Studies on Mutant Forms of Ubiquitin and Ubiquitin Activating Enzyme E1

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By

Brinda Panchamia

Under the supervision of

Prof. C Ratna Prabha

(Macromolecular Structure and Biology Laboratory)

Department of Biochemistry,

Faculty of Science,

The Maharaja Sayajirao University of Baroda,

Vadodara-390 002

Introduction

All proteins undergo synthesis and degradation i.e., they get hydrolyzed to release constituent amino acids and replaced by new synthesis. Regulation of cellular protein content and removal of misfolded protein is essential for cell survival. Majority of cellular proteins either go through lysosomal degradation or proteasome mediated degradation. Protein degradation is imparted with specificity through post-translational modification, where they get phosphorylated, glycosylated, ubiquitinated, methylated in order to achieve cellular homeostasis [1]. Many intracellular proteins are ubiquitinated and degraded by ubiquitin–proteasome System (UPS). UPS accomplishes this function by conjugating a small covalent modifier protein called ubiquitin (Ub) to substrate protein.

Ubiquitin has seven lysine residues (K6, K11, K27, K29, K33, K48 and K63) which take part in polyubiquitin chain formation. The branching of polyubiquitin chain resulting from the use of different lysine residues signals different functions, protein degradation by the 26S proteasome [2, 3], DNA repair, transcription regulation and others. Chain of at least four ubiquitin molecules linked through K48 linkages generally act as signal for targeted protein degradation. UPS pathway is controlled by a set of 3 enzymes: E1 (Ubiquitin activating enzyme), E2 (Ubiquitin conjugating enzyme) and E3 ligase. The resultant polyubiquitinated protein is degraded by 26S proteasome [4].

The first step in protein degradation process is to activate the C-terminal glycine residue of the ubiquitin in an ATP dependent process, called adenylation. After which a covalent thioester bond is formed between ubiquitin and catalytic cysteine of E1. Finally this activated Ub-E1 complex recruits specific E2 and transfers the activated ubiquitin to catalytic cysteine of E2 via transthioesterification reaction. The energy stored in Ub-E2 complex is then utilized in conjugating Ub with target protein to be degraded which is brought by E3 ligases. Polyubiquitinated substrates are then fragmented by 26S proteasome complex, where they are unfolded and degraded into small peptides. Later ubiquitin chains are reduced to single ubiquitin molecules by deubiquiting enzymes.

Ubiquitin is highly conserved and stable protein that adopts a compact β-grasp fold with a flexible six-residue long extended C-terminal tail [5]. Ubiquitin structurally consists of two helices, five mixed β sheets and two β bulges. To understand the importance of the conserved residues the gene for Ub was mutated in our laboratory and the protein is studied to understand structure–function relationships with the help of mutants. One of the β-bulge formed by Gln2, Glu64 and Ser65, which is entirely conserved, located adjacent to Lys63, was mutated as follows: Q2N, E64G and S65D [6,7]. At 64^{th} position glutamate to glycine substitution affected the overall structure by decreasing the helicity (4-5%), whereas 2^{nd} and 65^{th} position substitution also shows alteration in structural features [8,9]. Functional studies showed certain functional aberrations too.

Ubiquitin activating enzyme (E1) is first enzyme in the conjugation of degradation signal in proteasomal degradation pathways of protein. In yeast, E1 is encoded by 3075 base pairs long *uba1* gene [10], which translates into 1024 residue protein. It is about 110kDa monomeric multidomain protein. E1 is essential gene and deletion of the gene results in lethality [11]. Studies have showed that E1 plays a crucial role in binding of ubiquitin to correct E2 [11]. Moreover, sequence homology of yeast and human E1 is about 50% with all structural domains being highly conserved [14]. The crystal structure of yeast E1-Ub complex has already been resolved by x-ray crystallography [12] still the detailed mechanism of folding of domains is unknown.

E1 consists of six structural domains i.e. IAD (Inactive Adenylation Domain), AAD (Active Adenylation Domain), FCCH (First Catalytic Cysteine Half-Domain), SCCH (Second Catalytic Cysteine Half-Domain), 4HB (Four Helix Bundles) and UFD (Ubiquitin Fold Domain). UFD is linked to AAD by 18 residue long β-hairpin linker, which is called as UFD linker. FCCH is linked to IAD by two antiparallel β-sheets, while SCCH is linked with AAD by 18 residues cross over loop. AAD, FCCH, SCCH and UFD pack together to form a large canyon like structure where ubiquitin molecules are recruited. E1 contacts with Arg72 residue of ubiquitin [12].

