Chapter 7

Structure function correlation of proteasomal degradation

7.1 INTRODUCTION

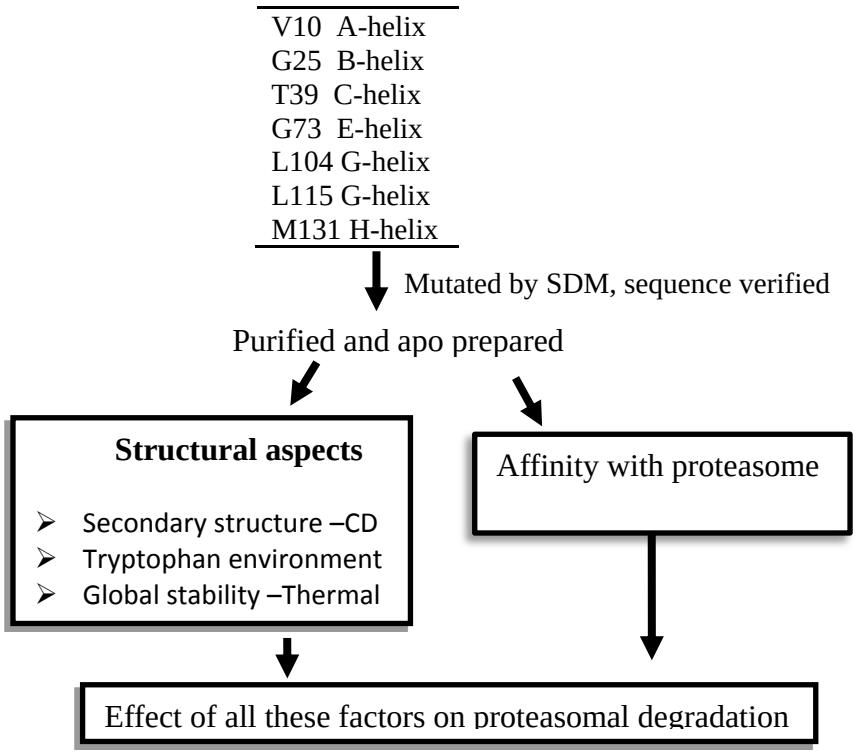
We have made the following observations so far: a) Ligand free form of Mb was recognized and degraded by the purified 26S proteasome. b) The trans-acting elements tested did not alter the half-life of the protein. c) Local stabilization of floppy helix increased the half-life of the protein. d) ApoMb interacts with proteasome mainly through A-helix and degradation starts most probably from the floppy helix.

One of the major observations was that the local secondary structure was important for proteasomal degradation. It would therefore be interesting to understand what happens when helix to disorder transitions are induced in other regions of apoMb. To answer this and to understand the role of local secondary structure, overall stability and the affinity of substrate with proteasomal degradation, we designed a multipurpose strategy. One buried residue of each of the Mb helices were identified by calculating the solvent accessibility surface area of each residue and mutated to Cys. Replacing buried residues from different helices to Cys would allow us to not only understand substrate requirement and identify rate limiting factors for proteasomal degradation but also would provide with a tool to monitor substrate unfolding. There were no Cys in Mb, introducing Cys in various helices may disrupt their helicity and also provide flexibility of monitoring each and every helix during unfolding or degradation. Thiol specific environment sensitive fluorophore may be utilized to monitor role of proteasomal ATPases.

7.2 MATERIALS and METHODS

7.2.1 Identification of buried residue in Mb helices: The relative solvent accessibility surface area (rSASA) was calculated using ASA-View server. This server presents solvent accessibility of each residue of the protein provided the structure is known. To

identify a buried residue from each of the Mb helices, PDB 2JHO was used and least solvent exposed residue was identified from various Mb helices (A-helix-V10, B-helix-G25, C-heilix-T39, E-helix-G73C, G-helix-L104 and L115, H-helix-M131). The work plane of Mb Cys mutants is summarized below-



Work flow of Mb Cys mutants

7.2.2 Mutagenesis, expression and purification: The buried residues identified from Mb helices were converted to Cys by site directed mutagenesis. Primer pair used for creating the mutations is listed in table 7.1. The mutations were confirmed by sequencing. Mutant proteins were expressed in DH5α and purified by cation exchange chromatography.

