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Glutamate64 to Glycine Substitution in G1 β-bulge of Ubiquitin Impairs Function and Stabilizes Structure of the Protein

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Ubiquitin is a globular protein with a highly conserved sequence. Sequence conservation and compact structure make it an ideal protein for structure–function studies. One of the atypical secondary structural features found in ubiquitin is a parallel G1 β-bulge. Glutamate at 64 is the first residue of this β-bulge and the third residue in a type II turn. However, glycine is seen in these positions in several proteins. To understand the effects of substitution of glutamate64 by glycine on the structure, stability and function of ubiquitin, mutant UbE64G has been constructed and characterized in *Saccharomyces cerevisiae*. The secondary and tertiary structures of UbE64G mutant protein are only marginally different from wild-type protein (UbWt) and fluorescent form of ubiquitin (UbF45W). The earlier studies have shown that the structure and stability of UbWt and UbF45W were similar. However, UbE64G has less surface hydrophobicity than UbWt. UbE64G is found to be more stable compared with UbF45W towards guanidinium chloride induced denaturation. *In vivo*, complementation shows substrate proteins with Pro as the N-terminal residue, which undergo ubiquitination, have extended half-lives with UbE64G. This altered preference for Pro as opposed to Met might be related to natural preference of glutamate at 64th position in ubiquitin.

Key words: G1 β-bulge of ubiquitin, mutations in ubiquitin, ubiquitin, ubiquitin function, ubiquitin structure.

Abbreviations: UbWt, wild-type ubiquitin; UbE64G, ubiquitin with Glu64 replaced by Gly; UbF45W, Ubiquitin with Phe45 replaced by Trp; *UBI4*, Polyubiquitin gene; Ub-X-β-gal, Ubiquitin-β-galactosidase fusion, where X is the first residue of β-galactosidase and it can be any of the 20 amino acids; DTT, Dithiothreitol; CD, Circular dichroism; ANS, 1-Anilino 8-naphthalene sulphonic acid; gdmCl, guanidinium chloride; TLTGK, Thr-Leu-Thr-Gly-Lys; NPDG, Asn-Pro-Asp-Gly.

The function of a macromolecule is a consequence of its structure and dynamics. A small, globular protein such as ubiquitin with extensively hydrogen bonded structure and evolutionary conservation presents an excellent model system to understand structure–function relationships. Ubiquitin has no cysteines, metal ions or cofactors. Ubiquitin has been isolated and sequenced from a variety of sources and has been found to be identical in all higher eukaryotes from insects to humans (*1–4*). In yeast (*5*) and oat (*6*) ubiquitin, amino acid replacement is seen in only three positions of the entire sequence. Ubiquitin is a remarkable protein from the functional point of view as well. It forms conjugates with histones playing a key role in cellular nucleic acid metabolism (*7*). Ubiquitination of many important cellular proteins marks them for ATP-dependent degradation mediated by proteasomes (*8*, *9*) and by lysosomes in case of certain membrane proteins (*10*). The indispensable role of this protein and its striking conservation in nature reflect the structural constraints imposed on almost every residue in the sequence of the protein and its interaction with enzymes involved in ubiquitin conjugation.

The X-ray crystallographic structure of ubiquitin reveals a globular α/β-structure with hydrophobic core surrounded by five strands of β-sheet and four turns of α-helix. The compact structure of ubiquitin has nine reverse turns (*11*). Pulsed H-D exchange NMR experiments of ubiquitin folding indicated that the backbone amide protons of N-terminal β-sheet and α-helix are protected early. The C-terminal half of the protein exhibits a relatively slow folding kinetics (*12*). Partially folded state of ubiquitin stabilized in methanol/water mixture revealed conservation of native secondary structural elements in the N-terminal half. But the C-terminal half, which is predominantly β-strand in character, undergoes a transition to helical state (*13–15*). While folding, the N-terminal portion of ubiquitin serves as an autonomously folding chassis, governing the folding of rest of the protein through tertiary interactions (*16*).

Strict conservation of ubiquitin sequence leaves no scope of comparison with its homologues from other species to understand the role of individual residues. However, site-directed mutagenesis has been successfully employed to elucidate specific functions of many residues (*17–19*). Ubiquitin does not contain any Trp residues.

