Chapter 3

Substrate recognition is an essential step in proteasomal degradation

3.1. Introduction

ApoMb was degraded by proteasome in an ATP dependent manner while holoMb was stable. There could be several possibilities for the stability of holoMb, such as a) it was not recognized by proteasome or b) it was structurally too stable to be unfolded by proteasome. To test whether holoMb was recognized at all by proteasome and to monitor the binding affinity we designed methods that could faithfully provide a measure of affinity of substrate recognition by 26S proteasome that can be delinked from the post recognition steps like unfolding and degradation. In case of Ub dependent proteasomal degradation, substrates are recruited to proteasome with the help of poly ubiquitin. At least two of the proteasomal subunits contain one or more ubiquitin binding domain (UBD) (Deveraux et al., 1994). Proteasome also degrade substrates through an ub independent pathway. In such cases substrates are thought to be recruited to proteasome through an adaptor protein. However, there is limited knowledge about the kinetics of substrate and proteasome interaction mainly because of the bulky, complex structure of proteasome and its tendency to dissociate into sub complexes. Qualitatively, interaction of poly Ub chain with proteasome has been shown by pull down, IP and density sedimentation assays. Binding has been estimated indirectly using enzyme kinetics and Ub competition assays. Affinity of ubiquitin for proteasome was obtained using Ub-fusion tags of DHFR and correlating the rate of degradation with affinity, assuming that the downstream processes are not a contributing factor (Thrower et al., 2000). Although this study provides the first evidence that at least four Ub tags are required for degradation of substrate, it does not present a clear idea about substrate and proteasome interaction kinetics (Thrower et al., 2000).

There are several techniques by which protein-protein interaction kinetics can be measured but requirements of each of these methods are unique and dependent on many

properties of the ligand and the receptor. One of the fastest methods that also provide kinetic measurement is the label free technique called surface plasma resonance (SPR). This technique not only provides dissociation constant but also rate of association and dissociation in real time. SPR has been successfully used to show the effects of nucleotide in 20S and PAN association (Smith et al., 2005). While binding of unstructured proteins like casein with 20S proteasome has been demonstrated (Hutschenreiter et al., 2004), to the best of our knowledge the affinity of substrate to the 26S proteasome has not been demonstrated till date. We standardized binding assay using anti-FLAG antibody to capture the 26S proteasome and tested holo and apoMb binding to the immobilized proteasome.

Interaction of some of the ATPases with their client substrate is accompanied by an increase in the rate of ATP hydrolysis (Benaroudj et al., 2003; Cashikar et al., 2002). This response may act as an indicator of substrate interaction by the proteasomes. We hypothesized that in the absence of substrate (apoMb) basal level of ATP hydrolysis would be observed while in presence of it, ATPase activity would increase. There are several ways by which ATP hydrolysis can be quantified. Most of these methods are based on quantitation of the amount of either ADP or Pi generated due to ATP hydrolysis. Such studies could provide newer insights into proteasomal degradation and help in screening of new binding partners or inhibitors.

3.2. MATERIALS and METHODS

3.2.1 Proteasome and Mb interaction: Binding affinity of apoMb and proteasome was determined be Enzyme-linked immunosorbent assay (ELISA). Multi-subunit complex of 26S proteasome was captured by antibody coated on ELISA plate and the amount of bound Mb was quantified using Mb specific antibody (**Fig. 3.1A**).

Materials: *ELISA plate (NUNC- Maxisorb), 3, 3′,5,5′-Tetramethylbenzidine (TMB substrate-Calbiochem).*

Antibodies: *Anti FLAG antibody (raised in rabbit–Sigma), anti-Mb antibody (raised in mouse-Cell Signaling), anti-mouse HRP (Amersham).*

Instrument: *Multi well plate reader* (Spectra max190).

Essential buffers:

Bicarbonate/carbonate coating buffer: 0.08 g Na_2CO_3 and 0.14 g $NaHCO_3$ were dissolved in 50 ml MQ water, pH ~9.6 (no need to adjust).

Note- coating buffer should be prepared fresh.

