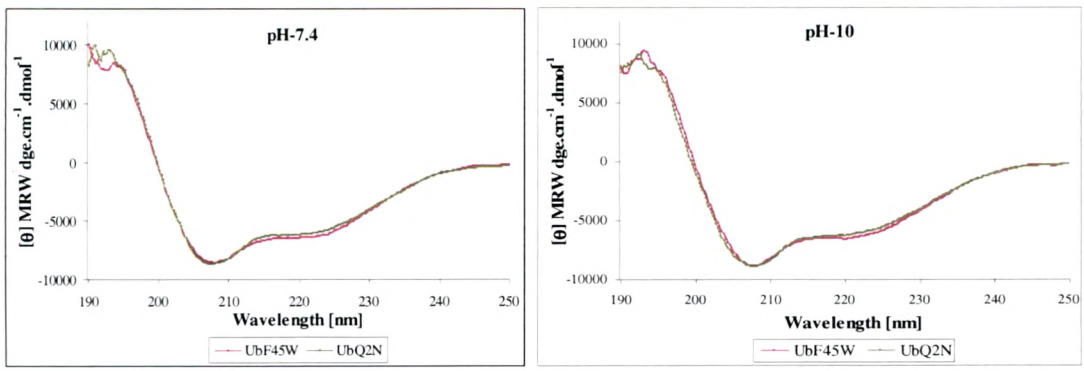



**Figure 4.5. Far uv CD spectra of the two variants of ubiquitin UbF45W and UbQ2N at various pH buffers 2.2, 5, 7.4 and 10.**

Based on near uv CD spectra, the tertiary structure of UbQ2N and UbF45W seem to be alike at pH 7.4 (Figure 4.6.). Though the tertiary structure content of the two variants seems to differ at other pH values.

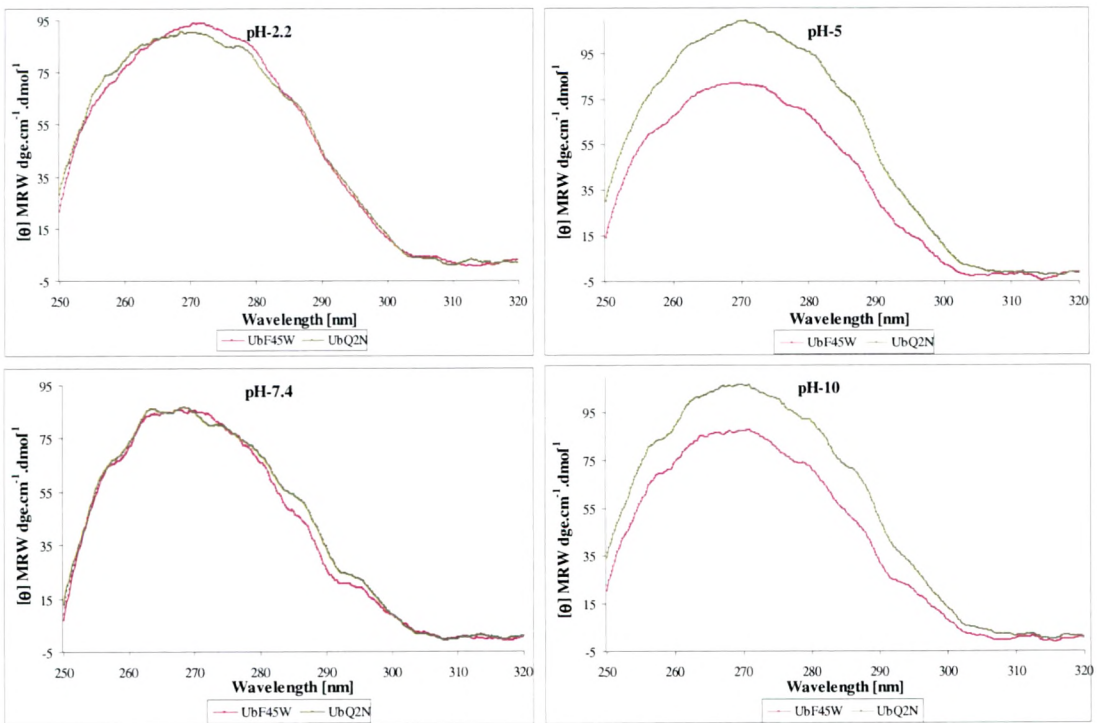
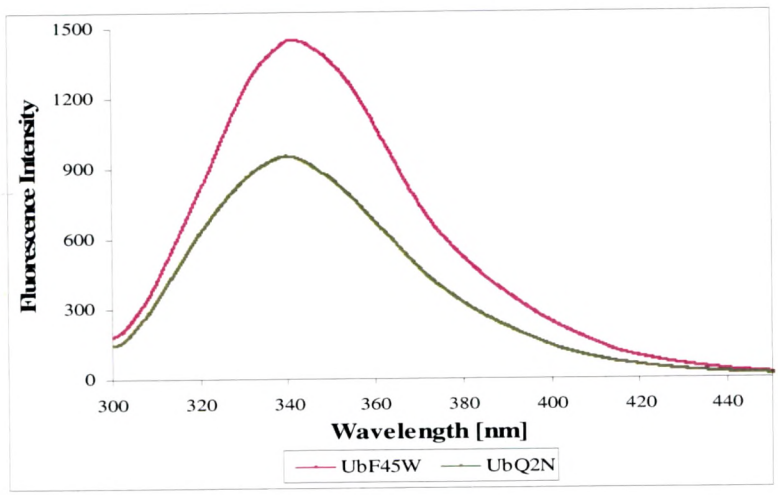


