CHAPTER 2

CONSTRUCTION, EXPRESSION, PURIFICATION OF UbE64G AND ITS STRUCTURAL ANALYSIS

2.1. INTRODUCTION

Ubiquitin is a protein present in eukaryotes. This protein is used to mark proteins for degradation by proteasomes. The substrates for ubiquitin are generally cell cycle switches, developmental regulators, antitumor proteins and apoptotic factors. Ubiquitin is a small, single domain, α/ β protein of 76 residues (Vijay-Kumar et al., 1987), showing almost total conservation from yeast to higher eukaryotes (Gavilanes et al., 1982; Watson et al., 1978; Schlesinger et al., 1975; Schlesinger and Goldsteiner, 1975). The reasons for total conservation can be attributed to constraints imposed by structure, function, folding and stability aspects. Ubiquitin mutants are therefore developed to understand the actual importance of the residues in certain crucial positions.

There are two β bulges in this protein, which partially overlap with β-turns. The first turn and the bulge have been studied in great detail. The second β-bulge also displays some unusual features. The β-bulge present at the C-terminal end is accompanying a type II turn and also occurring in a parallel β-sheet, both these incidences are rare in proteins (Chan et al., 1993). Glu64 is the third residue in a type II turn and the first residue in β-bulge respectively (Vijay-Kumar et al., 1987). Generally the first residue in a β bulge is a Gly. Presence of Glu64 is supported by unusual Φ and Ψ angles which are not under the allowed region of Glu in Ramachandran plot (Ramachandran, Ramakrishnan and Sasisekharan, 1963). Furthermore these angles are observed with Gly (Chan et al., 1993). Percentage frequency of Glu in a bulge is 0.23 which is much lower than that of Gly which is 11.3 as shown in Figure 2.1.

Amino acid	I	F	V	L	W	M	A	G	C	Y	P	T	S	H	E	N	Q	D	K	R
Faai/bulge	0.00	0.17	0.00	0.00	1.13	0.00	0.00	11.30	0.39	0.00	0.00	0.00	0.00	0.00	0.23	3.95	0.34	0.71	0.34	0.25
Naai	0	1	0	0	2	0	0	84	1	0	0	0	0	0	1	13	1	3	2	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 2.1. Percentage frequencies of amino acids in the first position of β-bulge. (Chan et al., 1993)

The percentage frequency of Gly is the highest and thus becomes first choice for replacement of Glu64. Moreover Gly is also observed at the 1st position in the structural homologs of ubiquitin. The comparison of sequences of ubiquitin and its homologs is shown in Figure 2.2. Furthermore, the structural homologs of ubiquitin RUB1 and NEDD8 display Gly in this position (Jentsch et al., 2000). The reasons for the anomaly, why nature selected Glu instead of Gly and conserved through evolution would be interesting to know.

Figure 2.2. Sequence comparison of ubiquitin with its homologs. (Jentsh et al., 2000)

Ile61 adjacent to Glu64 shows slower protection in kinetic refolding experiments (Briggs and Roder, 1992). Lys63, the immediate neighbour of Glu64 plays important role in some of the functions of ubiquitin such as UV induced DNA repair, post-replicative DNA repair and membrane protein degradation (Hofmann and Pickart, 2001; Galan and Haguenauer-Tsapis, 1997; Spence et al., 1995; Ulrich et al., 2002; Hicke et al., 2003). In view of this it would be interesting to understand the importance of this feature in folding, structural stability and function of ubiquitin. Hence in the present study, the mutant of ubiquitin UbE64G was constructed by replacing Glu64 by Gly, so that the secondary structure of the protein remains undisturbed, giving a scope for revealing the importance of the residue in the over all structure of the protein and its function. Circular dichroism (CD) and fluorescence techniques can be used for characterizing the structure and stability of UbE64G.

2.2. MATERIALS AND METHODS

2.2.1. Plasmid Constructs

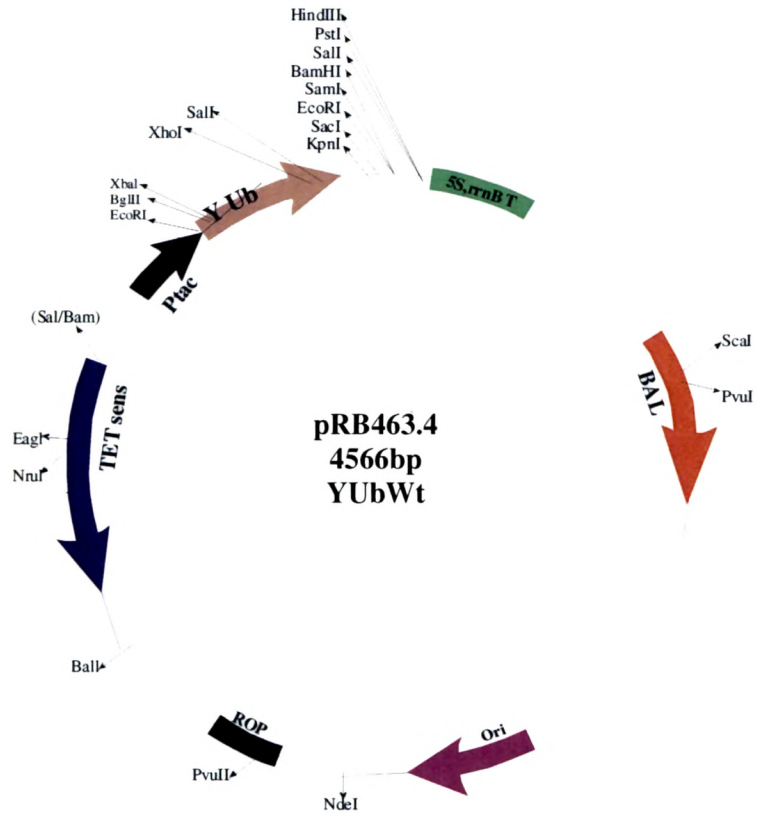


Figure 2.3. *E.coli* expression vector pRB463.4. The plasmid carries Yeast-wild type ubiquitin gene (YUbWt) under tac promoter

The DNA sequence of the *Eco* RI- *Eco* RI insert in pRB463.4

GAATTC ATTATG CAGATCT.....Yeast-UB....GGT GGT TGA GGTACC GAGCTC GAATTC
Eco RI Met *Bgl* II Gly Gly *Kpn* I *Eco* RI

Eco RI fragment (≈250bp) corresponding to the gene for UbWt was taken from Plasmid pRB457 and inserted into pKK261 in place of synthetic ubiquitin gene. The resultant plasmid construct was named pRB463.4 (Figure 2.3). Plasmid pRB463.4 (YUbWt) was a gift from Dr. Rohan Baker (The John Curtin School of Medical Research, The Australian National University, Australia). Expression of UbWt is under tac promoter. The plasmid was transformed into the Lac I^q strain of *E.coli* JM109. (Since DH5α strain of *E.coli*

is not a Lac I^q strain, it cannot be used for the expression of genes under tac promoter). Lac I^q strain over-produces lac repressor to control the expression of the gene under tac promoter stringently. The vector is isopropyl-β-D-1-thiogalactoside (IPTG) inducible at a final concentration of 0.1mM-1mM. The construct confers resistance to ampicillin at 50μg-100μg/ml final concentration due to β-lactamase gene (BAL) present on the vector.