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In order to study the structural properties of ubiquitin using intrinsic tryptophan fluorescence, tryptophan was introduced in the place of phenylalanine at position 45 in the ubiquitin sequence. The folding kinetics and stability of this protein were investigated using fluorescence spectroscopy, and it was found to behave like the wild-type protein (UbWt) (*20*). In most of the earlier studies, the structure and folding of mutant ubiquitins were investigated in great detail. However, the functional consequences of mutations in ubiquitin molecule were largely ignored.

Ubiquitin has two β-bulges. The first one is located in N-terminal region in the type I turn of the β-hairpin. This region displays native-like structure in early folding intermediates. Occurrence of β-bulge in type I turn, however, is uncommon (*11*). Hence, it became a topic for extensive studies. Peptides from N-terminal region of ubiquitin have been found to attain β-hairpin conformation autonomously in the presence (*21*) and in the absence of organic solvents (*16*), confirming the potential of this peptide to act as an initiation site for protein folding. The replacement of TLTGK sequence, which forms type I turn along with β-bulge, by another type I turn forming NPDG resulted in non-native strand alignment (*22*).

The second β-bulge also displays some unusual features. It is a parallel G1 β-bulge, which is very rare. Glu64 forms the third residue in a type II turn and first residue in the β-bulge (*11*). Generally first residue in a β-bulge is a Gly. Further, the homologues of ubiquitin of Rub1 and NEDD8 display Gly in this position (*23*) (Fig. 1A). This unusual feature and its conservation through millions of years of evolution make it an interesting protein for structure–function studies. Moreover, this acidic residue is adjacent to a basic residue Lys63, which has been found to be important for UV repair of DNA (*24*), resistance of cells to stress conditions (*25*, *26*) and endosomal degradation of certain proteins (*10*). Significance of this structural feature in ubiquitin biology

A

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UBI -   1MQIVKTLTGKTITLEVESSDTIDKVKSKIQDKEGIPPDQQRLIFA45
RUB1 -  MIVKVTLTGKEISVELKESDLVYEIKELLEEKEGIPPSQQRLIFQ
NEDD8 - MLIKVKTLTGKEIEIDIEPTDKVERIKERVEEKEGIPPQQQRLIYS

UBI -   46GKQLEDGRTLSDYNIQKESTLHLVLRLRGG76
RUB1 -  GKQIDDKLTVTDAHLVEGMQLHLVLTLRGG
NEDD8 – GKQNNDEKTAADYKILGGSVLHLVLALRGG
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B

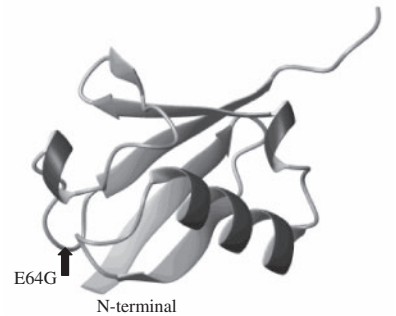

Fig. 1. **(A) The sequences of Rub1 and NEDD8 have been aligned with ubiquitin to show the preference for G in the first position of β-bulge (indicated in bold).** (B) The position of E64 in 3D structure of ubiquitin.

is the main focus of this work. In order to understand the importance of glu64 in the structure, stability and function of ubiquitin, a variant of ubiquitin (UbE64G) has been engineered using site-directed mutagenesis, characterized by circular dichroism (CD) and fluorescence spectroscopy. The position of this mutation in the 3D structure of the protein is shown in Fig. 1B. Its stability was evaluated by guanidine hydrochloride and thermal denaturation studies. Our results show that the mutant protein is structurally similar to UbWt, except that its surface hydrophobicity is reduced. In the present study, two forms of ubiquitin, namely the UbWt and fluorescent variant of ubiquitin (UbF45W), were used as controls. UbE64G was constructed from UbF45W, thus containing the double mutation.

