Synopsis

Homi Bhabha National Institute

Ph. D. PROGRAMME

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SYNOPSIS

Introduction:

For the healthy survival of every organism, all cellular processes must be stringently controlled. Most of these processes are spatiotemporally regulated by the 26S proteasome, a giant ATP dependent protease (Glickman and Ciechanover, 2002; Hanna and Finley, 2007; Wolf and Hilt, 2004). Proteasomes are multi subunit and multi specific proteases which are conserved across all kingdoms (Glickman and Ciechanover, 2002; Hanna and Finley, 2007; Wolf and Hilt, 2004). Deregulation in the proteasome machinery can result in several lethal diseases and disorders (Glickman and Ciechanover, 2002; Wolf and Hilt, 2004).

The most abundant functional complex of the proteasomes in eukaryotes is the 26S holo complex. 26S proteasomes are composed of 20S proteolytic core particle sandwiched by one or two 19S regulatory particles (Baumeister et al., 1998). The cylindrical 20S particle contains four heptameric rings around a central pore. Interior surface of the middle two rings (β-ring) have proteolytic activity (Groll, Ditzel et al. 1997). Only unfolded substrates gain access to catalytic sites via a narrow 13Å pore, which is guarded by a gate that can be opened by the interaction of 20S proteolytic core with the 19S regulatory particle (Glickman and Ciechanover, 2002; Groll et al., 2000; Groll et al., 1997). The 19S regulatory particle is composed of two distinct sub complexes called the base and lid. Base is composed of six homologous AAA^+ ATPases arranged as a spiral staircase that abuts the outer rings of the 20S. Lid is composed of at least ten non ATPases subunits. The 19S regulatory particle is thought to be responsible for substrate recognition, binding, unfolding and translocation (Henderson et al., 2011; Navon and Goldberg, 2001; Ogura and Tanaka, 2003).

Substrates for degradation are targeted via ubiquitin dependent or independent pathways. They are recruited to the proteasome either via multi-ubiquitin tag or an adaptor (trans-acting element) but before the substrate is subjected to degradation, these elements are removed. Beside these targeting molecules, an unstructured region in the substrate seems to be mandatory for proteasomal degradation (Janse et al., 2004; Lee et al., 2001; Prakash et al., 2009; Prakash et al., 2004). Although such observations have led to a better understanding of the mechanism of degradation, many of the fundamental questions regarding the mechanism are still unanswered. Some of the key questions that need answers are:

1. How are substrates recruited to proteasome?
2. What are the cis-acting elements in the substrates that help in substrate recognition?
3. If an unstructured region is necessary, how does it originate within substrate?
4. What are the sequence and structural requirements for protein degradation?
5. What are the rate limiting steps in proteasomal degradation?

We believe that these questions can only be answered if model systems capable of recapitulating the various hierarchical steps involved in the proteasomal degradation without the help of any trans-acting elements and composed only of purified components are created.

Objective:

Our aim therefore is to develop an *in vitro* model system for the characterization of substrate recognition, global unfolding and degradation by eukaryotic proteasome.

Results and discussion:

1. **Establishment of *in vitro* model system**

Selection of model substrate: A few well known factors that affect the half-life of proteins are ubiquitination, post-translational modification, misfolding of proteins and loss of one of the binding partners (Baumeister et al., 1998; Glickman and Ciechanover, 2002; Prakash et al., 2009; Prakash et al., 2004). It is reported that upon ligand removal some proteins become more labile for degradation by proteases. We chose to test myoglobin (Mb) as a 'model substrate' for the proteasome primarily because it can be readily obtained in both the holo and apo forms (heme free). Additional characteristics that make myoglobin an attractive model system are – a) Both holo and apo forms have well defined tertiary structure, b) crystal structure of holo and NMR structure of apo form is known, allowing targeted protein manipulations and structural interpretations possible (Arcovito et al., 2007; Eliezer and Wright, 1996; Phillips, 1980), c) protein is largely α-helical (less stable than the β-sheet), d) extensive studies have been performed to understand the thermodynamics and kinetic properties of apoMb unfolding (Feng et al., 1999; Jamin, 2005; Jamin and Baldwin, 1996; Lecomte et al., 1996; Onufriev et al., 2003; Samatova et al., 2010)

Purification and characterization of Myoglobin: Using cation-exchange chromatography we could achieve single step purification of holomyoglobin (holoMb). Purified protein was >95% pure on SD*S PAGE*. UV-visible spectrum showed the characteristic Soret peak for bound heme, indicating that the protein is well folded. Acid-acetone method was used to remove heme group of holoMb which resulted in loss of the soret peak.