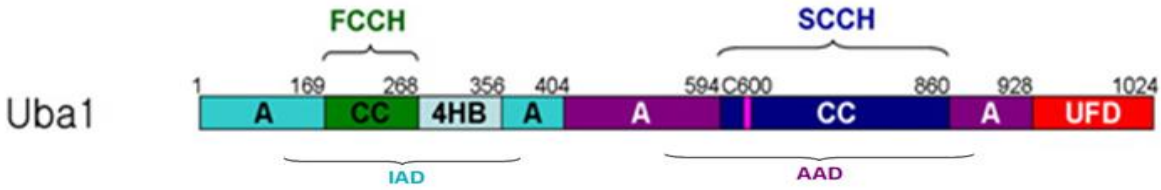

Figure 1: Domain arrangement in E1 [12]

Mutagenesis on E1 was carried out by McGrath for the first time by constructing 3 mutants, of which uba1-26 is temperature sensitive mutant widely used as control in experiments of ubiquitin proteasome system [13]. The mutants reported in Imsang's thesis are, D782A and D782N (Present in SCCH) showed little effect on Uba1~Ub thioester formation. E1004K, D1014K/E1016K and E1004K/D1014K/ E1016K mutations resulted in reduced formation of Ubc1-Ub thioester. I912P, A913P, S914P, A913P/S914P and I912P/S914PS resulted in modest reduction of UFD rotation [12]. Uba1-204 cells carry temperature sensitive mutant allele of *uba1* consisting of mutations at 8 different places [14].

Prokaryotic proteins MoaD and ThiS share structural homology with ubiquitin but are not involved in any degradation pathways. MoaD protein plays role in synthesis of molybdenum cofactor (MoCo), whereas ThiS protein in involved in thiamine biosynthesis. MoaD and ThiS have C-terminal glycine residue which gets adenylated prior to entering the pathways similar to ubiquitin. Interestingly, these reactions are catalysed by enzymes MoeB and ThiF respectively which show significant structural and sequencial similarity to adenylating

domains of E1 [15]. This suggests that Ubiquitin activating enzyme is a modular protein and each unit has assigned function and the domains are combined to do a complex function.

The UFD is situated at C-terminus of the protein consisting of residues from 928 to 1024. This domain can be superimposed with structure of ubiquitin. There is one study on Uba6 (second ubiquitin activating enzyme in some vertebrates and sea urchin) showed that UFD of E1 contributes in selection of E2 recruitment [14]. With this information available we decided to study folding of UFD fragment. There are few reasons which may lead to the assumption that UFD can fold independently. Crystal structure of E1 suggests that UFD is connected to the rest of the enzyme by an extended 18-residue linker (UFD linker) [12] implying that there is a room for accommodating conformational changes arising out of its function.

So far 4HB has not been much studied. It is formed by residues 269 to 356. It is present between FCCH and IAD. The interaction between Phe283, Ala284 of 4HB with Ala46 and Gly47 of ubiquitin respectively supports the binding of ubiquitin with E1. By the crystal structure of E1 it is only suggested that the helix bundle packs against the Rossmann like fold of IAD, which blocks the access of ubiquitin to this domain [12]. However the significance of its presence in the overall structure of the protein is unknown.

Fragments of a protein are studied to identify sites of initiation of folding of the protein. It would also extend the understanding to domain-wise organization in E1. Further E1 has a pseudo-dyad symmetry. However, only one site can bind ubiquitin covalently, while the other is blocked by 4HB. If we find that the structure can be maintained in this fragment independent of rest of the molecule [12], it may suggest the possibility that the domain was inserted from elsewhere during the course of evolution of E1.

SCCH is positioned from residues 594 to 860. It has ~80 residues long core motif which has catalytic cysteine residue at 600^{th} position, although the importance of the stretch other than the region forming core motif is unclear. The thioesterification reaction takes place at the catalytic cysteine, so this domain is core domain to the function of E1. This domain present in AAD is connected to neighbouring domains by flexible linker [12]. It is important for SCCH active site to be in close proximity to C-terminal of ubiquitin for the reaction to take place. Since crystal structure is present as Ub-Uba1 complex, it is interesting to check what would be the structural detail without ubiquitin in the vicinity.

FCCH is present from residues 175 to 265. It does not contain catalytic cysteine residue and its role has not been determined yet. However, this domain is also connected to both the neighbouring domain by two flexible linkers. It is not known if it undergoes conformational changes during translocation of ubiquitin from adenylation site to thioesterification site. Further, there are no studies on the structure formation in this domain.

With this information we planned our objectives as below:

Major Objectives

1. Cloning and characterization of domains of E1.
2. Studying interaction of β-bulge mutants of ubiquitin with E1.

Objective 1: Cloning and characterization of domains of E1.