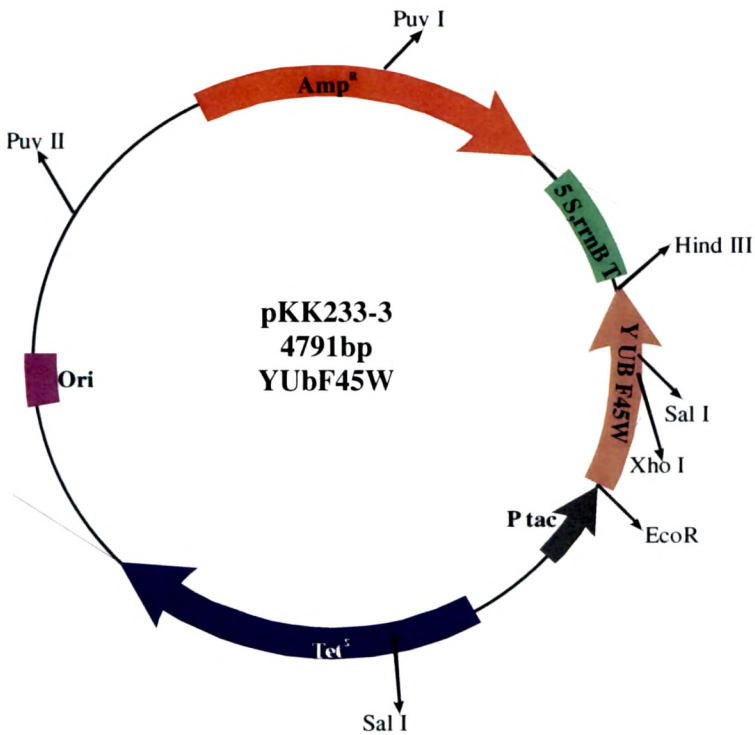


Figure 2.4. *E.coli* expression vector pKK223-3. The plasmid carries Yeast ubiquitin gene with F45W mutation (YUbF45W) under tac promoter. (Platt et al., 2003)

The DNA sequence of the *Eco* RI- *Hind* III insert in pKK223-3

GAATTC	ATTATGC	AGATCT	....Yeast-UB....	GGT	GGT	TGA	GGTACC
Eco RI	Met	*Bgl* II		Gly	Gly		*Hind* III

Plasmid pKK223-3 (YUbF45W) was constructed by cloning the ≈250bp *Eco* RI-*Hind* III fragment containing UbF45W gene sequence (Figure 2.4). Ubiquitin is expressed under a strong tac promoter. Downstream to the gene strong rrnB ribosomal terminator is present for the control of protein expression. pKK223-3 is also expressed in $LacI^q$ strain and

is IPTG inducible at final concentration of 0.1mM-1mM and displays ampicillin resistance at 50μg-100μg/ml.

2.2.2. Construction and expression of UbE64G - pKK 223-3

The wild type ubiquitin, UbWt does not contain any tryptophan residues, making intrinsic fluorescence studies impossible. In order to study the structural properties of ubiquitin using intrinsic tryptophan fluorescence, tryptophan was introduced in place of phenylalanine at position 45 in the ubiquitin sequence. The secondary and tertiary structures of fluorescent form of ubiquitin (UbF45W) are only marginally different from wild-type protein (UbWt). The earlier studies have shown that the structure and stability of UbWt and UbF45W were similar (Khorasanizadeh et al., 1993). Plasmid pKK 223-3 carrying mutant form of ubiquitin gene with F45W mutation was a generous gift from Prof. Mark Searle's laboratory (School of Chemistry, Nottingham, United Kingdom). The sequence of the gene is given in Figure 2.5. The gene from the plasmid (≈231 bp) was taken out by digesting it with *Eco* RI and *Hind* III and was subcloned into pUC18. For all the mutagenesis work carried out in Chapters 2, 3, 4 and 5 the gene carrying mutation for F45W was used as a template, as the resulting proteins can be studied using intrinsic fluorescence experiments.

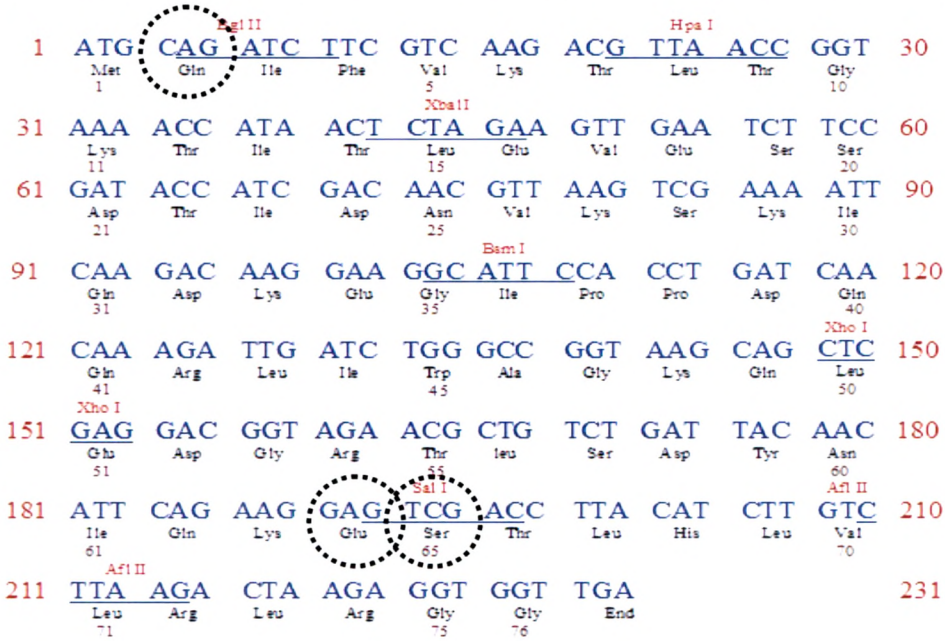


Figure 2.5. Sequence of synthetic yeast ubiquitin gene with mutation for UbF45W.

Mutation was introduced utilizing mutation primers following standard Genei in vitro site directed mutagenesis protocol. Introduction of mutation was confirmed by *Sal* I site destruction and subsequently by DNA sequencing. The sequence data confirmed introduction of mutation. The *Hind* III- *Eco* RI fragment from pUC18 vector was cloned back and pKK 223-3-UbE64G was obtained. Expression of ubiquitin was checked on 15% acrylamide gel after inducing in 0.5mM IPTG for two hours.