Wash buffer (TBST): 50 mM Tris pH 7.5,150 mM NaCl and 0.05 % Tween 20.

Blocking buffer: 2% BSA in TBST

Dilution buffer: 1 mM ATP, 5 mM $MgCl_2$ and 0.1 % BSA in TBST

Note: after adding ATP, dilution buffer must be stored at 4ºC (not more than 24 h).

Maxisorb ELISA plate wells were coated overnight at 4ºC with 2 μg/ml of rabbit anti FLAG antibody (90 to 100 μl/well). To avoid nonspecific binding each well were blocked by incubating with 200μl of blocking buffer for 1h at 37ºC. In dilution buffer 1 μg/ml (0.37 nM) of 26S proteasome was immobilized on plate. Varying concentrations of apo or holoMb (580 nM to 0.006 nM) were incubated with immobilized proteasomes. After washing the plate to remove unbound Mb, amount of 26S proteasome bound Mb was quantified by using 100 μl/well of anti-Mb antibody (1:500) followed by anti-mouse HRP antibody (1:5000, 100 μl/well). Wells were washed and incubated with 100 μl/well of peroxidase substrate TMB. TBM reacts with HRP to produce a blue byproduct the color intensity is proportional to the amount of 26S proteasome bound Mb. After 15 minutes, reaction was stopped using 50 μl/well 2 M H_2SO_4, and

absorbance was measured at 450 nm. All the washing steps were done thrice and except for the coating step, all other incubation steps were done at 37ºC for 1h.

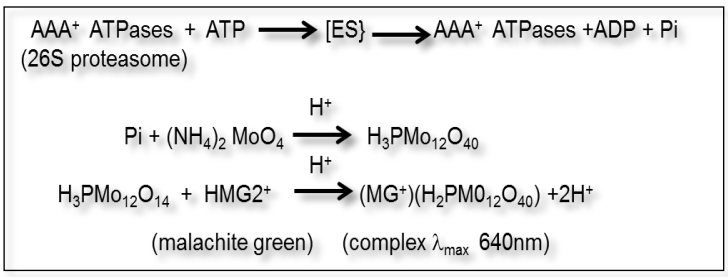
Note: A. All the washing steps were done by filling the wells with wash buffer. All solutions or washes were removed by flicking the plate over a sink and remaining drops are removed by patting the plate on a paper towel two to three times.

B. In case of apoMb, saturation binding could be achieved. This is very important aspect for reliable measurement of affinity and in our case shows that all the sites on 26S proteasome available for binding were occupied by apoMb.

C. Immobilized 26S proteasome was able to release AMC from fluorogenic substrate and degrade casein.

Dissociation constant (*Kd)* was calculated by fitting the data, using GraphPad Prism 5 assuming one site specific binding (the two 19S caps were considered to be equivalent).

3.2.2 Response of proteasomal ATPases upon substrate recognition: To test the hypothesis whether proteasomal ATPase activity will increase upon apoMb binding, ATPase assay was performed. Amount of Pi released due to ATP hydrolysis (ATP=ADP +Pi) was measured using acidified malachite green and ammonium molybdate (as shown below) (Tgavalekos et al., 2003).



Materials: *ATP (Sigma), HEPES (Sigma), $MgCl_2$, Malachite green, Ammonium molybdate, Polyvinyl alcohol (PVA).*

Essential buffers:

Assay buffers: 25 mM HEPES/NaOH pH7.5, 3 mM ATP, 15 mM $MgCl_2$.

Dye buffer: 3:1 mixture of 4.2 % Ammonium molybdate (in 5 M HCl) and 0.4 % Malachite green (in MQ water) supplemented with 0.3 % polyvinyl alcohol (PVA) as stabilizing agent.

A 50µl assay buffer containing 0.01 µg/µl 26S proteasome (3.7pM) with or without 5µg (580nM) of the substrate was incubated at 37ºC for 15 min. ATP only and protein only were taken as control. The amount of inorganic phosphate generated was measured by adding 450 µl of dye buffer and monitoring absorbance at 650 nm in 96 well plate (Spectra max190). Data was subtracted from ATP control, Pi released was measured calorimetrically and quantified using a standard graph. Data was represented as nanomoles of Pi released.