Purification and characterization of proteasome: 26S and 20S Proteasomes were affinity (3X FLAG tagged RPN11 or Pre1) purified using M2-agarose (Sone et al.,

2004). Purification steps were monitored using fluorogenic substrate Suc-LLVY-AMC. In-gel activity assay showed that purified 26S and 20S proteasomes were intact and active.

Degradation of apomyoglobin by proteasome: Upon reconstitution of pure myoglobin and the 26S proteasome apoMb was found to be degraded by proteasome while ligand bound holoMb was resistance to degradation. However, we were surprised to find that contrary to our expectation that ligand free apoMb an all helical protein would be degraded fast by the proteasome, the degradation was a slow process and it took 12h for the proteasome to degrade 50% of apoMb. Unfolded substrates can be degraded by 20S proteasome alone but globular proteins require 19S regulatory particle for unfolding and translocation. Purified 20S proteasome was unable to degrade apoMb indicating that recognition of apoMb it's unfolding and translocation to the catalytic core requires the 19S regulatory particle. The process of unfolding and translocation requires energy. Accordingly, in the presence of ATPγS (non-hydrolysable analogue of ATP), minimal degradation of apoMb was observed.

Taken together, the above findings suggest that apoMb was specifically degraded by purified proteasome in an ATP dependent manner. This is the first demonstration of the ability of 26S proteasome to degrade a globular protein *in vitro* in the absence of ubiquitin, adaptor or other trans-acting element.

2. Substrate recognition is an essential step in proteasomal degradation

One possible reason for the failure of the proteasome to degrade holoMb could be the failure to recognize this form of the substrate. To check whether holoMb and proteasome interacted and to assess the affinity of apoMb for proteasomes, we developed a quantitative binding assay based on ELISA. Proteasomes were immobilized by anti-Flag antibody and after incubation with holo or apoMb, bound amount of ligand

was quantified using standard steps involved in ELISA holoMb did not interact with immobilized proteasome while apoMb bound tightly (*Kd*=3.5nM). As reported for substrate of several chaperones and AAA ATPases, apoMb stimulated the ATPase activity of the proteasome. (Benaroudj et al., 2003; Cashikar et al., 2002). HoloMb however did not stimulate the ATPase activity again, suggesting that holoMb was not recognized and therefore was not degraded by proteasome. However, even after tight association and eliciting a response from proteasome, degradation of apoMb was a slow process (half-life = 12h). Contrary to the observation that substrate localization is sufficient for efficient degradation (Janse et al., 2004), our findings suggest that all encounters with substrate may not be productive; any of the downstream processes like chain unfolding or translocation could be rate limiting in proteasomal degradation. We conclude that substrate recognition by proteasome is essential, but may not be sufficient of efficient degradation.

In addressing the differential effect of proteasome on apo and holoMb, we have developed a method by which the affinity of proteasome and substrate interaction could be quantified independent of other steps required for proteolysis. This provides a novel method to discriminate between substrates and non substrates of the proteasome.

3. Effect of unstructured 'cis-acting trans elements' on substrate half-life

PEST fusion: A small number of protein sequences when fused in 'trans' shorten the half-life of protein. Although why certain sequences should behave so is not well understood, these sequences are utilized to study the mechanism of degradation, and are called as 'degrons'. Some proteins containing one or more regions rich in proline (P), glutamic acid (E), serine (S), and threonine (T) were found to exhibit less than 2 hours of intracellular half-lives (Rogers et al., 1986). These are generally called as PEST sequences. To test the effect of PEST sequences on apoMb half-life, PEST sequences

from GCN4 (apoMb PEST 1), Hac1 (apoMb PEST 2) and ABCA1 (apoMb PEST 3) were fused to the C-terminus of apoMb. Fusion of these PEST sequences did not affect the half-life of apoMb.