The gene for UbE64G was introduced into a suitable vector and expressed in UBI4 mutant of *Saccharomyces cerevisiae*, a polyubiquitin deletion mutant, to validate its functional integrity by complementation. The polyubiquitin gene *UBI4* is expressed under stress and rescues the organism in extreme conditions (*27*). The mutant protein UbE64G was found to be functionally complementary and rescued UBI4 mutants of *S. cerevisiae* under thermal stress (results not shown). The effect of mutation on the turnover of proteins was studied with two different constructs of ubiquitin–β-galactosidase fusion protein, having Met and Pro as the N-terminal residues of β-galactosidase in UBI4 mutant expressing UbE64G. According to N-end rule, proteins with Met as N-terminal have longer half-lives, where as proteins with Pro as their N-terminal are extremely short lived (*28*). Interestingly, our results show that proteins with Pro as the N-terminal residue have extended half-lives with UbE64G complementation compared with the UbWt control. Thus, a point mutation leads to altered substrate preference.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis, Construction of UbE64G Plasmid—Plasmid pKK223-3 carrying ubiquitin gene with F45W mutation was a gift from Prof. Mark Searle's laboratory (*22*). The ubiquitin gene from pKK223-3 was subcloned into pUC18. The mutation for UbE64G was introduced following Genei *in vitro* site-directed mutagenesis protocol. Introduction of mutation was confirmed by sequencing. The mutated fragment from pUC18 vector was cloned back and pKK223-3-E64G was obtained.

Purification of UbWt, UbF45W and UbE64G Proteins—The proteins UbWt, UbF45W and UbE64G (double mutant) were purified using the methods given in literature (*29–31*). Since UbWt is known to be a heat-stable protein, the heat step was employed during the purification of the protein. The supernatant that was collected, after cell lysis and precipitation of DNA, was heated at 85–90°C in the presence of 1 mM DTT for 15 min. The supernatant contained ubiquitin while most of the proteins coagulated and were removed by centrifugation (*29*). This step was adapted for the purification of UbF45W earlier (*20*). This purification step based on the thermal stability of ubiquitin could be adapted to the purification of UbE64G as well. The following changes

were made in the protocol. After cell lysis, the lysate was fractionated (*31*) up to 85% ammonium sulphate precipitation. After dialysis, ubiquitin containing fraction was purified on Sephadex G-50 column. The protein was concentrated by precipitation and dialysis. The protein stocks were stored in 10% glycerol at −20°C. Protein concentration for F45W was determined using its extinction coefficient of 6744/M/cm at 280 nm (*32*). Mutant ubiquitin UbE64G was purified using the same protocol that was used for the UbWt.

CD and Fluorescence Spectroscopy of UbF45W and UbE64G—CD spectra were recorded using a Jasco J-715 spectropolarimeter. Far-UV CD spectra were recorded with 1 mm path length cells. Spectra were recorded in the range of 200–250 nm with a scan speed of 50 nm/s. The spectra were accumulated for six times and averaged to improve the signal-to-noise ratio. Protein solutions were prepared in 10 mM Tris–HCl, pH 7.4 and concentration of protein was 0.25 mg/ml. The near-UV CD spectra of the proteins were recorded between 250 and 320 nm. Path length was 1 cm and protein concentration was 0.774 mg/ml. Appropriate 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 using excitation and emission bandpasses of 5 nm. Protein concentration was 0.1 mg/ml. Samples were excited at 295 nm to record the intrinsic tryptophan fluorescence in the range of 300–400 nm.

1-Anilino 8-naphthalene sulphonic acid (ANS) was used as an extrinsic fluorophore at a concentration of 50 μM. ANS was excited at 390 nm and emission was recorded between 450 and 600 nm. Slit width was 5 nm. Protein concentration was 0.2 mg/ml. Protein solutions were prepared in Tris–HCl buffer, pH 7.4.

Construction of Yeast Vector Carrying UbE64G Gene—Yeast–bacteria shuttle vector pUb175-E64G was constructed from pUb175 by replacing *Ub* gene under CUP-1 promoter with *UbE64G* gene, which can be induced in yeast by 10–100 μM copper sulphate. The mutant ubiquitin gene from pKK223-3-E64G was PCR amplified. The resultant DNA was subcloned into pUB175 yeast expression vector. The plasmid pUB175 was a kind gift from Prof. Daniel Finley. Similarly, the gene for UbF45W was also cloned to have a control for fluorescence studies. The plasmid pUB175-E64G was digested with restriction enzymes, and later it was sequenced for confirmation.

The vector was transformed into yeast UBI4 mutant, which lacks the polyubiquitin gene. The gene for UBI4 expresses under stress and mainly responsible for the survival of the organism under stress conditions. In the absence of this gene, the organism can grow at 30°C (permissive temperature), but fails to grow at ≥39°C. Complementation by functional ubiquitin restores wild-type phenotype to the organism. This system was used for complementation tests with UbE64G mutant.