As we discussed above E1 is multidomain monomeric protein, we planned to dissect the domains individually and study their structure.

To conduct structural studies, all the domains had been cloned in bacterial expression vector pET28a.

A) Cloning and structural characterization of domain SCCH
   Cloning: The SCCH fragment was amplified with site specific primers. It was then ligated in bacterial expression vector pET28a. Primary confirmation was done by PCR later the clone was confirmed by sequencing.
   Protein expression and purification: The cloned SCCH was then transformed in BL21 DE3 cells for purification. Cells were treated with 1mM IPTG overnight at 37°C in shaking condition for overexpression. Expression was confirmed by loading on SDS PAGE. After which protein was purified by Ni-NTA affinity chromatography under native conditions. Protein was purified in bulk quantity for further analysis.
   Structural characterization: The Purified SCCH was treated with various concentrations of guanidine hydrochloride to study the unfolding of peptide. Intrinsic fluorescence emission spectra were recorded and change in fluorescence intensity was observed as a result of difference in the concentrations of guanidine hydrochloride, also we noticed red shift indicating change in the position and exposure of aromatic amino acids with respect to environment.
   Further to study further details of structure far UV CD (Circular Dichroism) spectra were recorded, which showed prominent helical structure in the separated domain. It was analysed by BestSel software which indicated it is made up of approximately 48% helices and 26% anti-parallel β-sheets.

B) Cloning and structural characterization of domain FCCH
   Cloning: The FCCH fragment was amplified with site specific primers. It was then ligated in bacterial expression vector pET28a. Primary confirmation was done by PCR later the clone was confirmed by sequencing.
   Protein expression and purification: The cloned FCCH was transformed in BL21DE3 cells for purification. Cells were treated with 1mM IPTG overnight at 37°C in shaking condition for overexpression. Expression was checked by loading on SDS PAGE. After which protein was purified by Ni-NTA affinity chromatography under native conditions. Protein was purified in bulk quantity for further analysis.

Structural characterization: The Purified FCCH was treated with various concentrations of guanidine hydrochloride to check compactness of the peptide. Emission spectra for intrinsic fluorescence were recorded and it showed change in fluorescence intensity at different concentrations of guanidine hydrochloride, also we noticed red shift indicating change in position of aromatic amino acids with respect to polar environment.

Further to study details of structure far UV CD (Circular Dichroism) spectra were recorded, which showed no specific secondary structure.

C) Cloning and structural characterization of domain UFD

Cloning: The UFD fragment was amplified with site specific primers. It was then ligated in bacterial expression vector pET28a. Primary confirmation was done by PCR and later the clone was confirmed by sequencing.

Protein expression and purification: The cloned UFD was transformed in BL21 DE3 cells for purification. Cells were treated with 1mM IPTG overnight at 37°C on shaking condition for overexpression. It was checked by loading on SDS PAGE. After which protein was purified by Ni-NTA affinity chromatography under native conditions but it did not go into soluble fractions. Further denaturing conditions were applied to protein for purification, after which salts were removed by gradual decrease in salt concentration by dialysis. Then the protein was purified in bulk quantity for further analysis.

Structural characterization: The purified UFD was treated with various concentration of guanidine hydrochloride to check compactness of peptide. Fluorescence emission spectra of intrinsic fluorescence were recorded and it showed change in fluorescence intensity at different concentrations of guanidine hydrochloride, also we noticed red shift indicating change in position of aromatic amino acids from hydrophobic to polar environment.

To study details of position of aromatic amino acids, the peptide was treated with extrinsic fluorophore ANS (8-Anilinonaphthalene-1-sulfonic acid), which detects hydrate hydrophobic residues. The result showed more fluorescence under native condition. Further characterization of the peptide is underway.

D) Cloning and structural characterization of domain 4HB

Cloning: The 4HB fragment was amplified with site specific primers. It was then ligated in bacterial expression vector pET28a. Primary confirmation was done by PCR later the clone was confirmed by sequencing.

Protein expression and purification: The cloned 4HB was transformed in BL21 DE3 cells for purification. Cells were treated with 1mM IPTG overnight at 37°C on shaking condition for overexpression. Expression was checked by loading on SDS PAGE. After which protein was purified by Ni-NTA affinity chromatography under native conditions. Protein was purified in bulk quantity for further analysis.

Structural characterization: The Purified UFD was treated with various concentrations of guanidine hydrochloride to check presence of structure in the peptide. Fluorescence emission spectra were recorded and it showed change in fluorescence intensity at

difference concentrations of guanidine hydrochloride, also we noticed red shift indicating change in the position of aromatic amino acids.