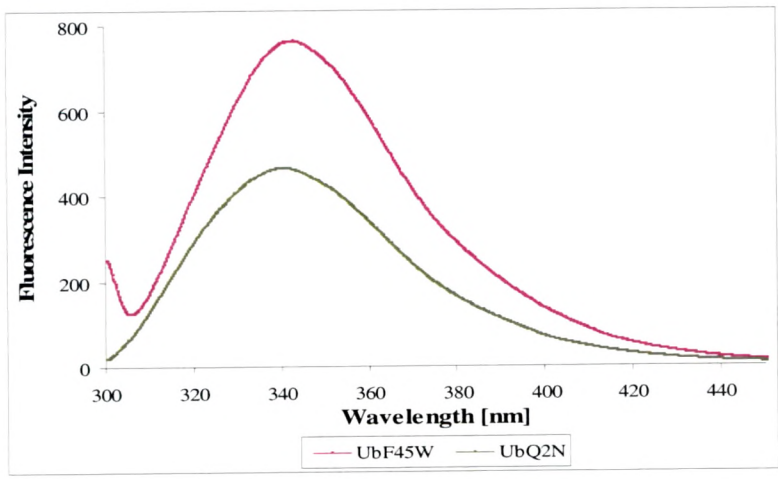

**Figure 4.6. Near uv CD spectra of UbF45W and UbQ2N at various pH values 2.2, 5, 7.4 and 10.**

### 4.3.4. Fluorescence spectrum of UbF45W and UbQ2N

Fluorescence spectra of UbF45W and UbQ2N were recorded by exciting the proteins at 280nm. UbQ2N and UbF45W show difference in the intensities of fluorescence, indicating that the environment around aromatic amino acid residue is effected by the substitution. This fact is supported by the spectra obtained by exciting Trp at 295nm. The $\lambda_{max}$ of emission of UbQ2N in both cases shows red shift with respect to UbF45W (Figure 4.7.).