Effect of the Ubiquitin Mutation on Substrate Protein Turnover—SUB60 (*MAT*a *ubi4-*Δ*2::LEU2 lys2-801 leu2-3,112 ura3-52 his3-Δ200 trp1-1*) and SUB62 (*MAT*a *lys2-801 leu2-3,112 ura3-52 his3-Δ200 trp1-1*) strains of *S. cerevisiae* (*33*) were used for *in vivo* studies. SUB60 and SUB62 strains of *S. cerevisiae* were transformed with yeast plasmid Yep96 (*24*) carrying the three variants of ubiquitin gene, namely wild-type or UbWt, UbF45W and UbE64G, and the recipients were referred to as Yep96/wt, Yep96/F45W and Yep96/E64G. SUB60 double transformed with pUB23, a 2 μ-based vector expressing ubiquitin-X-βGal (Ub-X-βGal) fusion gene under the control of galactose inducible GAL10 promoter with X position as Met and Pro in independent sets. SUB62 cells were also transformed with pUB23 (*28*) with X position as Met and Pro. *Saccharomyces cerevisiae* transformants were grown in synthetic galactose media at 30°C, conditions under which Ub-X-βGal is constitutively expressed. The cells were grown with and without 100 μM of $CuSO_4$ in two independent sets for the induction of UbWt, UbF45W and UbE64G from Yep96. β-Galactosidase activity assay was used as a measure of protein stability in this set of experiments.

RESULTS

CD and Fluorescence Spectra of the Three Variants of Ubiquitin—Far-UV CD spectra were recorded to study the changes in secondary structure due to the mutation in the sequence of ubiquitin. The far-UV CD spectrum of UbE64G shows slight changes in secondary structure content, with respect to both UbWt and UbF45W (Fig. 2). Percentages of various components of secondary structure in far-UV CD spectra of UbWt, UbF45W and UbE64G were calculated using CD Pro software (*34*) and are shown in Table 1. The values obtained with UbWt are in agreement with X-ray crystallographic data (*34*). The values observed with the two variants UbF45W and UbE64G appear to be similar to those observed with UbWt. However, UbE64G shows 4–5% decrease in

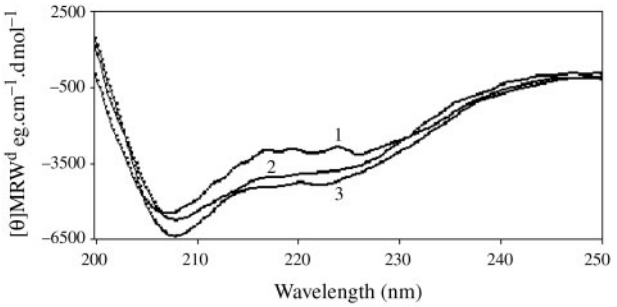

Fig. 2. **Far-UV CD spectrum of three forms of ubiquitin: UbWt (1), UbF45W (2), UbE64G (3).**

Table 1. **Secondary structural analysis of far-UV CD spectra using CD Pro software.**

Type of secondary structure	UbWt[a] (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

[a]The values reported by Chyan *et al.* (*34*).

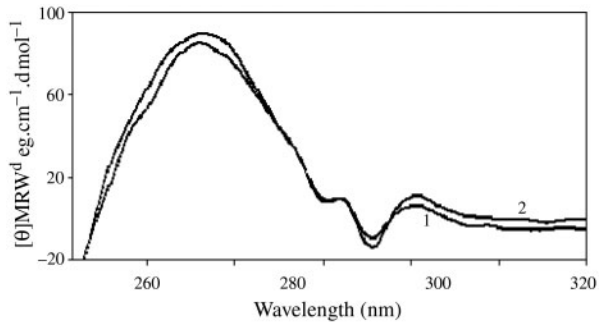

Fig. 3. **Near-UV CD spectrum: UbF45W (1), UbE64G (2).**

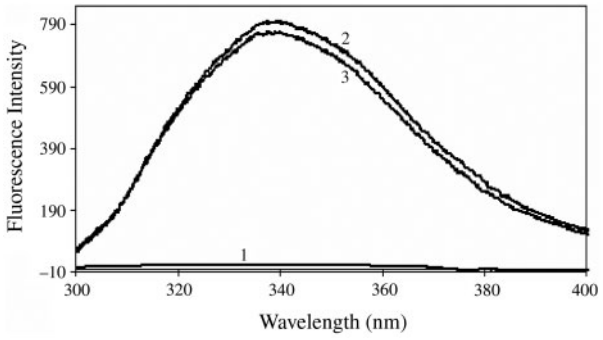

Fig. 4. **Fluorescence emission spectra of UbWt (1), UbF45W (2), UbE64G (3) recorded after exciting the protein at 280 nm.**

α-helical content and 3% increase in β-sheet. The changes in secondary structure of UbE64G could be due to enhanced flexibility introduced by the substitution.