7.2.3 Secondary structure of Cys mutants of Mb: Mutating buried residues of Mb to Cys might destabilize the protein structure locally as well as globally. Cys disulfide crosslinking also might affect the secondary structure of the proteins. Apo form of mutant and wt proteins was treated with 2 mM DTT at 37ºC to reduce the oxidized

disulphide. DTT treated proteins were centrifuged and used for all biophysical experiments. The secondary structure of all the Cys mutants were measured by Far-UV CD in 20 mM sodium phosphate buffer pH 7.5 and 1 mM DTT. DTT absorbs strongly below 200 nm, to minimize this 1 mm path length cuvette was used for CD experiment. The secondary structure component was calculated by SELCON and CONTIN using Dichroweb server.

Table 7.1 Oligonucleotides used for Cys mutagenesis

Mb mutant	**Prime sequences**
V10C F	GAATGGCAGCTGTGCCTGCATGTTTGGGC
V10C R	GCCCAAACATGCAGGCACAGCTGCCATTC
G25C F	CGTCGCTGGTCATTGCCAGGACATCTTGATTCG
G25C R	CGAATCAAGATGTCCTGGCAATGACCAGCGACG
T39C F	CTCATCCGGAATGCCTGGAAAAATTCGATCG
T39C R	CGATCGAATTTTTCCAGGCATTCCGGATGAG
G73C F	GTTAACTGCCCTATGCGCTATCCTTAAGAAAAAAGG
G73C R	CCTTTTTTCTTAAGGATAGCGCATAGGGCAGTTAAC
L104C F	AGAGATGAATTCGCAGTATTTGATCGG
L104C R	CCGATCAAATACTGCGAATTCATCTCT
L115C F	CGATCATCCATGTTTGCCATTCTAGACATCC
L115C R	GGATGTCTAGAATGGCAAACATGGATGATCG
M131C F	CTCAGGGTGCTTGCAACAAAGCTCTCGAG
M131C R	CTCGAGAGCTTTGTTGCAAGCACCCTGAG

7.2.4 Tryptophan Fluorescence of Cys mutants of Mb: To understand the effect of mutation on tryptophan environment, tryptophan fluorescence of all the mutants were collected and represented as relative fluorescence.

7.2.5 Thermal stability of Mb mutants: The global stability of mutants might be different than wt due to rewiring of tertiary contacts. The melting temperature of Mb mutants was derived from thermal denaturation curve as described earlier.

7.2.6 Affinity of Mb mutants with proteasome: The mutated residue might be directly interacting with proteasome or other changes might affect the affinity of these mutants with proteasome. The affinity of Mb mutants was measured in binding assay, using immobilized 26S proteasome.

7.2.7 Proteasomal degradation of Mb mutants: Mb mutants were subjected to proteasomal degradation. The effect of secondary structure, tertiary structure, global stability and affinity was correlated with the half-life of proteins.

7.3 RESULTS and DISCUSSION

7.3.1 Identification of buried residues in Mb helices: Using ASA-View server and holoMb crystal structure (PDB 2JHO), the relative solvent accessibility of each residue of Mb was calculated. We identified buried residue from various helices of Mb. The following buried residues- V10 (A-helix), G25 (B-helix), T39 (C-helix), G73 (E-helix), L104, L115 (G-helix) and M131 (H-helix) were then used to test the effect of structure (secondary as well as tertiary structure and global stability) and binding affinity on proteasomal degradation. In holoMb the identified residue interacted with residues from different helices (**Table 7.2**).

Table 7.2 Cys mutant, their helical interface and neighboring residues in 3D structure

Protein	Helix interface	Residues around 4ºA
V10C	A-G	A130, M131, A134
G25C	B-E	G65, L69
T39C	C-B and C-G	L32, F33,Y103
G73C	E-A	W14
L104C	G-H	F138, R139, I142
L115C	G-A and G-H	V13, F123, M131
M131C	H-A and H-G	V10, I112, L115