**(a)**

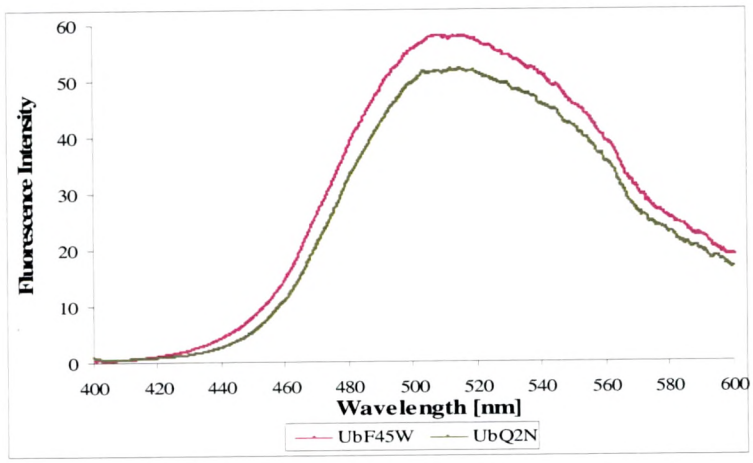



**(b)**

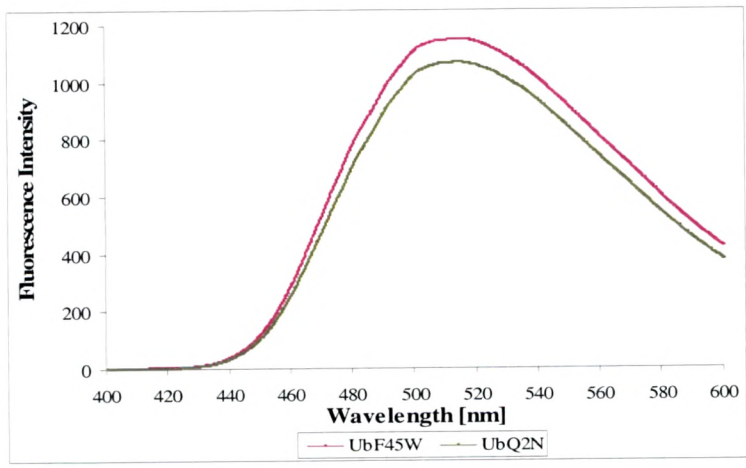
**Figure 4.7. Fluorescence emission spectra of UbF45W and UbQ2N recorded after exciting the protein at 280nm (a) and 295nm (b)**

### 4.3.5. Comparison of the content of hydrated hydrophobic residues in UbF45W and UbQ2N

Fluorescence spectra of ubiquitin with extrinsic fluorophore ANS and Bis-ANS showed marginally less intensity with UbQ2N compared to UbF45W. ANS is known to bind hydrated hydrophobic residues and emit fluorescence. Almost equal intensity of ANS and Bis-ANS fluorescence as a result of binding to UbF45W and UbQ2N indicates no change in the exposure of hydrophobic residues to the surface after substitution (Figure 4.8.).




**(a)**




**(b)**

**Figure 4.8. Fluorescence emission spectra of extrinsic fluorophores (a) ANS and (b) Bis- ANS, bound to UbF45W and UbQ2N.**

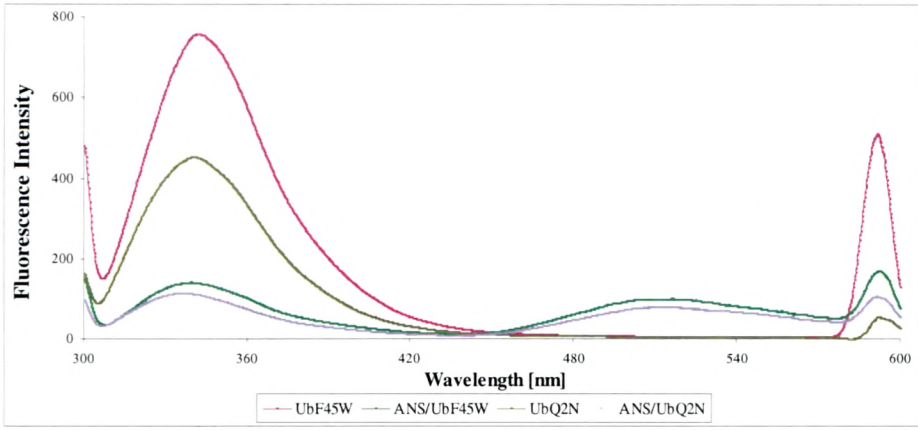



**Figure 4.9. Fluorescence resonance energy transfer spectra between tryptophan of UbF45W and UbQ2N in absence and presence of ANS.**

Fluorescence resonance energy transfer spectra demonstrating energy transfer between Trp and ANS do not show much difference between UbQ2N and UbF45W. The results support the observations made with extrinsic fluorescence establishing that exposed hydrophobic residue content with both variants is equal (Figure 4.9.). Interestingly, the hydropathic indices of the residues Gln and Asn are identical at -3.5 (Kyte and Doolittle, 1992).