The CD spectrum of a protein in the near-UV spectral region (250–320 nm) can be sensitive to certain aspects of tertiary structure. At these wavelengths, the chromophores are the aromatic amino acids and disulphide bonds, and the CD signals they produce are sensitive to the overall tertiary structure of the protein. Hence, tertiary structure is a qualitative measure of the overall conformation of the molecule (*35–38*). Based on near-UV CD spectra, the tertiary structures of UbE64G and UbF45W appear to be similar, an indication that the conformation of the protein around the aromatic residue is unaffected by this mutation (Fig. 3).

Due to lack of tryptophan, UbWt does not display any fluorescence properties. On the other hand, UbE64G and UbF45W display very similar fluorescence properties, indicating that the environment around aromatic amino acid residue Trp45 is almost identical (Fig. 4).

UbWt Shows Greater Surface Hydrophobicity than UbE64G—Fluorescence spectra of ubiquitin with extrinsic fluorophore ANS showed higher intensity with UbWt compared with UbE64G. ANS is known to bind to the exposed hydrophobic surfaces of proteins and show enhanced fluorescence compared with its weak fluorescence in aqueous media. Figure 5 shows that the intensity of ANS fluorescence is higher when bound to

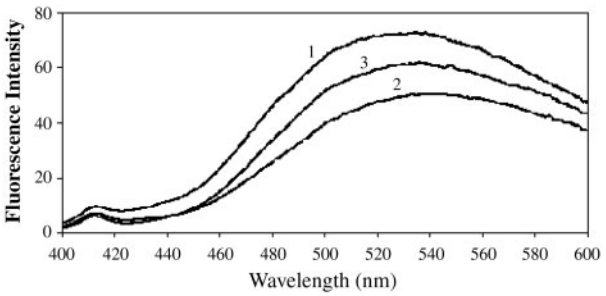

Fig. 5. **Fluorescence emission spectra of the three forms of ubiquitin, UbWt (1), UbF45W (2) and UbE64G (3), bound by extrinsic fluorophores ANS recorded after exciting the ANS at 280 nm.**

UbE64G, compared with that in the case of UbF45W and this indicates that UbE64G has more exposed hydrophobic surface than UbF45W. However, higher intensity of ANS fluorescence observed with UbWt is due to greater exposure of hydrophobic residues to the surface in the UbWt compared to UbE64G (Fig. 5). Similar results were obtained with two other extrinsic fluorophores, Bis-ANS and pyrene (results not shown). 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 with G, which differ in hydropathy index, E is −3.5 while G is −0.4 (*39*).

Thermal denaturation and renaturation studies showed that the two proteins follow the same pathway during folding and unfolding experiments and do not show much change in their conformation. In retrospective, it could be reasoned that it was possible to use the thermal treatment step in the protocol for purification of UbF45W for UbE64G as well without any modification, due to their similar thermal stabilities.

However, 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 3.6 and 3.24 M (Fig. 6C). The far and near-UV CD spectra recorded in the absence and presence of 4 M guanidine hydrochloride suggest that UbE64G retains some amount of its secondary and tertiary structure, whereas UbF45W loses tertiary structure completely (Fig. 6A and B) in the presence of 4 M guanidine hydrochloride.

In Vivo *Studies—UbI4* gene cluster has four to five copies of ubiquitin expressed as a single polypeptide chain, which is processed into ubiquitin molecules post-translationally. SUB60 mutants lacking *UbI4* fail to withstand stress conditions, but grow normally at 30°C. SUB60 mutants lacking *UBI4* gene and SUB62 wild-type cells were transformed with yeast plasmids (carrying variants of ubiquitin gene as indicated), Yep96/wt, Yep96/F45W and Yep96/E64G. Yep96/wt, Yep96/F45W and Yep96/E64G are plasmids carrying ubiquitin gene under CUP-1 promoter and have tryptophan auxotrophy for selection. These transformants were co-transformed by pUB23, a plasmid with UbWt gene fused to *lacZ*.

