Summary

Summary

Ubiquitination is an important posttranslational modification regulating various cellular processes including cell cycle regulation (Koepp et al., 1999), inflammatory response (Ghosh et al., 1998), developmental processes, proteasomal degradation (Hough et al., 1986) of proteins amongst many. Ubiquitin proteasome degradation system is one important process that is involved in modeling the intracellular protein make up. The Ubiquitin proteasome degradation pathway comprises ubiquitin, group of three enzymes i.e., E1, E2 and E3, proteasome and target protein (Pickart, 2001).

The ubiquitin-activating enzyme Uba1 (E1) set up the first step in the cascade of ubiquitin (Ub) mediated modification of target proteins. Structurally, E1 is a large multidomain monomeric protein that has weakly conserved two-fold geometry. The second half, which is actively involved in the enzyme's activity, is identical with the first half of the enzyme. There are six in the protein namely, IAD, FCCH, 4HB, AAD, SCCH and UFD. It has a complex arrangement due to the discontinuous and interspersed organization of these six domains. E1 enzyme's modular structure is associated with activities specific to different domains, 1) adenylation is carried out by AAD, 2) thioester reaction takes place on SCCH, and 3) recruitment of specific E2s is accomplished by UFD. Moreover, the arrangement of the domains UFD, FCCH and SCCH are crucial for the overall tertiary structure of E1. They are connected to their respective adjacent domains by long flexible linkers (Lee and Schindelin, 2008).

Taking all this information into account, we opted for structural characterization of 4 domains of ubiquitin activating enzyme namely, FCCH, 4HB, SCCH and UFD as independent units. Modular organization of the protein and presence of long flexible linkers between domains indicates large conformational independence of individual domains, suggesting the possibility of characterizing their structure and function individually.

Summary

The DNA fragments encoding the four domains of E1 namely, FCCH, 4HB, SCCH and UFD were cloned individually into a bacterial expression vector pET28a. All four domains were expressed and purified using chromatographic techniques. Purification of the UFD domain posed a problem as the peptide molecules aggregated. Urea was used to elute the peptide, after several attempts at standardization of purification.

Purified peptides were studied for their secondary structural characteristics by far-UV-CD spectroscopy and the spectra were analyzed using BestSel software. The spectrum of FCCH revealed the presence of mostly β-sheet structure with very little α-helical structure. 4HB domain displayed mostly helical structure in its far-UV-CD spectrum. The structure of SCCH also has been found to be mostly helical. The spectrum of UFD revealed that the peptide has a mixed α/β structure. Results obtained from these spectra were compared with the structure of crystallized yeast E1 and the information available in the literature for SCCH and FCCH domains of mouse E1. Further, the results obtained here were compared to the predicted secondary structures using PSI-Pred.

The secondary structure of the domains obtained from spectra was in agreement with the secondary structure the same domains obtained from the crystallographic studies of an intact form of E1. However, the values of secondary structure content differ slightly. Yeast E1 and mouse E1 share an overall 52% sequence identity. Far-UV-CD spectra of mouse FCCH revealed to have more of β-sheet (Szczepanowski et al., 2005), which is comparable to the spectrum we obtained for yeast FCCH. Similarly, the secondary structural features of mouse SCCH showed prominent helical nature of the domain. Our experiment also showed a similar result with the yeast SCCH domain. Since there is not much information available on 4HB and UFD domains of the mouse, the structure of these domains of the mouse was predicted using PSI-Pred and compared with the results obtained with yeast 4HB and UFD domains. In the case of 4HB, the structure predicted for the mouse model was

mostly helical. The values obtained from the far-UV-CD spectrum of yeast 4HB also showed a helical structure. The last domain of E1, i.e. UFD was identified to have a mix of α/β structure, which again was in agreement with the structure predicted for mouse UFD.

Overall, secondary structural features were retained by all domains even when they were present as single independent units.

The fluorescence denaturation spectra were carried out for all four domains FCCH, 4HB, SCCH and UFD, with aromatic amino acid residues as the intrinsic fluorophores as well as by adding extrinsic fluorophore. Denaturation of these peptides by guanidine hydrochloride revealed the existence of these peptides in compactly folded conformations. A prominent red shift was observed in most of the peptides indicating exposure of aromatic amino acids to the polar environment as a consequence of a change in the overall structure of the domains. All four peptides were treated with the extrinsic fluorophore ANS (1-Anilino 8-naphthalene sulphonic acid). ANS is used to check surface hydrophobicity of protein, as it is known to bind exposed hydrophobic residues in a protein and emit fluorescence of higher intensity. Peptides FCCH, 4HB and SCCH showed higher intensity with increasing concentration of guanidine hydrochloride. This indicated the presence of hydrophobic residues in the core of the folded domains initially, suggesting that the peptides have adopted a native-like folded state. For UFD peptide the intensity was higher at a lower concentration of guanidine hydrochloride pointing out the presence of hydrophobic residues on the surface. This observation is in agreement with the difficulties encountered during purification.