### 4.3.6. Thermal denaturation/ renaturation profiles of UbQ2N and UbF45W

Thermal denaturation experiments showed that the two proteins follow same path during unfolding (Figure 4.10.).

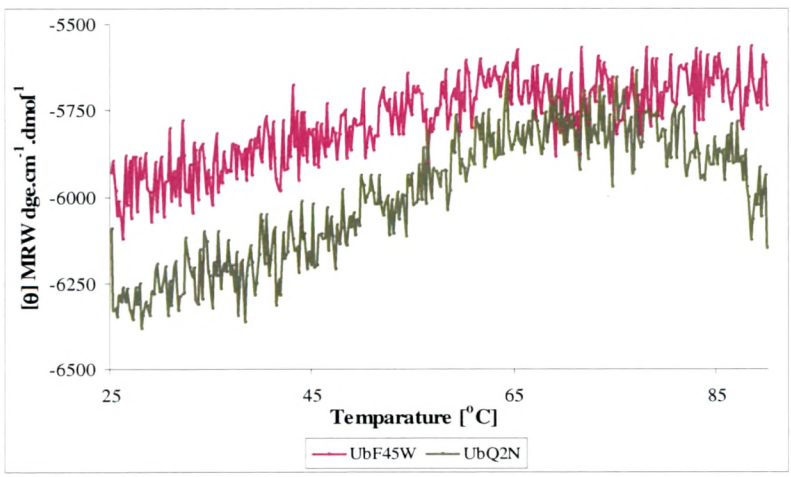
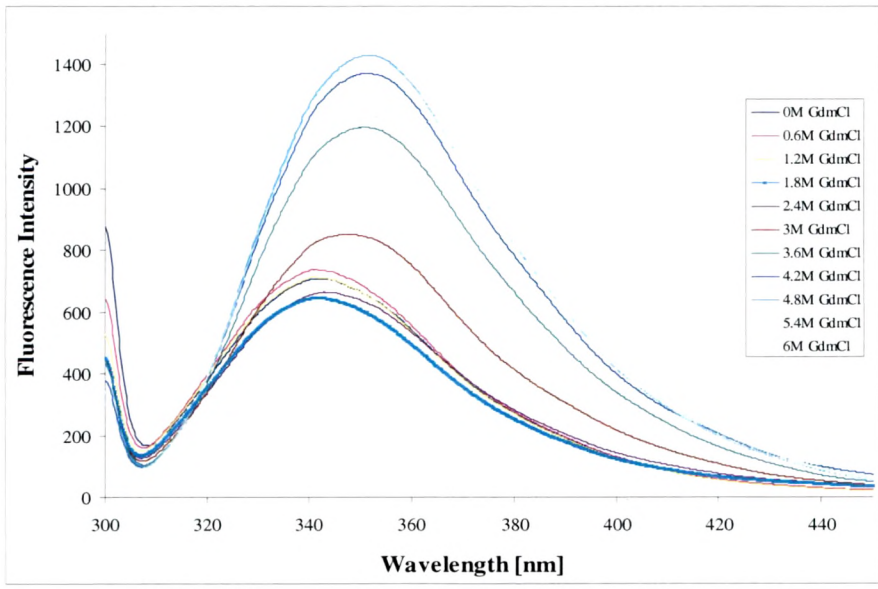