7.3.2 Effect of structure on proteasomal degradation: Mb does not contain any Cys residue. Therefore, replacing a buried residue with bulky and charged Cys might affect local secondary structure and/or overall stability of the protein. The secondary structural characterization of Mb mutants indicated that apo form of G25C, G73C, L115C and L104C were less helical than that of apowt (**Fig. 7.1A and table 7.3**). The helical content of apoV10C, T39C and M131C were similar to apowtMb (**Fig. 7.1A**). The tertiary structure characterization by tryptophan fluorescence indicated that the

tryptophan environment of all cysteine mutants was affected. Around 20% decrease in fluorescent intensity was observed in V10C, T39C, L104C, L115C and M131C mutants indicating at least one of the Trp (W7 or W14) was partially solvent exposed in the mutants (**Fig. 7.1B**). The melting temperature (the temperature at which half the protein molecules are unfolded) of mutant proteins was monitored by thermal denaturation. Tm of V10C and T39C was partially affected while L104C, L115C, M131C showed about 10ºC change in Tm (**Fig. 7.1C**).

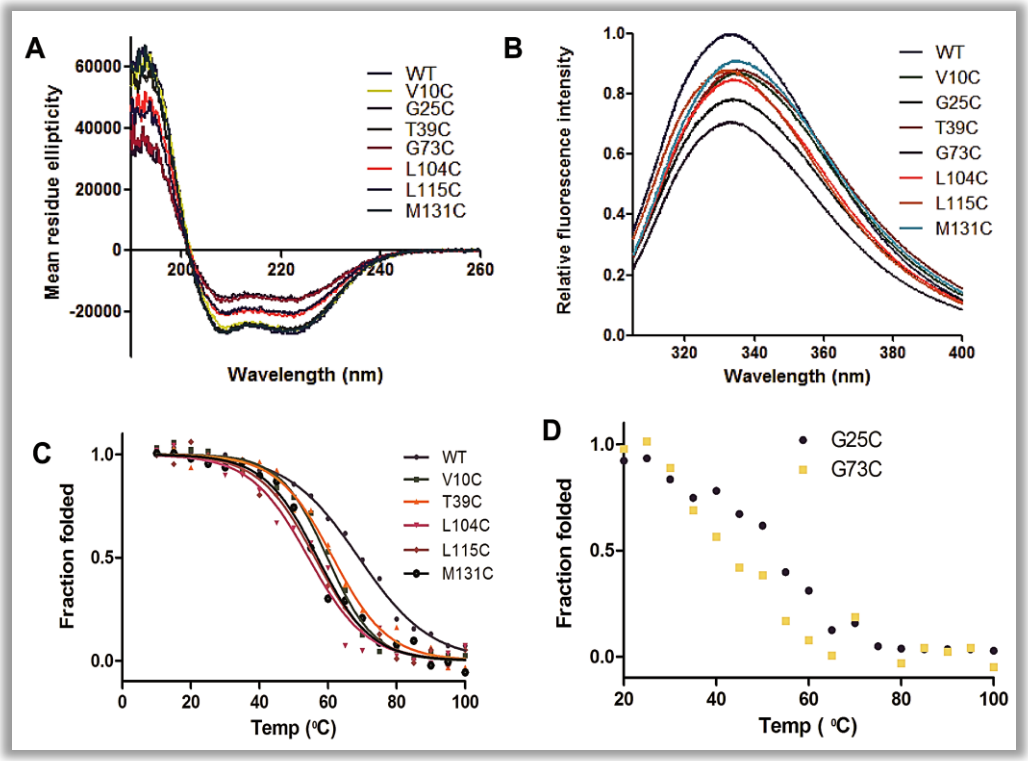


Figure 7.1 Effect of Cys mutation on secondary structure, Trp environment and Tm of Mb: *The far-UV CD spectrum was collected for wt and Cys mutants in presence 1 mM DTT at pH 7.5 (A). Trp fluorescence of wt and Cys mutants were collected, protein sample was excited at 295 nm and emission spectrum was collected from 305 to 400 nm (B). Thermal stability of wt and Mb mutants were determined by collecting far-UV spectrum as a function of increasing temperature and ellipticity at 222 nm was used to determine fraction folded (C and D).*

ApoG25C and G73C behaved like unstructured or molten globule protein. These proteins were less than 50% helical, displayed more than 30% decrease in Trp

fluorescence and most importantly did not follow two state transition during thermal denaturation (**Fig. 7.1 A, B and D**). Besides, they had the tendency to precipitate during incubation. Because of the above reasons G25C and G73C proteins were not included in analysis.