Peptides were found to be retaining their secondary structural characteristics, indicating that they are attaining native like structure as isolated domains. These observations suggested the possibility for the peptides FCCh, 4HB, SCCH and UFD to act as functional moieties and hence studies

were carried out to check for the effect of their presence in the cell as individual units. Under cellular stress conditions, like heat stress or antibiotic stress, the population of unfolded and misfolded proteins increases. To get rid of these misfolded or unfolded proteins, UPS would work more and try to retain balance in the cell by eliminating this unwanted protein chunk (Finley et al., 1994). Hence, the effect of expression of the peptides in yeast cells was tested under normal as well as stress conditions. If the domains are functional, they have the potential to interact with their respective binding partners and interfere or slow down cellular machinery by competing with the E1 enzyme. Under normal conditions, the expression of domains led to no change in cell growth. Domains FCCH and 4HB showed no effect on cell growth even under heat and antibiotic stresses. SCCH domain affected cell survival under heat stress and Hygromycin B antibiotic stress. UFD also showed a moderate effect on cell survival in the presence of heat stress and antibiotic stress.

The role of ubiquitin as a tag in posttranslational modifications is pivotal to cell metabolism due to its involvement in multiple cellular processes. It is a small globular protein with a high degree of sequence conservation throughout Eukaryota (Vijay-Kumar et al., 1987). It is composed of a 5-stranded β-sheet, a short 3_{10} helix and a 3.5-turn α-helix along with a C-terminal tail and seven internal lysine residues (Vijay-Kumar et al., 1987). The C-terminal glycine and internal lysine residues are important in forming bonds with substrate protein as well as with other ubiquitin molecules to form polyubiquitin chains. Ubiquitin is found to be extremely stable even at high temperatures due to the presence of extensive hydrogen bonds. The compact structure of ubiquitin showed the presence of two β-bulges (Vijay-Kumar et al., 1987). First, β-bulge is present near its N-terminus made up of Gly10, Lys11 and Thr7 residues, whereas the second β-bulge was present near C-terminus composed of residues Glu64, Ser65 and Gln2. The N-terminal β-bulge has been studied extensively (Cox et al., 1993b), on the other hand very few studies were done on C-terminal β-bulge.

Summary

Our laboratory focused on the parallel β-bulge of ubiquitin, which is present in continuation with a type II turn and located in a parallel β-sheet. This kind of parallel β-bulge is rarely found in proteins. Further, the residues present in this β-bulge are less preferred and uncommon for their respective positions. Interestingly these residues have been conserved totally in ubiquitin during the course of evolution (Chan et al., 1993). To understand the importance of these residues at their positions, site-directed mutagenesis was carried out. Earlier in our laboratory, residues of the β-bulge were replaced by residues of greater propensity at their positions resulting in mutants Q2N, E64G and S65D (Mishra et al., 2009, 2011). Results showed in stress sensitive polyubiquitin deletion mutants lacking the *UBI4* gene, the three mutant forms of ubiquitin namely UbE64G, UbS65D and UbQ2N could not complement the cells and resulting in decreased survival of yeast cells. Experiment with Ub-Pro-β galactosidase, which acted as a substrate for degradation by ubiquitin fusion degradation (UFD) pathway, also showed a drop in the rate of degradation. However, cells survived under heat stress and mutant proteins remained unaffected under high temperatures. These β-bulge mutants also showed similar structural characteristics as wild-type ubiquitin protein (Mishra et al., 2009, 2011).

One of the objectives of the current study was to check the activation efficiency of β-bulge mutants Q2N, E64G and S65D of ubiquitin by ubiquitin activating enzyme E1. To conduct activation assay, all three mutant proteins were purified and incubated with the E1 enzyme in the presence of ATP and buffer system. Later this mixture was loaded on SDS PAGE and transferred to nitrocellulose membrane for Western blot analysis. As the antibody used was anti-ubiquitin antibody, the formation of the ubiquitin-E1 complex could be identified on the blot. Results clearly showed, all three β-bulge mutants Q2N, E64G and S65D were able to form a bond with E1 with the same efficiency as wild type ubiquitin. This showed all three mutants were functionally active like the wild type ubiquitin.

One more experiment was carried out to check the stability of these β-bulge mutants with a range of different pH conditions by recording far-UV CD spectra in different pH buffers. At pH 2, the E64G mutant showed some alteration in structure, whereas, pH 2 and pH 4showed little effect on the structure of S65D and Q2N.

In summary, ubiquitin activating enzyme E1 is a modular protein with different domains acting as independent units. DNA sequences encoding four domains of E1 namely, FCCH, 4HB, SCCH and UFD were individually cloned into a bacterial expression vector to check the structural integrity of these domains. CD spectra of FCCH showed the presence of mostly β-sheets, whereas 4HB and SCCH were found to have prominent helical structures and lastly UFD revealed to have mix α/β structure. Secondary structural characteristics of individual domains were similar to structural characteristics observed when they were an integral part of the protein E1. Fluorescence emission studies of guanidine hydrochloride denaturation of these domains showed indicated that these domains were able to attain native like folded structures independently. Further, studies with extrinsic fluorophore ANS established the presence of hydrophobic core in FCCH, 4HB and SCCH. Only the UFD domain showed the presence of hydrophobic residues on the surface even in the absence of denaturant. Studies on ubiquitin mutations and their interactions with E1 being one of the objectives of this work, experiments of ubiquitin activation were carried out with E1 and β-bulge mutants Q2N, E64G and S65D. Results of the study showed all three mutants could interact with E1 and form a covalent linkage with its active site cysteine. The intensity of bands obtained in western blots showed that they can be activated at the same rate by E1 in spite of the mutation. In continuation of previous studies from the laboratory, CD spectra of β-bulge mutants Q2N, E64G and S65D were recorded under different pH conditions. Exposure to different pHs indicated the stability of β-bulge mutants under extreme pH conditions.