Note: A. Dye buffer should be filtered before use. Dye buffer should not be stored for long time as molybdate tend to oxidize. Care must be taken when using dye buffer, it is highly acidic and gives permanent color. All reagents should be prepared carefully. Phosphate based buffer must not be used for ATPases assay.

3.3. RESULTS and DISCUSSION

3.3.1 Proteasome and Mb interaction kinetics: For determining the kinetics of Mb and proteasome interaction, we tried surface plasma resonance (SPR) using biacore 3000 (GE Amersham) as well as proteon XPR36 (Bio-Rad) platforms but found it was difficult to keep 26S proteasome intact on chip during regeneration step; also the size of the complex was too big to avoid bulk effect. To obtain affinity constants of interaction we standardized a simple, reproducible and robust method based on ELISA. We immobilized 26S proteasome on ELISA plate using anti FLAG antibody. To test

whether holoMb was interacting with proteasome and to measure the affinity of apoMb different concentrations of holo or apo Mb was incubated with immobilized 26S proteasome. Unbound Mb was washed and the amount of bound Mb was quantified. ApoMb interacted very tightly with 26S proteasome (*Kd*=3.5±1 nM), while in case of holoMb saturation was not achieved at the highest concentration tested (570nM) (**Fig. 3.1B**). The observation that degradation resistant holoMb was not interacting with proteasome suggested that substrate interaction could be a most important step in proteasomal degradation. However, the fact that despite being bound tightly to the proteasome with very high affinity, apoMb was degraded with a half-life of 12 h, illustrates that although substrate interaction was essential but not sufficient for efficient degradation.

Our data differs from the observation that localization of substrate to the proteasome is sufficient for efficient degradation (Janse et al., 2004) and suggest that any of downstream steps like unfolding, translocation, initiation of degradation could be rate limiting.

3.3.2. Response of proteasome upon substrate recognition: Several ATPase elicits response in the form of elevated rate of ATP hydrolysis upon substrate recognition. If proteasome is behaving similarly in presence of Mb or known substrate, rate of ATP hydrolysis should increase (**Fig. 3.1C**). ATPase assay was performed with or without Mb and amount of Pi released was measured. Basal ATP hydrolysis was observed with 26S proteasome alone and with holoMb, the activity was elevated several fold with apoMb and casein (**Fig. 3.1D**). This observation further provided evidence that holoMb was not recognized by proteasome.