To study the structural details with the help of position of aromatic amino acids the peptide was treated with extrinsic fluorophore ANS. The result showed more fluorescence intensity under denaturing conditions, which indicated presence of hydrophobic residues in the core region due to structure formation.

Objective 2: Studying interaction of β-bulge mutants of ubiquitin with E1.

As it was discussed earlier our lab had generated β-bulge mutations viz, Q2N, E64G and S65D to study importance of these residues in the structure and function of the protein. Here, we decided to check if these mutants can participate efficiently in the reaction with E1. An in vitro assay is planned to check chain formation where mutant ubiquitin would be added to ubiquitin activating enzyme E1, buffer and ATP. Then these samples would be run on SDS PAGE and will be analysed by western blotting. Ubiquitin mutants have been purified by size exclusion chromatography and E1 is purified by affinity chromatography for assay. Currently, the study on interaction of E1 with ubiquitin mutants is underway.

Conclusion

Structural studies of domains of E1 showed that these peptides are capable of folding on their own. SCCH domain showed change in fluorescence intensity with different concentration of chaotropic agent indicating the peptide folded independent of rest of the enzyme. It also showed red shift indicating change in the exposure of aromatic amino acids to polar environment. When SCCH was measured by far UV CD spectra it showed prominent helical structure, which is similar to the crystallized SCCH part of E1-Ub structure. Although when this domain was present with rest of the enzyme it had 70% helical structure, when present as independent unit it showed around 50% helicity. These results suggest SCCH folds to native like state independent of rest of the enzyme but probably when bound to ubiquitin it showed more helicity due to more compactness because of binding. The next domain FCCH also showed change in fluorescence intensity as well as red shift, again indicating presence of folded peptide independent of rest of the enzyme. The far UV CD spectra of FCCH peptide did not show any prominent structure in contrast. These results suggest although FCCH is acquiring some compactness but it is not same as in Ub-E1 crystal complex. The peptides UFD and 4HB were also exposed to different concentrations of guanidine hydrochloride. Both of them showed change in fluorescence intensity. These results indicate both the peptides are capable of folding independently. The red shift pointed the change in positions of aromatic amino acids. These peptides were treated with extrinsic fluorophore ANS as well, which binds to hydrated hydrophobic residues. In case of UFD, under native state ANS showed higher emission indicating presence of hydrophobic residues on the surface whereas with 4HB, after denaturation i.e. at higher concentration of guanidine hydrochloride, ANS showed more emission

indicating presence of hydrophobic residues buried in the core region under native state.

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Poster Presentations and Conferences Attended During Ph.D.:

1. **Poster** presented as first author on **"Structural Study on SCCH Domain of Ubiquitin Activating Enzyme E1 of *Saccharomyces cerevisiae*"** at 86th conference of Society of Biological Chemists (SBC) on Emerging Discoveries in Health and Agricultural Sciences, 16-19 November 2017, organised by School of Life sciences, Jawaharlal Nehru University, New Delhi, India.

2. Abstract **"Structural Insight of SCCH Domain of Yeast Ubiquitin Activating Enzyme E1"** is published by **Willey** as a special edition of Protein Science, page 110. **DBT-CTEP international travel grant** was allotted to present this work.

3. **Poster** presented as first author on "**Structural characterization of SCCH domain of yeast ubiquitin activating enzyme E1**" at Indo-US conference on Advances in Enzymology: Implications in Health, Disease and Therapeutics, 17-19 January 2017, ACTREC, Navi Mumbai, India.

4. **National symposium** on **"Omics… to Structural Basis of Diseases"** organized by Department of Biochemistry, M. S. University of Baroda in 2016.

5. **Workshop** on **Science Communication** organized by the Welcome Trust/ DBT India at Department of Microbiology, M. S. University of Baroda in 2016.

6. **National Symposium** on **"Emerging Trends in Biochemical Sciences"** organized by Department of Biochemistry, M. S. University of Baroda in 2014.

7. **National seminar** on **"Molecular Basis of Diseases"** organized by Department of Biochemistry, M. S. University of Baroda in 2014.

8. Participated in **Open house Science Fair** held at M. S. University of Baroda in 2012.
9. **XXVI Gujarat Science Congress** 2012 held at M. S. University of Baroda.
10. **OSDD science conference** organized by OSDD and CSIR India at Malabar Christian College, Calicut in 2010.
11. **GARUDA BOOT CAMP** organized by OSDD and GARUDA CDAC at Malabar Christian College, Calicut in 2010.

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Signature of candidate
(Brinda Panchamia)

Guide
(Prof. C. Ratna Prabha)

Head
Department of Biochemistry

Dean
Faculty of Science