Quest to find the probable 'degron': Although the role of 'degron' in the degradation step is not clear, few recent observations suggest that long unstructured regions (90-120 residue) when fused to protein (as cis-acting trans elements), render them susceptible for degradation (Prakash et al., 2009; Prakash et al., 2004). These unstructured regions may help in initiation of degradation (Prakash et al., 2009; Prakash et al., 2004). Since presence of such long unstructured regions in proteins is not very common we tested the ability of short sequences to act as 'degrons' by fusing them to the C-terminus of Mb. These sequences were- a) C-terminus of ODC– essential for ubiquitin independent degradation of ODC, was able to shorten the half-life of GFP when fused to its C terminus (MbODC) (Corish and Tyler-Smith, 1999); b) C-terminus of E12 – know to interact with PSMD9, a non-ATPase subunit of proteasome (Mb E12) (Thomas et al., 2009); c) Short regions from N and C-terminus of proteins– In indigenous screening these were found to interact with immobilized 26S proteasome (Mb P8 and Mb P13).

Compared to wt, none of the fusion proteins could significantly shorten the half-life of Mb. Fusion of short sequences which are 'trans in origin' did not facilitate Mb degradation. Reasons could be: a) Due to very high affinity of apoMb (*Kd*=3.5 nM) for proteasome, additional interactions as a result of fusion of these elements might not facilitate degradation; b) Local structural changes may be more important than fusing unstructured sequences to the termini

3. Importance of local secondary structure in apoMb degradation

In order to understand the reason behind failure of proteasome to recognize and to degrade holoMb, crystal structure of holoMb and NMR structure of apoMb were

analyzed. Heme pocket in holoMb is surrounded by B, C, E helices from top and side and F helix from the bottom. Upon heme removal, dramatic structural change was observed in the F-helix (K79 to F106) while, structure of other helixes involved in heme binding were unaffected. The F-helix in apoMb is in conformational equilibrium between partially folded and unfolded state. Circular dichroism data reflected that apoMb was 18% (F-helix contribute 17% of total helicity of holoMb) less structured than that of holoMb. This unstructured region in the protein might act as recognition element or initiator of degradation. We hypothesized that stabilization of F-helix would affect the half-life of Mb. We modified G80 (EF loop), P88 and S92 (F-helix) to Ala and H97 (FG–loop) to Asn with the intention to stabilize the F-helix even in the absence of heme. The Wt and mutant F-helix (G80A P88A S92A and H97N) were analyzed by AGADIR, a program based on helix/coil transition theory that provides residue level helicity. As compared to wt, mutant F-helix was more helical in the 78 to 106 region. Quick molecular dynamics simulation of ligand free wt and mutant F-helix protein for 2.8ns at 400K showed that F-helix melts almost immediately in wt whereas in the mutant, the helix was stable and remained so until the end of simulation. These modifications were incorporated in Mb cDNA. F-helix mutant protein was purified, secondary structure of holo as well as apo form were determined by CD. No remarkable change was observed in holo form of wt and F-helix mutant protein by CD spectroscopy. On the other hand, apoF-helix mutant was 9±2% more helical than that of apowtMb. Since, increase in secondary structure due to incorporation of mutations may affect the overall stability of the protein, tryptophan fluorescence, thermal denaturation and chemical denaturant induced unfolding experiments were performed. There was no significant change in melting temperature, tryptophan environment and the thermodynamic stability (ΔG of urea unfolding) of apowt and F-helix mutant Mb,

suggesting that overall fold of apo F-helix mutant is similar to that of apowtMb. Structural stabilization was most likely confined to local region in the protein (F-helix). To further prove the stabilization of F-helix, we took the help of limited proteolysis, where a specific protease is allowed to act on a protein at suboptimal conditions. Apowt generated two fragments with trypsin (nick after K96) and chymotrypsin (nick after L89) indicating that F-helix was floppy in the wt structure. But, F-helix mutant was relatively resistance to cleavage by these enzymes thereby proving that F-helix was stable in the mutant protein. Apowt and F-helix mutants were tested for proteasomal degradation. As hypothesized, mutant protein was relatively stable for proteasomal degradation. Interestingly the affinity of mutant protein was not compromised and mutant like the wt apoMb structure was able to stimulate proteasomal ATPases.