2.2.3. Purification of UbWt, UBF45Wand UBE64G proteins

The proteins UbWt and UbF45W were purified using the methods given by Ciechanover et al., 1980 and Ecker et al., 1987. A pKK223-3 plasmid construct containing the yeast ubiquitin gene was used to express the UbWt and UbF45W protein in *Escherichia coli* strain BL21 (DE3) under the control of IPTG inducible p*tac* promoter. Cells were grown at 37°C in Luria broth (LB) containing ampicillin (100µg/ml) to mid-log stage and harvested after 4hours of induction (0.5mM IPTG). Cells were resuspended in lysis buffer pH7.8 containing 5% glycerol, 50mM Tris and 2mM EDTA. Cells were treated with lysozyme (1mg/ml) for 30 minutes. at 37°C. Lysed cells were sonicated (50 duty cycles for 15 sec/3-4 times in an interval of 5 sec) and centrifuged at 8,000g for 10 min. The cell lysate was treated with protease inhibitor cocktail and phenylmethylsulfonyl fluoride (PMSF) (1mM final). Following centrifugation the supernatant containing the remaining soluble cellular proteins was collected. The collected supernatant was treated with 0.2% polyethyleneimine (PEI) and the precipitates were removed by centrifugation at 16,000g for 30 minutes. As ubiquitin is a heat stable protein, the supernatant collected from the above step is heat treated with 1mM dithiothreitol (DTT) at 85-90°C for 15 minutes. The coagulated proteins were removed by centrifugation at 16,000g for 30 minutes. Ubiquitin is further precipitated out of the supernatant at 45-85% ammonium sulphate. After dialysis ubiquitin containing fraction was purified on Sephadex G-50 column. The protein was concentrated by precipitation and dialysis.

Protein concentration for F45W was determined using its extinction coefficient 6744 M^{-1} cm^{-1} at 280 nm according to the method of Gill and von Hippel (1989). Mutant ubiquitin UbE64G could be purified using the same protocol used for purification of UbWt and

UbF45W. This fact suggests the mutant ubiquitin is as stable as wild type and structurally similar.

2.2.4. Circular dichroism and fluorescence spectroscopy

CD spectra were recorded using a Jasco J-715 spectropolarimeter. For far uv CD spectra (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. Five spectra were accumulated to reduce the noise. Protein solutions were prepared in 10mM Tris HCl, pH7.4 and concentration of protein was 0.25mg/ml (29.5μM). The near uv CD spectra of the proteins were recorded between 250 and 320nm. Path length was 1cm and protein concentration was 0.774mg/ml (90.56μM). Spectra for far and near uv were also recorded in buffers of pH2.2 (glycine-HCl), pH5 (sodium acetate buffer) and pH10 (glycine-NaOH) at 10mM concentration. Proper blanks were prepared for all samples and the spectra of the samples were blank corrected.

Flourescence spectra were recorded using Hitachi F-4010 fluorescence spectrophotometer. Slit width was 5nm. Protein solutions were prepared in 10mM Tris HCl, pH7.4 and concentration of protein was 0.1mg/ml (11.7μM). Samples were excited at 295nm to record the intrinsic fluorescence due to tryptophan (Trp). Emission was recorded between 320-440nm.

4,4'-Bis (1-anilinonaphthalene 8-sulfonate) (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 pH7.4. The experiment was repeated with 50μM 1-Anilino 8-Naphthalene sulphonic acid (ANS). Other parameters were not changed. Another extrinsic fluorophore pyrene was used at a concentration of 2μM. In this experiment the samples were excited at 335nm and emission was recorded between 350-500nm and slit width was 2.5nm.

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. Protein solutions were prepared in Tris HCl buffer pH7.4. The experiment was repeated with 50μM ANS. Other parameters remained the same.

2.3. RESULTS

2.3.1. Expression and purification of UbWt and UbF45W

Wild type ubiquitin (UbWt) was expressed using plasmid pRB463.4 (YUb). The plasmid was a generous gift from Dr. Rohan Baker (The John Curtin School of Medical Research, The Australian National University, Australia). UbWt was expressed and after purification was resolved on 15% SDS-PAGE (Figure 2.6). UBF45W protein was expressed using pKK 223-3 vector and purification was done by the same method as used for UbWt and checked for purity on 15% gel of SDS-PAGE.

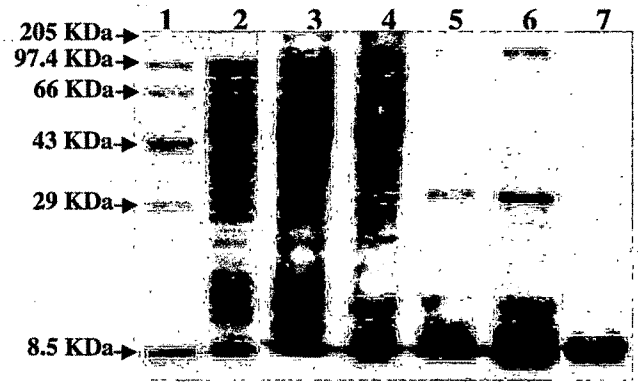
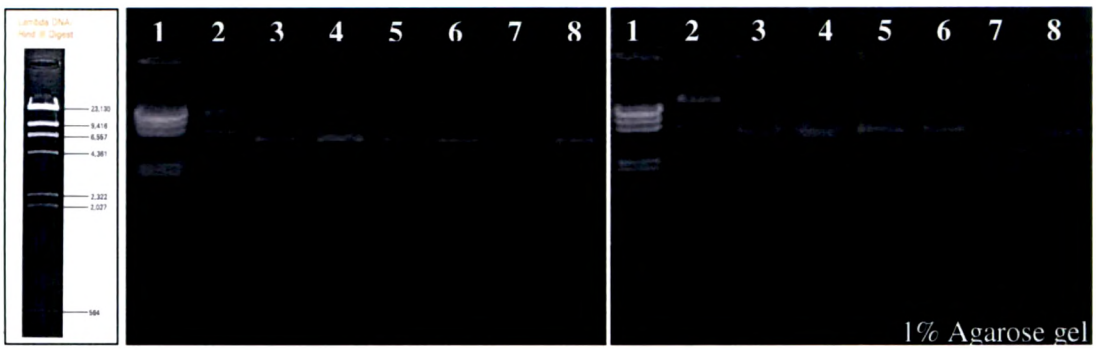


Figure 2.6. Ubiquitin expression and purification gel profile. Lane 1. Shows the protein molecular weight marker. Lane 2. Shows protein profile of JM109 cells transformed by pRB463.4(YUbWt) induced with IPTG. Lane 3. Shows supernatant of cell lysate after lysozyme treatment and sonication. Lane 4. Shows 0.2% polyethyleneimine (PEI) treated supernatant. Lane 5. Shows heat treated supernatant. Lane 6. Shows protein precipitated by 85% ammonium sulphate, loaded after dialysis and Lane 7. Shows the pure ubiquitin obtained after Sephadex G-50 column.