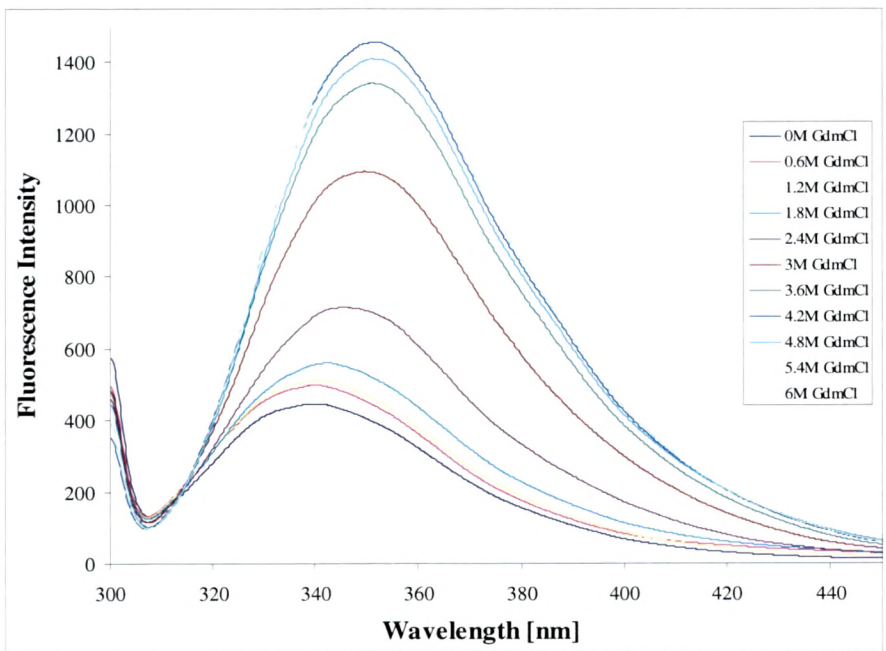

**Figure 4.10. CD spectra showing thermal denaturation of UbF45W and Q2N.**

### 4.3.7. Gaundine hydrochloride denaturation of UbQ2N and UbF45W

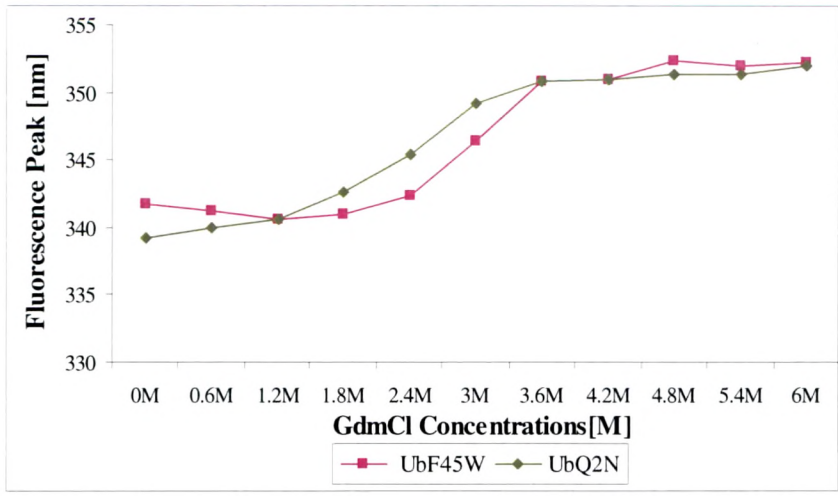
The guanidine hydrochloride denaturation of UbQ2N and UbF45W were studied at different concentration of guanidine hydrochloride to study the stability of the protein (figure 4.11.).




**(a)**




**(b)**




**(c)**

**Figure 4.11. Intrinsic fluorescence spectra for guanidine hydrochloride denaturation of (a) UbF45W and (b) UbQ2N. (c) Guanidine hydrochloride denaturation curves of UbF45W and UbQ2N.**

## 4.4. DISCUSSION

From the results it is obvious that substitution Gln by Asn has not only been accommodated well locally, also does not seem to effect the overall conformation of the protein,. However there is a minor alteration suffered by UbQ2N in the environment around Trp as a result of the substitution. In addition, this mutation makes the protein UbQ2N less stable in guanidine hydrochloride under equilibrium conditions than UbF45W.

In conclusion, replacing the conserved Gln at position 2 with Asn does not lead to any major changes in secondary and tertiary structures of the protein. This study demonstrates a point mutation produces slight structural alteration decreasing the stability of the protein.