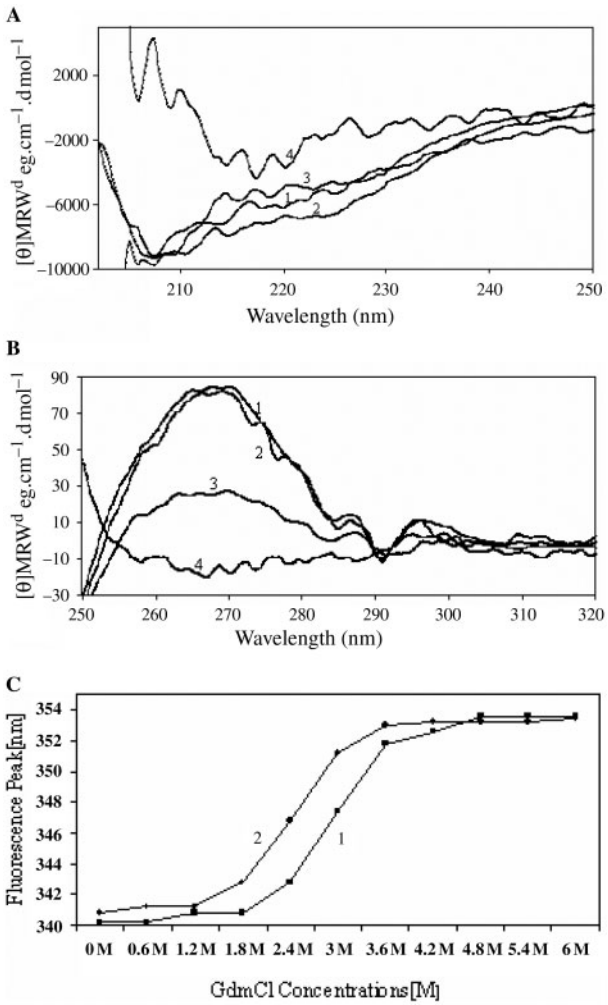

Fig. 6. **(A) Far-UV CD spectra of UbE64G and UbF45W recorded in 0 M guanidine hydrochloride (1 and 2 respectively) and 4 M guanidine hydrochloride (3 and 4 respectively).** (B) Near-UV CD spectra of UbE64G and UbF45W recorded in 0 M guanidine hydrochloride (1 and 2 respectively) and 4 M guanidine hydrochloride (3 and 4 respectively). (C) Guanidine hydrochloride denaturation curves of UbE64G (1) and UbF45W (2).

According to N-end rule, the stability of a protein is determined by its N-terminal residue. β-Galactosidase fusion with stabilizing N-terminal residues like Met is cleaved by ubiquitin hydrolases releasing a free β-galactosidase with longer half-life. These fusions with destabilizing residues such as Pro are not processed and a polyubiquitin chain is built subsequently on the ubiquitin in the fusion protein, resulting in degradation of the β-galactosidase. The ubiquitin–β-galactosidase fusion is under pGAL10 promoter. β-Galactosidase activity assay has been used as a measure of protein stability. Our results show that β-galactosidase activity remained more or less unchanged in Yep96/wt, when Met (stabilizing residue) was present as the N-end residue. β-Galactosidase activity showed a decline, when Pro was present as the N-end residue. However, the β-galactosidase activity increased for Pro-β-galactosidase in the UbE64G mutant, compared with UbWt and UbF45W in UbI4 mutant strain SUB60 (Table 2).

DISCUSSION

Proteins showing complete conservation of their sequence through millions of years of evolution are examples of optimal balance of structure and function. Ubiquitin is one such protein, showing no change in sequence from insects to humans and only three substitutions in yeast. A close look at its structure indicates that in the second β-bulge, there are certain unusual features. The first residue in the β-bulge has a glutamate in place of glycine, the later being a preferred choice in such structures. Structural homology searches also revealed that close structural homologues of ubiquitin, NEDD8 and Rub1 (*40*), and ubiquitin-like protein from baculovirus *Autographa californica* (*41*) have glycine at the same position. Interestingly, those structures are identical.