2.3.2. Construction, expression and purification of UBE64G from pKK 223-3

Construction of pKK 223-3-UbE64G was done by using standard Genei in vitro site directed mutagenesis protocol. The ubiquitin gene thus constructed is a double mutant at 45th and 64th position. Incorporation of E64G mutation was screened by loss of *Sal* I site (Figure 2.7). UbE64G protein was expressed by using pKK 223-3-UbE64G vector and purification was done by same method as used for UbWt and purity of protein on 15% SDS-PAGE.



(a) **(b)**

Figure 2.7. Restriction digestion patterns of (a) pKK223-3-UbF45W and (b) pKK223-3-UbE64G. Lane 1. Contains λ *Hind* III digest as marker. Lane 2. Contains undigested plasmid of pKK223-3-UbF45W (a) and pKK223-3-UbE64G (b). Lane 3. Contains *Eco* RI digest. *Eco* RI has a unique restriction site. Lane 4. Contains *Sal* I digest. *Sal* I has two sites in (a) pKK223-3-UbF45W and releases a 728bp fragment and (b) the site is lost in pKK223-3-UbE64G after subcloning of UbE64G resulting in no fragment release. Lane 5. Contains *Xho* I digest and gives a single band of 4791bp fragment with pKK223-3-UbF45W (a) and also for (b) pKK223-3-UbE64G, which is the actual size of the vector. and Lane 6. Contains *Hind* III digest. *Hind* III also has a unique restriction site. Lane 7. Shows the vector fragmentation with *Eco* RI and *Pvu* II releasing 2848bp and 1943bp fragment. Lane 8. Contains *Pvu* II digest. *Pvu* II also has a unique restriction site.

2.3.3. Sequence analysis of UbE64G gene in pKK223-3

Introduction of the mutation was also confirmed by sequencing of the UbE64G gene.

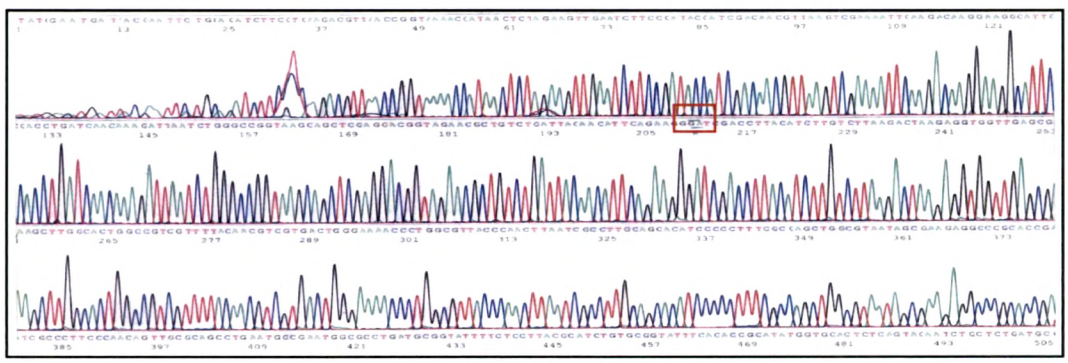


Figure 2.8. Electrophoretogram of UbE64G gene in pKK223-3 vector. The marked region confirms the E64G substitution.

2.3.4. Far and near UV CD spectra of the three variants of ubiquitin: UbWt, UbF45W and UbE64G

Far uv CD spectra were recorded to study the changes in secondary structure due to mutations in the sequence of ubiquitin. UbF45W was used as a reference in far and near uv CD spectra. UbE64G shows a slight change in secondary structure with respect to both UbWt and UbF45W. The CD spectrum of UbE64G shows a change in secondary structure and indicates that there is an increase in helicity, when compared to native protein (Figure 2.9.). Replacement of Glu64 by Gly, being a structurally conservative substitution, was not expected to change secondary structure of the protein, since Gly can readily take up the conformation needed to form β-turn and β-bulge, in which this residue is found. Far uv CD spectra were analyzed for secondary structural content using CDPro. The results are presented in Table 1.1.

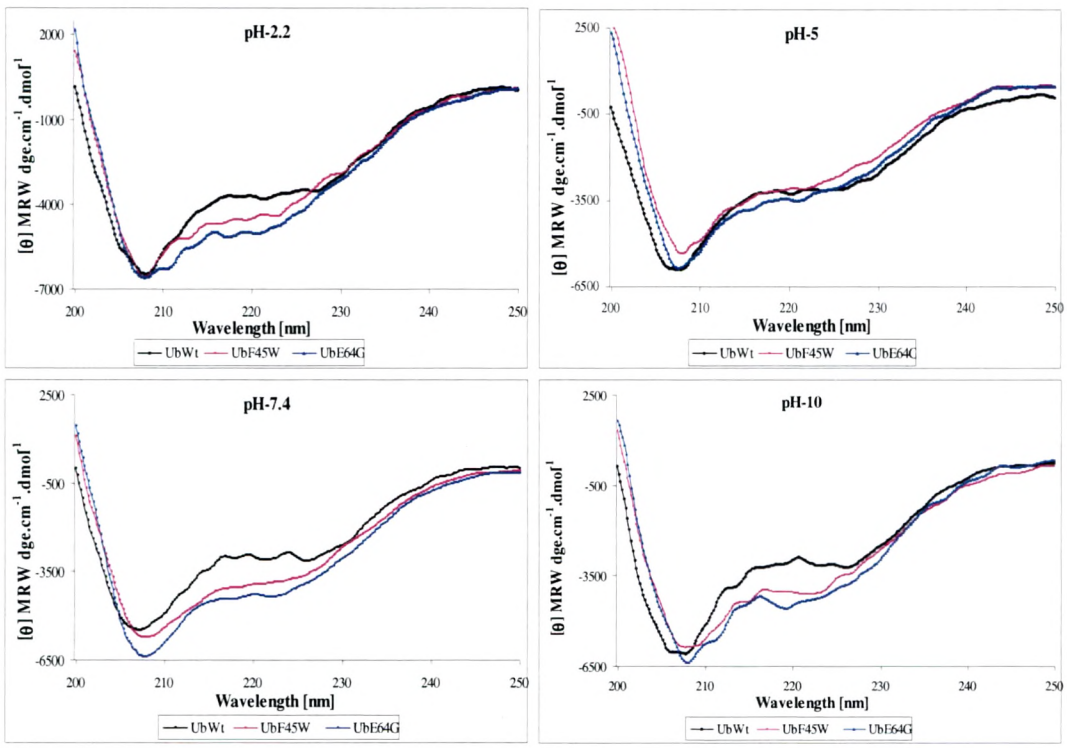


Figure 2.9. Far uv CD spectra of three forms of ubiquitin UbWt, UbF45W and UbE64G at various pH buffers 2.2, 5, 7.4 and 10.

