# Chapter 8

Conclusions

CONCLUSIONS

In order to dissect the hierarchical steps in proteasomal degradation, identify the sequence and structural requirements as well as the rate limiting steps in proteasomal degradation, we have developed an *in vitro* model system using purified 26S proteasomes and apoMb. To the best of our knowledge, this is the first report wherein we successfully demonstrated the inherent ability of purified 26S proteasome to degrade a globular protein (apoMb) *in vitro* in the absence of ubiquitin, adaptor or other trans-acting elements. The key observations have been summarized in the form of a model that provides insights into the process of proteasomal degradation of apoMb (**Fig. 8**). Briefly, heme bound form was not recognized and degraded by proteasome; 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. Degradation is initiated by insertion of the floppy F-helix in the form of a loop into the central channel. Adjacent helices are unraveled by AAA^+ ATPases of proteasome to generate an unstructured region long enough to reach the active site chamber. Stabilization of an unfolded intermediate seems to slow down degradation.
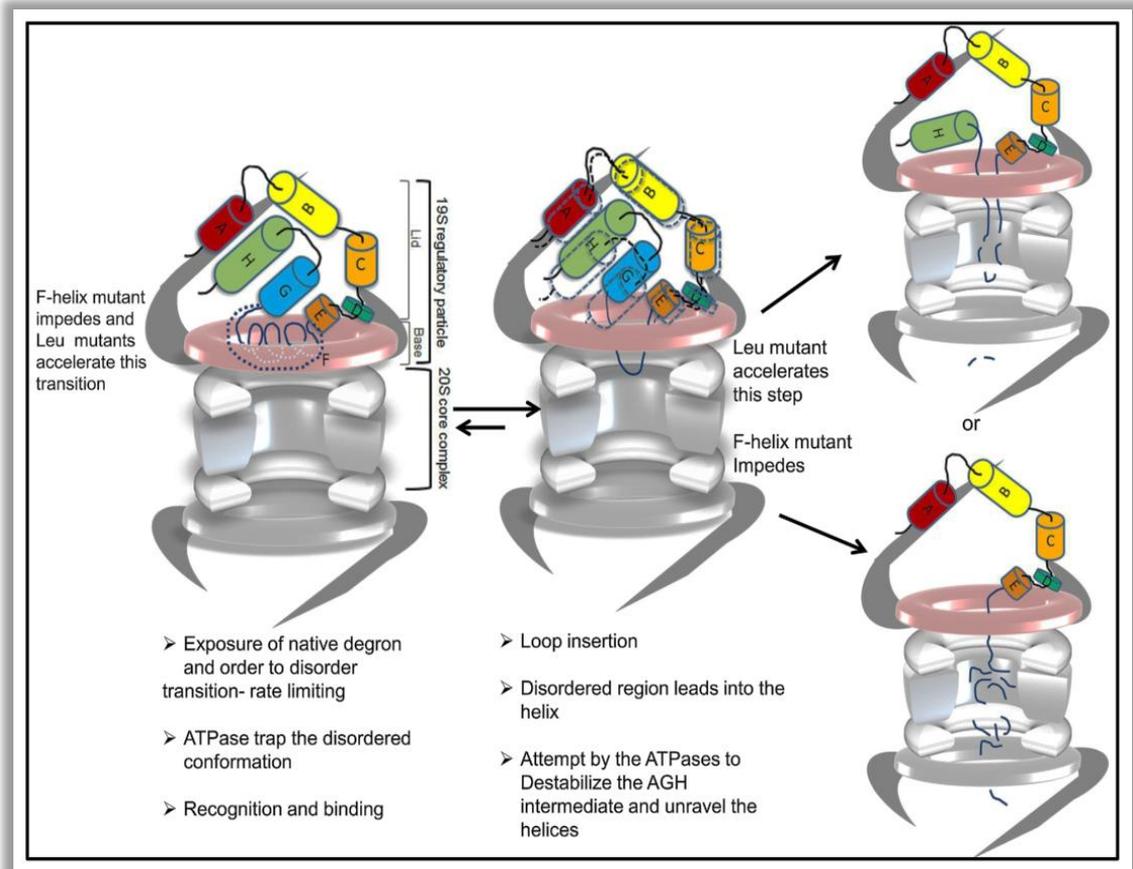
Figure 8 A model for the mechanistic steps involved in the proteasomal degradation of apoMb: *Removal of heme exposes a previously buried F-helix which is in a dynamic equilibrium between a partially folded and unfolded structure. This transition is a rate limiting step. Exposure of this floppy helix sensitizes the proteasome to the presence of the substrate. ApoMb is anchored to the proteasome by interactions primarily through the A-helix additional interactions originating from B-helix and CD loop stabilize it further. Degradation is primed by the insertion of the floppy helix in the form of a loop into the central channel that runs across the proteasome. An intermediate composed of AGH helices is likely to be formed. Melting of this intermediate by the ATPases to generate an unstructured region long enough to reach the active site is a likely rate limiting step. Mutations can stabilize or destabilize this unfolding intermediate affecting the rate of degradation.*

Our model system provides the following new insights:

a) Although the requirement of unstructured region for efficient proteasomal degradation is well known but, how such regions originate in the substrate was

not clear. We have shown that ligand removal (and similar changes) can originate the unstructured or floppy region of the protein.

b) Identified an intrinsic degradation signal in the substrate.

c) Substrate recognition is essential step in proteasomal degradation, but all encounters of substrate with proteasome may not be productive. Substrate binding and downstream event should be coupled for productive substrate interaction and that ATPases may be a key determinant in linking these steps.

d) Direct interaction between proteasome and its substrate as well as the determinants of protein-protein interactions between the two have not been reported till date. With the help of peptide panning, inhibition studies and structure guided approach, we identified some of the key residues in apoMb that directly interact with proteasome.

e) In addition to the structure adjacent to the degradation signals, the unfolding intermediates formed in globular proteins regardless of their secondary structural status may be key determinants of the rate of degradation.

Using structure guided design, site directed mutagenesis, parallel biophysical studies and proteolytic susceptibility as a probe for protein dynamics we showed the mechanism of degradation of all helical protein apoMb. It is clear from our observation that degradation of even a small protein is a well-controlled but complex process. Some of rate limiting steps in proteasomal degradation that emerge from our study are-

a) Substrate recognition- essential but may not be sufficient for efficient degradation. Exposure of 'cis-acting element'.

b) Floppy/unstructured region of sufficient length for initiation of degradation.

c) Substrate unfolding through chain unraveling.

d) Stabilization of unfolding intermediate.

e) Translocation to catalytic core.

SIGNIFICANCE OF THE STUDY

ApoMb 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 might open new quest for 'cis-acting elements' in other ATPase dependent systems. The interaction study can be optimized for other labile multi-subunit complex system. The peptide panning, competition and structure guided approach can be used to identify protein-protein interaction surfaces in other system; peptides derived from different proteins can be used to screen binding partners and peptide mimetic or other inhibitors can be designed from the information derived from this study.