Taken together, the above results provide following insights

1. F-helix was stabilized in mutant protein.
2. Local secondary structural alterations can determine the life time of a protein.
3. Floppy F-helix (78-106) was essential for apoMb degradation.

These observations provide the first evidence for structure function correlation of *in vitro* proteasomal degradation and identification and origin of intrinsic degradation signal (cis-acting element) in the substrate.

4. Recognition element in apoMb

The floppy F-helix of apoMb may act as recognition element and/or as an initiator of degradation. To test this and identify the interacting surface on apoMb, overlapping peptides that pan entire 153 amino acid sequence were synthesized with biotin at the N-terminus and screened for binding to the proteasome by ELISA. Peptide from A-helix not only bound tightly to the proteasome but was also able to compete for apoMb

binding (*Ki*=0.8±0.4μM). Other regions in Mb (B-helix and CD-loop) also interact with the proteasome but with much lower affinity and unlike the A-helix peptide, these peptides were unable to abolish binding of apoMb. A specifically designed 23 residue peptide covering the sequence from 77-100 in the F-helix region could not inhibit binding of apoMb to the proteasome. In presence of MG132 (proteasomal inhibitor), a short stretch of floppy F-helix, residues 69-83 (C-termini of E-helix and EF loop) and 90-104 (C-termini of the F-helix, FG-loop and N-termini of the G-helix) were able to bind and partially inhibit apoMb proteasome interaction. It is likely that floppy F-helix enters the central channel of the 20S proteasome in the form of a loop and acts as an initiator of degradation.

Using structure as a guide we identified few solvent exposed residues in the A-helix that may interact with the proteasome. These were mutated to Alanine and binding studies indicated that two of the residues in A-helix provide bulk of the binding energy.

Taken together, due to heme removal F-helix of Mb becomes floppy and apoMb with the floppy F-helix and not the holo protein was degraded by proteasome. This is supported by the behaviour of the F-helix stabilized mutant which was degraded slower than the wt. It seems that the floppy F helix mediates initial encounters between apoMb and proteasome. Strong interaction originated from A-helix of apoMb then strengthens these interactions. Floppy F-helix thus anchored and may act as initiator of degradation.

5. Strategies to understand structure function correlation- unfolding intermediates as a rate limiting step

To better understand the role of local secondary structure, overall stability and the affinity of substrate for proteasomal degradation, one buried residue in each of the helices of Mb was replaced by a cysteine (A-helix-V10C, B-helix-G25C, C-heilix-T39C, G-helix-L104C and L115C, H-helix-M131C). Structural characterization of these

proteins indicated that secondary structure of apo G25C, L115C and L104C was compromised. However, tertiary structure characterization by tryptophan fluorescence indicated that at least one of the tryptophan was solvent exposed in all mutants. Overall stability of these mutant proteins was monitored by thermal denaturation. ApoG25C was least stable while stability of apoL104C, L115C and M131C was compromised to some extent. ApoG25C can be treated as molten globule as this protein was less than 50% helical; tryptophan environment was severely affected in this mutant and did not follow two state transition during thermal denaturation. All cys mutants were tested for proteasomal degradation, only two mutant proteins apoL115C and L104C (both mutations in the G-helix) were found to have a shorter half-life (8hr) than apowt. We could not find direct correlation between binding affinity and half-life of the cysteine mutants tested.

Once recognized other processes like unfolding are likely to be more rate limiting than affinity per se. One of the rate limiting steps would be the creation of a disordered region that can enter the catalytic chamber. If F-helix was entering the central channel as a loop, 29 residue long floppy F-helix will be 5.3nm (3.8Å/residue) but the length required to reach catalytic core is 15 nm. Destabilizing G-helix (apo L104C and L115C) adjacent to the floppy F-helix propagates the disorderness and facilitates degradation. Creating such a long loop catalyzed by proteasomal AAA^+ ATPases would be a key rate limiting step.