Table 2.1. Secondary structural analysis of far-uv CD spectra using CD Pro software

Type of secondary structure	UbWt* (X-ray)	UbWt	UbF45W	UbE64G
α-helix	16	17	16	11.5
β-sheet	32	31	32	35
Turns and random coil	52	52	52	53.5

*The values reported by Chyan et al., 2004

Based on near uv CD spectra, the tertiary structure of UbE64G and UbF45W seem to be alike, an indication that the conformation of protein around the lone aromatic residue is unaffected by this mutation (Figure 2.10.).

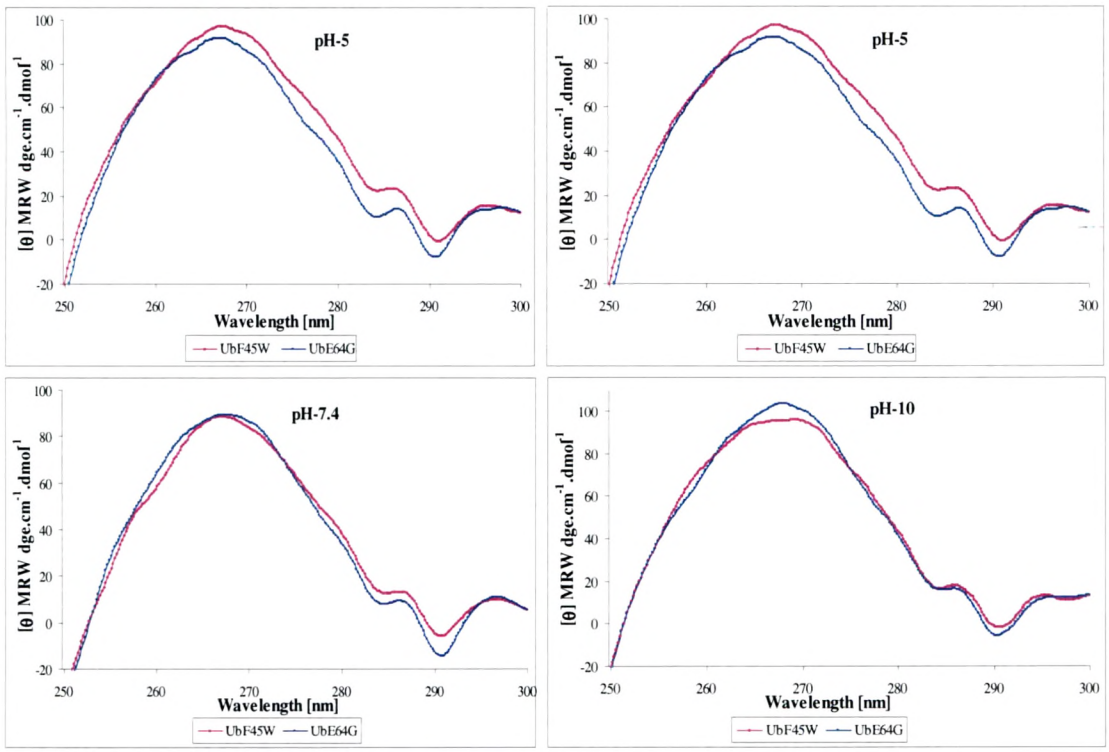
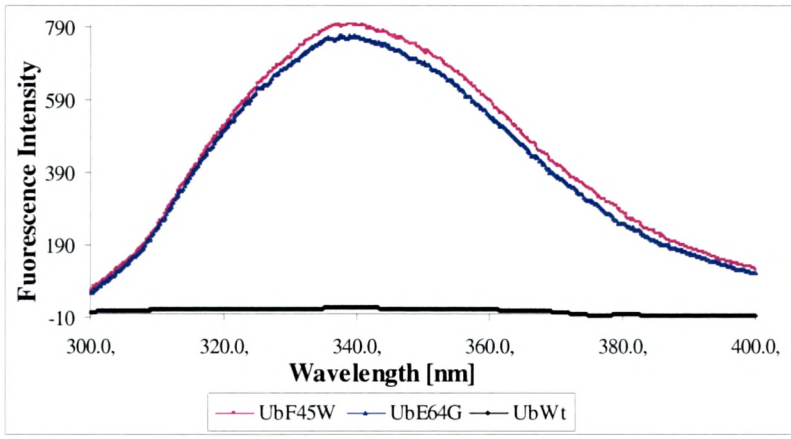


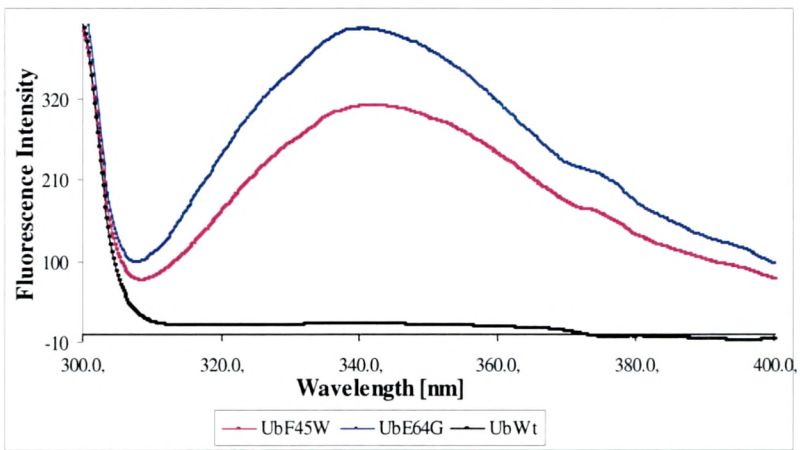
Figure 2.10. Near uv CD spectra for UbF45Wand UbE64G at various pH buffers 2.2, 5, 7.4 and 10.

2.3.5. Fluorescence spectrum of the three variants of ubiquitin UbWt, UbF45W and UbE64G

Fluorescence spectra of the three variants of ubiquitin were recorded by exciting the protein at 280nm. UbWt does not show any peak as it lacks Trp. UbE64G and UbF45W show almost identical intensities of fluorescence, indicating that the environment around aromatic amino acid residue is almost identical. This fact is supported by the spectra obtained by exciting Trp at 295nm. Though, there is a marginal difference in intensities of the spectra, indicating a slight difference in the local environment of Trp induced by this substitution (Figure 2.11.).