The mutant proteins were tested for proteasomal degradation. The helical content, Tm and half-life of V10C and T39C mutants were comparable with that of the wt. On the other hand, Tm of L104C, L115C and M131C were reduced by ~10ºC while secondary structure was adversely affected in L104C and L115C. Notably, only L104C and L115C (less helical than wt) were found to have a shorter half-life (<8hr) as compared apowt (12h) (**Fig. 7.2 A and B**). To show that our observation was indeed correct, the degradation experiment was repeated several times in presence or absence of proteasome inhibitor MG132. Limited proteolysis of apoL104C and L115C indicated that trypsin or chymotrypsin sites were more exposed in these mutants (**Fig 7.2C**). The Tm of Leu mutants was ~10ºC less than that of wt, this is mainly contributed by change in secondary structure of these mutants. To show that Leu mutants were not behaving like intrinsically disordered protein, apoL104C, L115C mutants and known unstructured protein casein was incubated with 20S proteasome. Casein was degraded by 20S proteasome in 3h, while G-helix mutant L104C was stable towards 20S degradation (**Fig.7.2D**). This observation further supported that degradation of apoL104C and L115C was tightly regulated by 19S RP. Although, Tm of M131 mutant was affected while helicity as well as half-life was comparable to that of wt indicating the local secondary structure rather than overall stability is more important for proteasomal degradation.

Interestingly, the L104 is part of the floppy F-helix (78-106) while L115C was at the C-terminal of G-helix. In holo Mb L104 interacts with residues from H-helix while

L115 with those of A and H-helix. As compared to apowt, L104C and L115C were 17 and 21% less helical respectively. It is most likely that disorderness of floppy F-helix has been propagated to the adjacent helices which facilitated degradation by creating a loop of sufficient length. If F-helix was entering the central channel as a loop, 29 residues long floppy F-helix would have been 5.3 nm (3.8Å/residue) but the length required to reach catalytic core is 15 nm. Therefore, the disorderness gets propagated to the adjacent G-helix (apo L104C and L115C) which in turn might facilitate degradation. Hence, in substrates wherein the degradation starts from an internal region creating a long loop, catalyzed by proteasomal AAA^+ ATPases, could be a key rate limiting step.