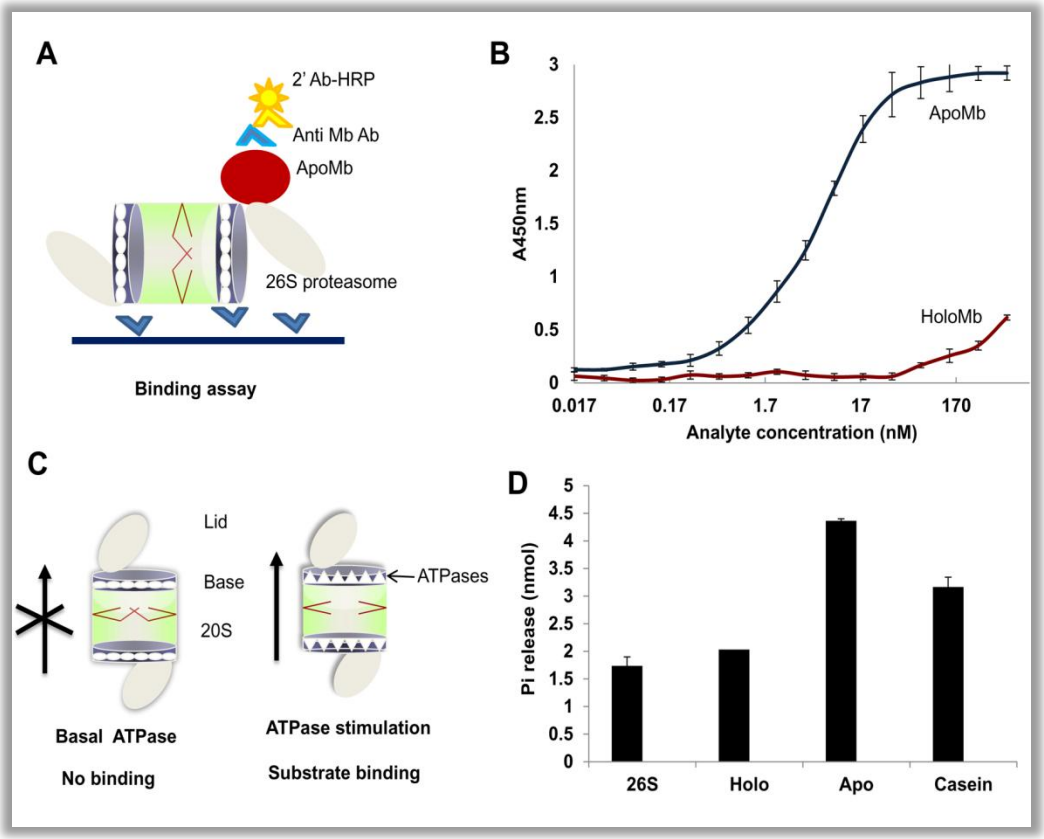


Figure 3.1 ApoMb was recognized by 26S proteasome: *Immobilized 26S proteasome was incubated with varying concentrations of apo or holoMb, the amount of proteasome bound Mb was quantified calorimetrically using antiMb antibody and HRP conjugated secondary antibody system (A). ApoMb bound tightly to the proteasome, all the binding sites available on 26S proteasome was saturated with apoMb (B). We hypothesis in absent of Mb and 26S proteasome interaction basal level of ATP hydrolysis will take place while if Mb was recognized by proteasome it will elicited response in the form of elevated ATPases activity (C). ATPases activity of 26S proteasome with or without Mb was monitored calorimetrically. ApoMb was able to activate proteasomal ATPases. Casein was taken as positive control (D).*

ApoMb was able to activate the AAA^+ ATPases of proteasome either by direct interaction with one or more of them and bring about conformation changes or apoMb interacts with non-ATPase subunits and relays the signal to the ATPase subunits eventually resulting in elevated ATPase activity. Several lid subunits have been found to interact directly with AAA^+ domains of the Rpt subunits. Rpn7 interacts with the AAA^+ domains of Rpt2 and Rpt6, while Rpn6 and Rpn5 interact with Rpt3 (Lander et

al., 2012). It was proposed that AAA^+ ATPases of proteasome (abundant in base sub complex) form a ring and sit on top of the 20S proteasome. Two of the non ATPases subunits are known to interact with poly Ub tag but till date it's not clear how non Ub substrates are recognized by 26S proteasome. Recent high resolution cryo-electron microscopy structure of 26S proteasome clearly demonstrated that proteasomal ATPases form spiral staircase like structure (Beck et al., 2012; Lander et al., 2012), this will give them more surface area for substrates to interact or sense substrate interaction. Three protruding flexible coils coil of proteasomal ATPase may also directly bind with substrates (Beck et al., 2012; Lander et al., 2012).

ApoMb was interacting with high affinity with 26S proteasome. Proteasomal ATPases elicited response of substrate recognition in the form of elevated rate of ATP hydrolysis while degradation was a slow process. We wanted to test whether this slow degradation was due the destabilization of apoMb due to the long incubation and also whether 26S proteasome was intact during long incubation. 26S proteasomes was incubated in assay buffer (3 mM ATP and 3.5% glycerol) at 37ºC for 12 h and analyzed by native PAGE and In-gel activity. From In-gel activity assay it was clear that 26S proteasome was intact even after 12 h of incubation. Moreover, after 12 h ~ 90% of the LLVY-AMC cleavage activity of proteasome was retained (**Fig. 3.2A**). At various time points (0h-12h) far-UV circular dichroism or tryptophan fluorescence of apoMb was compared. No dramatic change in the secondary structure or tryptophan fluorescence of apoMb was detected even after 12 h incubation while half of the apoMb was degraded by this time (**Fig. 3.2B**).