(a)

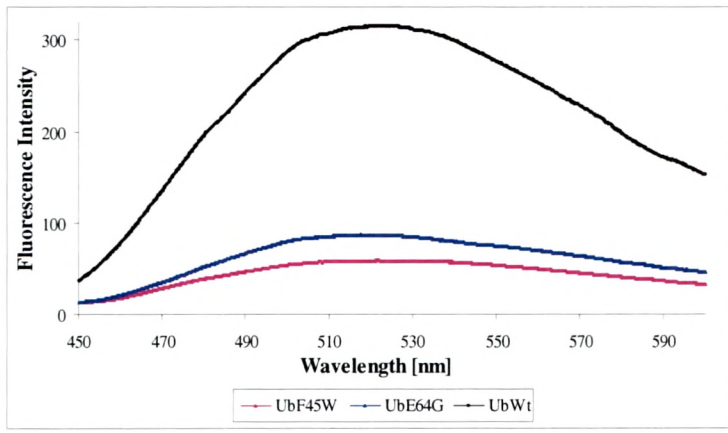


(b)

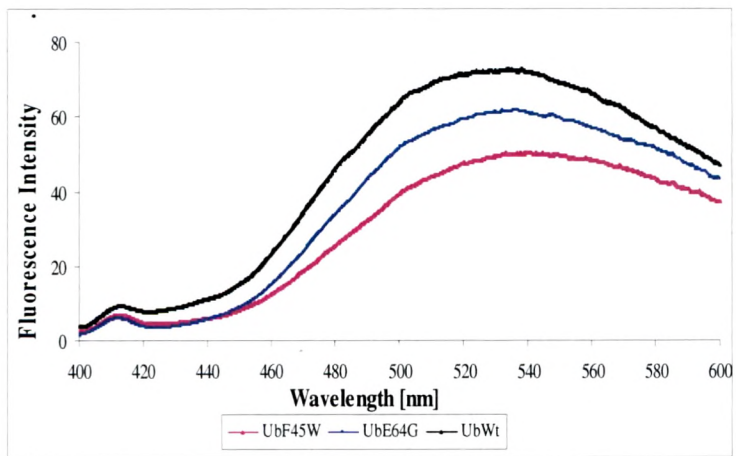
Figure 2.11. Fluorescence emission spectra of UbWt, UbF45Wand UbE64G recorded after exciting the protein at (a) 280nm and (b) 295nm

2.3.6. UbE64G shows greater content of hydrated hydrophobic residues

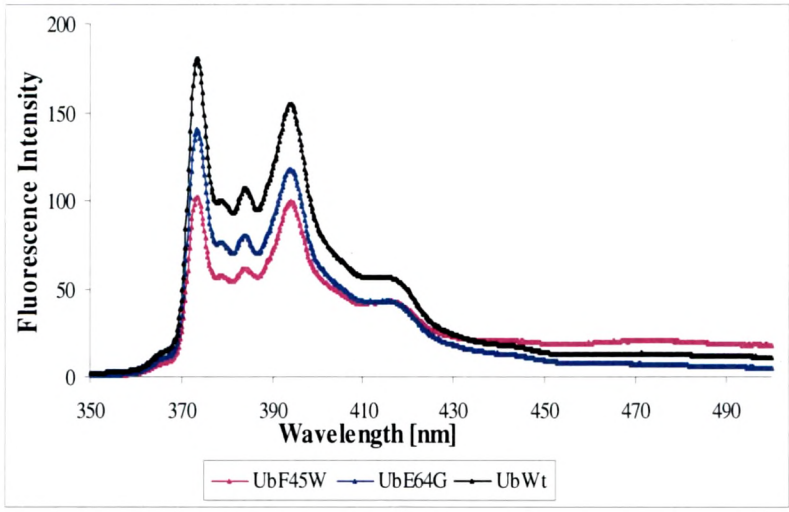
Fluorescence spectra of ubiquitin with extrinsic fluorophore ANS, showed higher intensity with UbE64G compared to UbWt. ANS is known to bind hydrated hydrophobic residues and emit fluorescence. Higher intensity of ANS fluorescence is due to greater exposure of hydrophobic residues to the surface in the mutant protein. Same result was observed with two other extrinsic fluorophores, Bis-ANS and pyrene (Figure 2.12.). The resultant differences seen in surface hydrophobicity of UbE64G perhaps can be attributed to minor alterations in structure leading to changes in the extent of exposure of hydrophobic residues. In addition the mutant replaces E (Glu) with G (Gly) which differ in hydropathy index, E is -3.5 while G is -0.4 (Kyte and Doolittle, 1992).



(a)



(b)



(c)

Figure 2.12. Fluorescence emission spectra of extrinsic fluorophores ANS, Bis-ANS and Pyrene are shown in panels (a), (b) and (c) respectively. The emission spectra of UbWt, UbF45Wand UbE64G are shown in each panel.

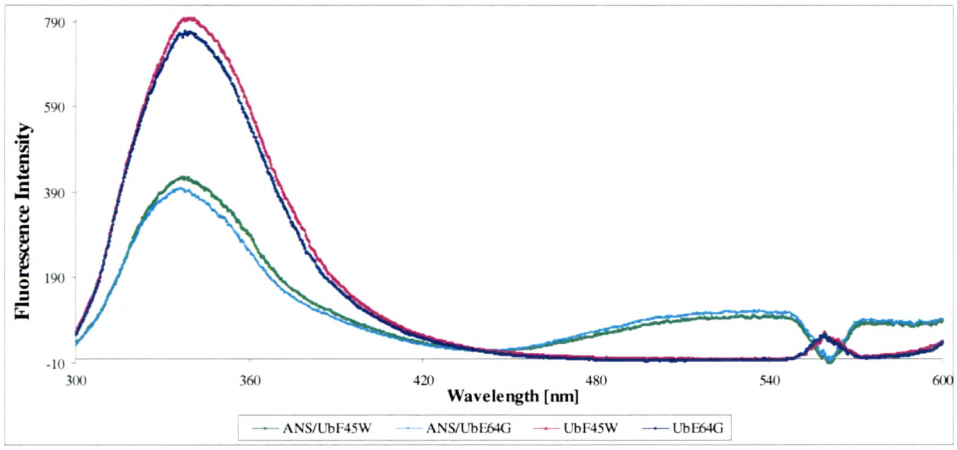


Figure 2.13. Fluorescence resonance energy transfer spectra of UbF45W and UbE64G recorded in absence and presence of ANS.

Fluorescence resonance energy transfer spectra demonstrating energy transfer between Trp and ANS were recorded, by exciting Trp at 280nm (Figure 2.13). Successful

transfer of fluorescence energy to ANS and identical peaks show that the two proteins have identical distances between Trp and ANS binding sites. The extent of ANS binding is also equal.

2.3.7. Thermal denaturation/ renaturation profiles of UbE64G and UbF45W are identical

Thermal denaturation and renaturation experiments showed that the two proteins follow same path during folding and unfolding. The process of unfolding was not complete owing to extreme thermal stability of the protein (Figure 2.14.).