From our results, it is clear that this substitution has been well-accommodated locally. However, replacement of glutamate by glycine 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 (pH 7), 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 4 M guanidine hydrochloride, UbF45W loses all its tertiary structure content, whereas UbE64G retains about half of it. Secondary structure of UbE64G remains unchanged even at 4 M guanidine hydrochloride (Fig. 6A). On the other hand, UbF45W lost most of its secondary structure. It was observed earlier that removal of surface charges stabilized the protein (*42*, *43*) and removal of certain charged residues influenced the pKa values of neighbouring residues (*43*).

As described under EXPERIMENTAL PROCEDURES section, UbE64G could be purified by adapting the heat treatment, used for purification of UbWt and UbF45W, because of its thermal stability. Since UbE64G and UbF45W are thermostable, it appears that thermal treatment does not lead to complete denaturation (Fig. 7). The thermal denaturation profiles appear similar. Thermal unfolding and refolding of UbE64G also did not differ from that of UbF45W.

The changes in structure of the molecule, which are evident *in vitro*, appear to have a bearing on the function of the molecule. Interestingly, UbE64G has extended the half-life of unstable substrate proteins with N-terminal Pro residue.

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, increased stability towards guanidinium chloride denaturation and more importantly altered substrate interaction. The UbWt leads to slower turnover of protein with N-terminal Met residue, compared with the proteins with N-terminal Pro residue. The mutant has reversed behaviour; it indeed extended the half-life of protein with

Table 2. **Effect of UbE64G on the half-life of proteins in the UbI4 background.**

Strains of *S. cerevisiae* used in the experiment	Ub-Met-β Gal nanomoles of ONPG/min/mg protein		Ub-Pro-β Gal nanomoles of ONPG/min/mg protein	
	After induction by copper sulphate	Without copper sulphate	After induction by copper sulphate	Without copper sulphate
SUB60	−8.283 ± 1.161	−11.4667 ± 1.180	−1.653 ± 0.231	−2.283 ± 0.234
SUB62	−11.627 ± 1.871	−14.08 ± 1.624	−2.323 ± 0.372	−2.750 ± 0.361
SUB60 β-Gal	55.303 ± 8.026	49.717 ± 9.027	16.843 ± 1.740	16.613 ± 2.054
SUB62 β-Gal	55.963 ± 8.792	46.873 ± 9.071	2.038 ± 0.901	1.937 ± 0.236
SUB60 β-Gal/UbWt	48.747 ± 2.941	39.480 ± 4.842	4.653 ± 0.738	7.183 ± 0.639
SUB60 β-Gal/F45W	48.373 ± 7.338	41.293 ± 4.905	6.297 ± 0.702	7.983 ± 0.623
SUB60 β-Gal/E64G	48.713 ± 10.745	40.017 ± 6.380	12.417 ± 0.453	12.5 ± 1.241

Saccharomyces cerevisiae strains SUB60, SUB62, SUB60 transformed by plasmidsYep96/UbWt, Yep96/F45W and Yep96/E64G expressing the three forms of ubiquitin, namely UbWt, F45W and E64G. These cells were also transformed by pUb23 expressing Ub-βGal fusion with Met and Pro as the N-terminal residues. In SUB62, cells were co-transformed by plasmids Yep96/UbWt, Yep96/F45W, Yep96/E64G and pUb23 expressing Ub-βGal fusion with Pro as the N-terminal residue. SUB60 and SUB62 were used as controls.

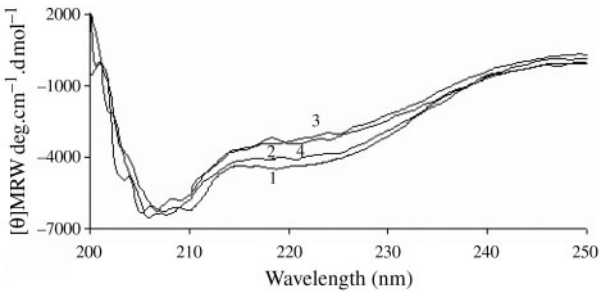

Fig. 7. **Far-UV CD spectra of UbF45W and UbE64G before thermal denaturation (1 and 2 respectively) and after thermal denaturation (3 and 4 respectively).**

N-terminal Pro, which should have been removed from the system faster.

This study provides an interesting example of a point mutation with subtle structural alteration and with a significantly altered function.

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CONFLICT OF INTEREST

None declared.

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