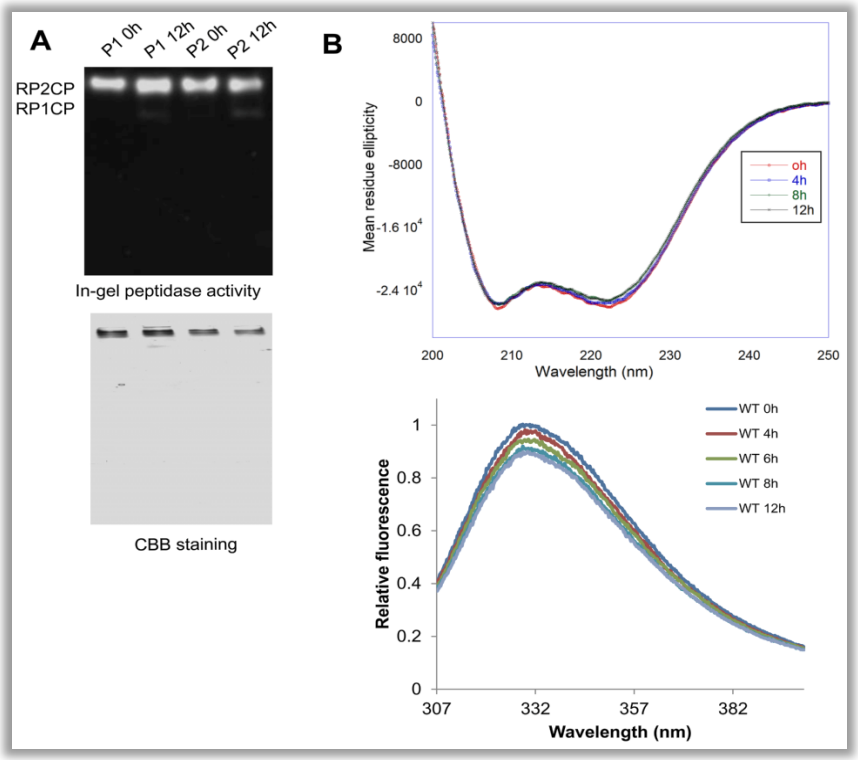


Figure 3.2 Stability of 26S proteasome and apoMb: *Two different preparations (P1 and P2) of 26S proteasome were incubated at 37°C for 12h in assay buffer. In gel activity and coommassie staining of native gel was performed (A). ApoMb was incubated at 37°C and at the indicated time CD and fluorescence spectra were collected (B).*

3.4 SUMMARY

We have successfully demonstrated for the first time, the affinity of substrate and immobilized proteasome interaction without the presence of ubiquitin or adaptors. A very high affinity was observed between apoMb and proteasome but the effect was not reflected in degradation. The possible explanations could be-

a) 'Off rate' of apoMb and proteasome interaction could be high.

b) Downstream processes like translocation, unfolding and initiation of degradation could be rate-limiting.

We optimized ELISA to immobilize the proteasome and keep it intact. This method could be utilized for screening an array of proteasomal substrates and inhibitors.

Although the generality of ATPase response needs to be investigated, nevertheless it gives a way to screen for potential proteasome substrates and inhibitors that would artificially keep proteasomal ATPases busy. It will also be interesting to monitor ATPases response for several forms of Ub modifications. For instance, whether ubiquitinated substrates also elicit the ATPase response? Several heavily ubiquitinated proteins interact with proteasome but do not get degraded- is it because of their inability of these proteins to stimulate ATPase activity.

Methods described above for studying substrate protein interaction provide two different aspects of this recognition step- a ELISA based method provide binding affinity of apoMb with 26S proteasome, while proteasomal ATPase stimulation showed the response of proteasome upon substrate recognition. Development of these methods have opened new dimension in understanding proteasomal degradation and screening of new binding partners or inhibitors.

Together, these observations suggest that substrate recognition is essential step in proteasomal degradation but all encounters of substrate with proteasome may not be productive. It seems that binding and downstream event should be coupled for the substrate and proteasome interaction to be productive and ATPase stimulation may be a key determinant in linking these steps.