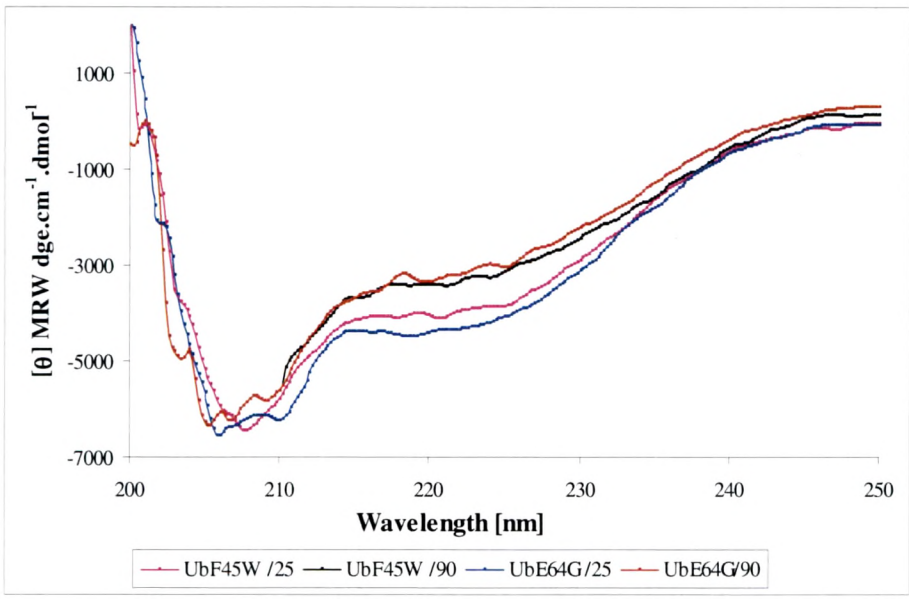
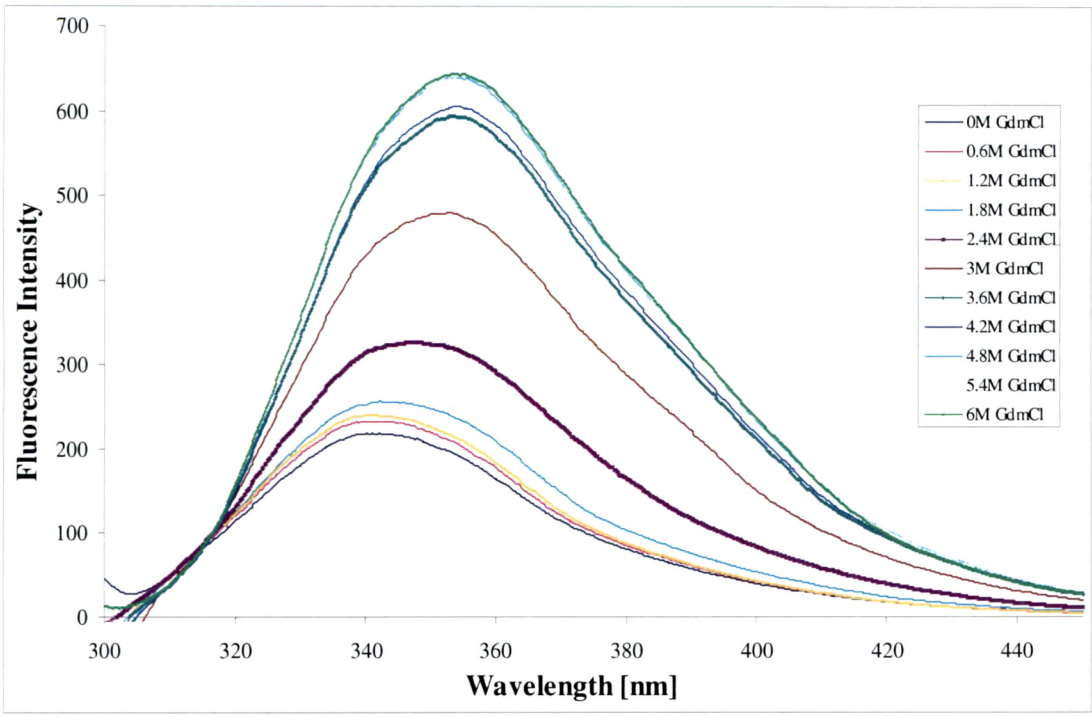


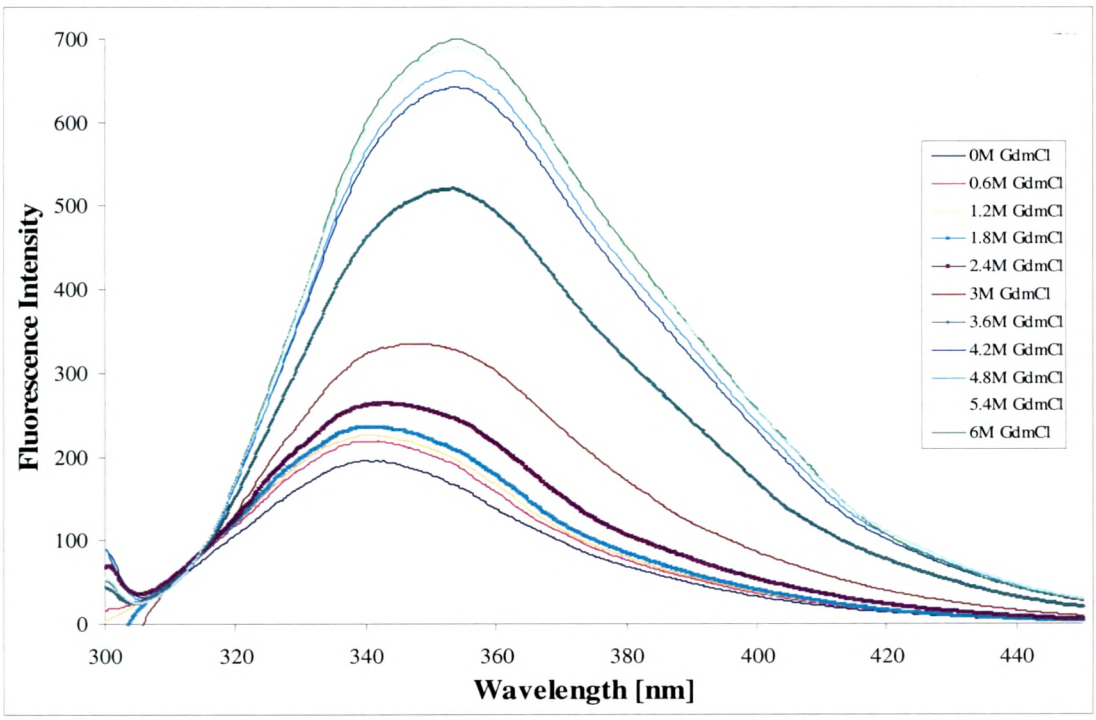
Figure 2.14. CD spectra showing thermal denaturation of UbF45W and UbE64G at 25°C and 90°C.