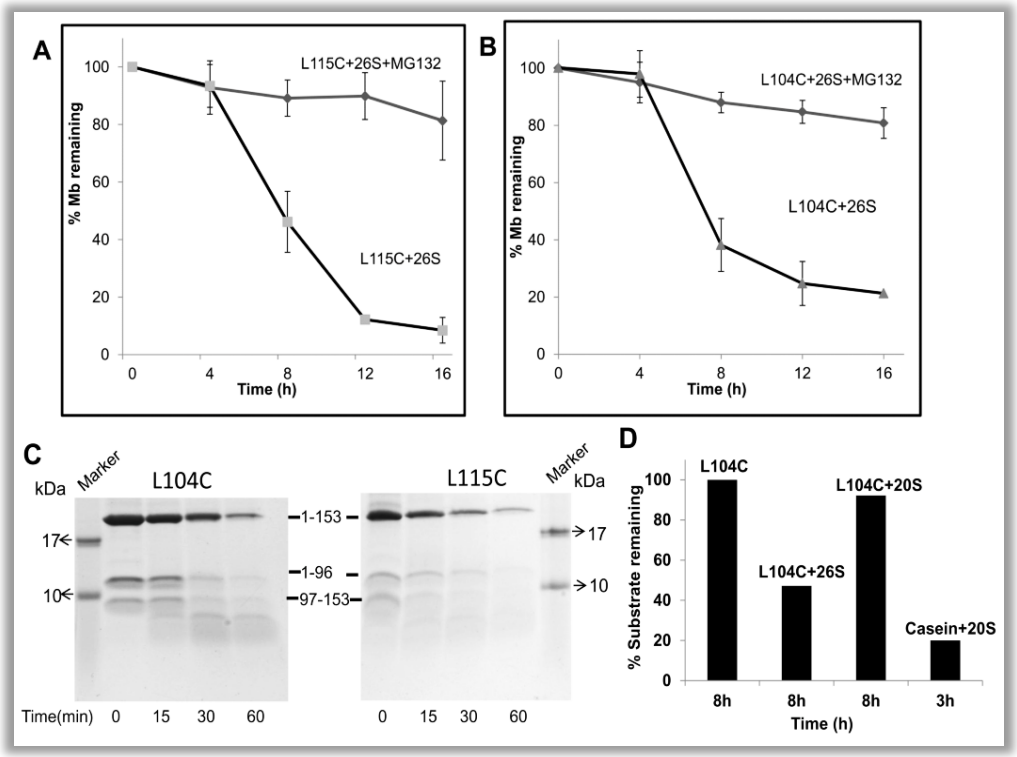


Figure 7.2 Mutation of buried Leu residues in the G-helix shortens the half-life of apoMb: *Apo L115C (A) and L104C (B) mutant proteins were incubated with proteasome in the presence or absence of MG132. Rate of degradation was followed by SDS-PAGE. Data represent mean values of at least three independent experiments ± S.D. Limited proteolysis of Leu mutants was done using chymotrypsin and resolved on Tricine-SDS PAGE (C). ApoL104C and unstructured casein was incubated with 20S proteasome, substrate remaining was quantified as described in methods L104C was stable for degradation while casein was degraded by 20S proteasome (D).*

7.3.3 Effect of affinity of substrates for proteasomal degradation: The affinity of Cys mutants with proteasome was measured by ELISA. The affinity of V10C, L104C, and L115C with 26S proteasome was not compromised (**Table 7.3**). While, T39C and M131C bound less tightly to proteasome. The holo Mb structure indicated that T39 in C-helix interacts with residues within the CD loop while M131 interacts with residues from A and G-helix. From peptide panning and competition experiment it was clear that A-helix and CD-loop were involved in holding apoMb to proteasome. Probably T39C and M131C were influencing the proteasome interacting residues in these helices or affecting the conformation of A-helix and CD loop. When affinity with proteasome was correlated with degradation, we could not find direct correlation with binding affinity and half-life of the Cys mutants of Mb.

Table 7.3 Effect of secondary structure, Trp environment, melting temperature (Tm) and affinity (Kd) of apoMb on proteasomal degradation

Protein	**% Helix***		**Relative Trp fluorescence**	**Tm (°C)**	**Affinity to proteasome (Kd nM)#**	**Half-life (h)**
	By 222nm $	SELCON3 ©				
Holowt	92	94	ND	80	ND	ND
Apowt	76	79	1	63	3.5±1	12
V10C A-helix	74	79	0.82 No shift	60	1.25±0.3	12
T39C C-helix	74	79	0.82 1nm red shift	62	19.5±7	12
Stabilized F-helix	85	87	1 No shift	63	0.57±0.1	16
L104C G-helix	60	62	0.82 1nm blue shift	54	0.6±0.5	6-7
L115C G-helix	58	58	0.83 1nm blue shift	56	0.63±0.3	7-8
M131C H-helix	75	79	0.89 2nm red shift	55	45±20	12

ND= not determined
**2-4% variation in helicity was observed in three independent experiments.*
© Data for CONTIN (not shown) was similar to SELCON 3.
 # Kd represents mean value of three independent experiments ±S.D.
$ Fractional helical content = ([Θ]222 – 3,000)/(-36,000 – 3,000) (Morrisett et al., 1973).

The degradation of small, all helical proteins by 26S proteasome was a slow process, although, these were recognized by proteasome quickly. Floppy F-helix would be captured by AAA^+ ATPase of proteasome and due to the pulling force applied by ATPases, loop long enough to reach active site would be created. Alternatively, due to the cooperative nature of protein unfolding, the unfolding of substrate by proteasomal ATPases might collapse entire structure of protein. However, in case of apoMb degradation this process does not seem to be that straight forward. Even the denaturant induced unfolding of apoMb is a complex process due to accumulation of stable long lived intermediate formed by AGH helices (**Fig. 7.3A**). It is possible that similar unfolding intermediate is abundant during proteasomal AAA^+ ATPase mediated apoMb unfolding and that destabilizing such an intermediate would be the key rate limiting step. Unfolding intermediate would be less stable in short lived G-helix mutants L104C and L115C while much more stable in F-helix mutant.