Although degradation of 153 residues (all helical) long apoMb was a slow process, recognition was fast. Floppy F-helix would be captured by AAA+ ATPase of proteasome either the ATPases would pull at the protein and create a loop long enough to reach the active site or due to the cooperative nature of protein unfolding the entire protein might collapse to initiate degradation. However, this process in apoMb does not

seem to be that straight forward. Parallel observation in literature indicate that even denaturant induced unfolding of apoMb is complex due to the formation of a stable long lived intermediate by the AGH helices. It is possible that similar unfolding intermediate is abundant during proteasomal AAA^+ ATPase mediated apoMb unfolding and destabilizing of such an intermediate would be the key rate limiting step. Unfolding intermediate would be less stable in short lived G-helix mutants like L104C and L115C.

It is to be pointed out that there are not many examples in literature where degradation of substrate starts from an internal region leading to complete degradation. If degradation is not initiated from the internal loop, there are two possibilities- a) One of the termini would be pulled into the catalytic chamber and degradation would take place in either N to C or C to N direction, b) Due to the destabilization of adjacent helices, a loop long enough to reach catalytic site would be created and degradation might start with a cut to generate two termini resulting in simultaneous degradation of both the polypeptides.

In the latter case, two polypeptides would enter the catalytic core and might compete with each other for translocation. Apart from the above stated rate limiting steps, this translocation step could also berate limiting in the substrates where degradation does not start from termini.

Summary:

In order to dissect the various hierarchical steps in proteasomal degradation, to identify motifs for substrate recognition and identify the rate limiting steps we have developed an *in vitro* model system using purified 26S proteasomes and apoMb. We report for the first time that purified 26S proteasome have the ability to directly recognize and degrade a globular protein (apoMb) in the absence of ubiquitin, extrinsic degradation tags or adaptor proteins. Removal of heme exposes a previously buried F-helix which is

dynamic in nature. This Floppy F-helix sensitizes the proteasomal ATPases to the presence of the substrate. ApoMb is then anchored to the proteasome primarily through A-helix; it is further stabilized by additional interactions with B-helix and CD-loop. Floppy F-helix enters the central channel in the form of a loop and acts as initiator of degradation. Adjacent helices are unraveled by AAA+ ATPases of proteasome to generate an unstructured region long enough to reach the active site. Stabilization of unfolding intermediate seems to slow down degradation.

It is clear from our observation that degradation of even a small all helical protein is a well-controlled but complex process. Some of rate limiting steps in proteasomal degradation that we recognize from our model system are -

a) Substrate recognition is essential but does not ensure efficient degradation
b) Initiation requires exposure of a 'cis-acting element'
c) Floppy/unstructured region of sufficient length is required for initiation of degradation
d) Substrate unfolding probably occurs through chain unraveling
e) Unfolding intermediates my accumulate
f) Translocation to catalytic core may be competitive depending on the directionality of degradation

Significance:

Apomyoglobin emerges as a new model substrate for in depth study of ubiquitin independent degradation. It can be used to investigate sequence, structure, thermodynamic and kinetic aspects of not only proteasomal degradation but also for other compartmentalized proteases. Our finding will open new quest for 'cis-acting elements' in other ATPase dependent systems. Our interaction study can be optimized for other labile multi-subunit complex systems.

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Publications:

Publication from thesis

a. Published:

Amit Kumar Singh Gautam, Satish Balakrishnan, Prasanna Venkatraman (2012) Direct and Ubiquitin independent recognition and degradation of a folded protein by the eukaryotic proteasomes- origin of intrinsic degradation signals. PLoS ONE 7(4): e34864. Doi:10.1371/journal.pone.0034864.

Other publications

b. Vinita Wadhawan, Yogesh A Kolhe, Nikhil Sangith, **Amit Kumar Singh Gautam** and Prasanna Venkatraman (2012).Biochemical Journal. From prediction to experimental validation-Desmoglein 2 is a functionally relevant substrate of matriptase in epithelial cells and their reciprocal relationship is important for cell adhesion.

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