2.3.8. Guanidine hydrochloride denaturation of UbE64G and UbF45W

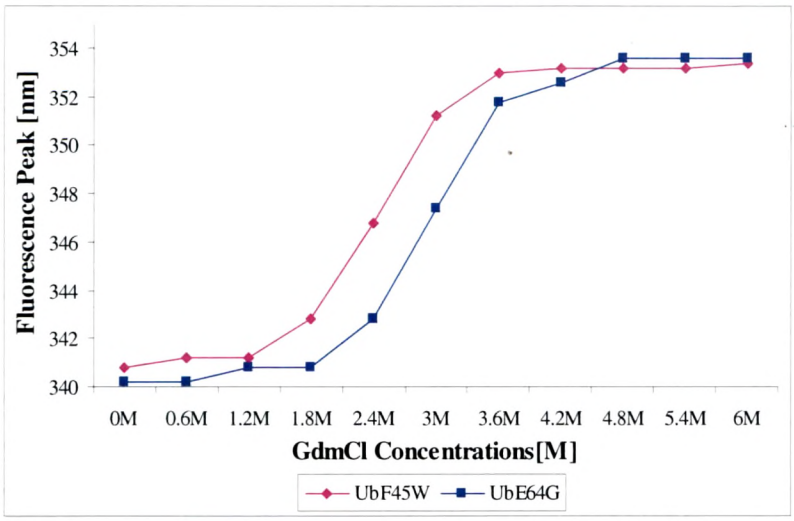
Guanidine hydrochloride denaturation of UbF45W and UbE64G were studied by fluorescence. Increasing concentration of guanidine hydrochloride causes loss of protein secondary and tertiary structures and subsequent increase in the fluorescence intensities, along with a red shift in λ_{max} of emission. The mutant protein UbE64G was found to be more stable than UbF45W (Figure 2.15.).


Fluorescence Intensity
Wavelength [nm]
0M GdmCl
0.6M GdmCl
1.2M GdmCl
1.8M GdmCl
2.4M GdmCl
3M GdmCl
3.6M GdmCl
4.2M GdmCl
4.8M GdmCl
5.4M GdmCl
6M GdmCl

(a)


Fluorescence Intensity
Wavelength [nm]
0M GdmCl
0.6M GdmCl
1.2M GdmCl
1.8M GdmCl
2.4M GdmCl
3M GdmCl
3.6M GdmCl
4.2M GdmCl
4.8M GdmCl
5.4M GdmCl
6M GdmCl

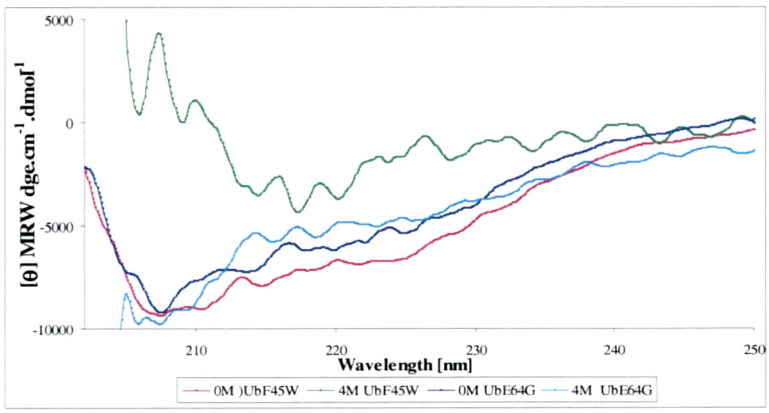
(b)



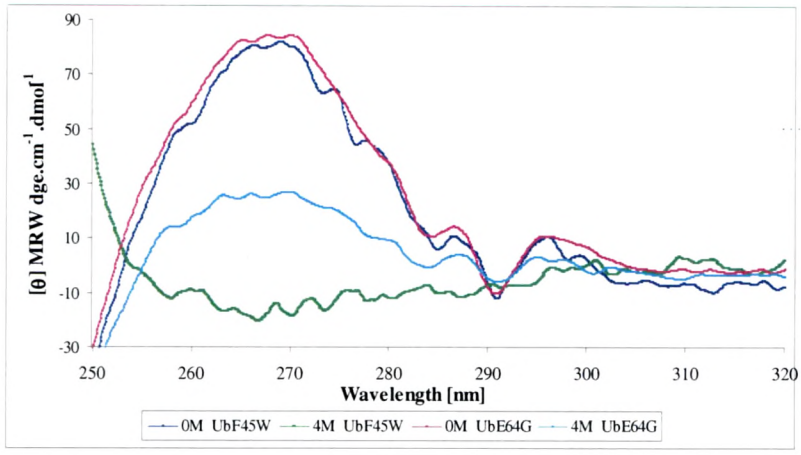
(c)

Figure 2.15. Intrinsic fluorescence emission spectra for guanidine hydrochloride denaturation of (a) UbF45Wand (b) UbE64G and (c) denaturation curves of UbE64G and UbF45W.

The guanidine hydrochloride denaturation curves of UbE64G and UbF45W were not coincident. The $C_{1/2}$ values of guanidine hydrochloride (GdmCl) for UbE64G and UbF45W were 3.6M and 3.24M. The far and near uv CD spectra were recorded with 0M and 4M guanidine hydrochloride. UbE64G retained its secondary and tertiary structure even after UbF45W loses its tertiary structure.



(a)



(b)

Figure 2.16. Guanidine hydrochloride denaturation curves of UbE64G and UbF45W. (a) Far uv CD and (b) Near uv CD spectra of UbF45W and UbE64G recorded in 0M guanidine hydrochloride and 4M guanidine hydrochloride.

2.4. DISCUSSION

From the results it is clear that this substitution has been well accommodated locally. However, replacement of Glu by Gly has affected the overall conformation of the protein, by altering the ionic and hydrophobic interactions, and changing the surface hydrophobicity. In addition, this mutation makes the protein UbE64G less stable in phosphate buffer (pH7), precipitating it from solution (results not shown). Besides, the protein UbE64G was found to be more stable than UbF45W towards guanidine hydrochloride denaturation under equilibrium conditions. In 4M guanidine hydrochloride UbF45W loses all its tertiary structure content, whereas UbE64G retains about half of it. Secondary structure of UbE64G remains unchanged even at 4M guanidine hydrochloride (Figure 2.16.a.). On the other hand, UbF45W loses most of its secondary structure. It was observed earlier that removal of surface charges stabilized the protein (Makhatadze et al., 2003; Sundd and Robertson, 2003) and removal of certain charged residues influenced the pKa values of neighbouring residues (Sundd and Robertson, 2003).

In conclusion, replacing the conserved glutamate at position 64 with glycine leads to subtle changes in structure as indicated by 4-5% decrease in helicity and increased stability towards guanidine hydrochloride denaturation. This study demonstrates a point mutation produces slight structural alteration changing the stability of the protein.