There are not many examples where degradation of substrate starts from an internal region and results in complete degradation. If degradation is not starting from the termini, there are two possibilities for initiation of degradation-

a) One of the termini will be pulled in 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, loop of sufficient length to reach the 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 later case two polypeptides would simultaneously translocate to the catalytic core and might compete with each other. Apart from the above stated rate limiting steps, this

translocation step could also be rate limiting in case of substrates where degradation does not start from the termini.

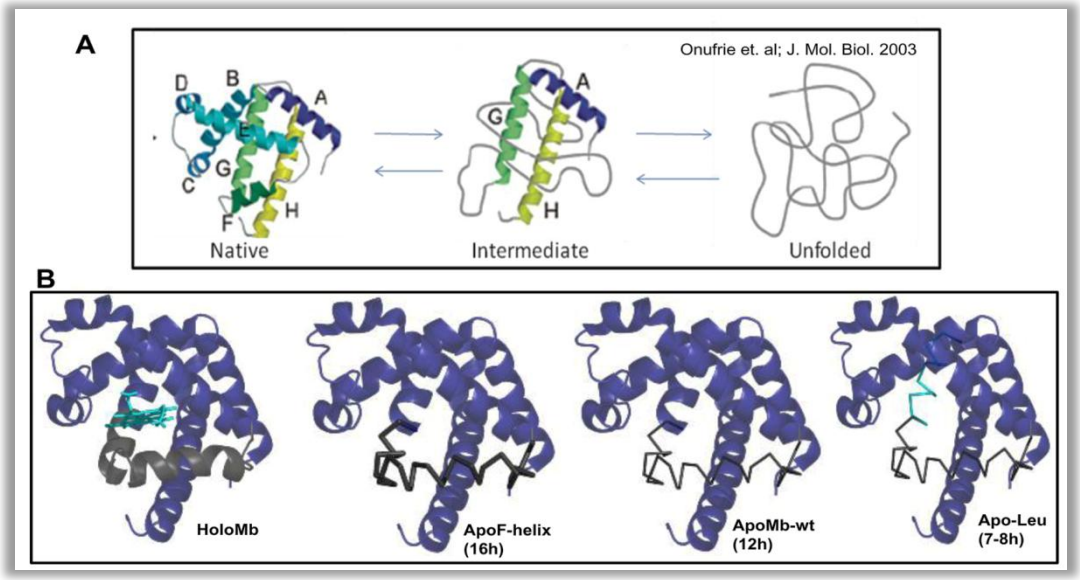


Figure 7.3 Unfolding intermediates of apoMb and Mb F-helix conformation variants: *The denaturant mediated apoMb unfolding goes through a stable unfolding intermediate enriched in AGH-helices (A). The conformation of F-helix (region 76-106, gray) affects the half-life of Mb. In holoMb it was buried and protein was not degraded by proteasome, due to heme removal it got exposed and apoMb was recognized and degraded by proteasome. When the helix was engineered to stabilize it, the half-life increased while in case of Leu mutants disorder was propagated in other helices that resulted in faster degradation (B).The variation in half-life could be due to the varying stability of unfolding intermediates. In case of F-helix mutant, the intermediate might be more stable while in case of Leu mutants it would be less stable.*

7.4 SUMMARY

In order to understand the structure function correlation and role of local conformation in proteasomal degradation of a substrate, buried residues from various Mb helices were identified and converted to Cys. The effect of Cys replacement on secondary structure, Trp environment, thermal stability and affinity with proteasome was determined and correlated with the half-life of the Cys mutant (**Table 7.3**). Out of the 5 Cys mutants, the half-life of two proteins 'both from G-helix' was substantially shorter than wt. Some of key observations are as follow

a) Proteasome is more sensitive to local structural changes rather than overall stability.
b) Floppy or unstructured region of sufficient length was required for efficient degradation.
c) Substrate recognition is a necessary step but high affinity binding may not always facilitate degradation, as downstream processes could be rate limiting.
d) Formation of stable unfolding intermediate could be rate limiting in proteasomal degradation.
e) Degradation initiation site might affect post recognition processes in proteasomal degradation.