

## Chapter 5

# Importance of local secondary structure in apoMb degradation

## 5.1 Introduction

In the previous chapter we described the effect of some trans-acting elements in apoMb degradation. None of these elements tested were able to affect the half-life of apoMb. We have also observed that holoMb was neither recognized nor degraded by the proteasome. There is no change in the amino acid sequence between holo and apo form. This throws open the following questions: is it the structure or conformation of apoMb which makes it susceptible for degradation? The crystal structure of holo Mb showed that out of the eight helices of Mb, four of them are involve in formation of heme pocket- helices B, C and E make top and side while F forms the bottom of this pocket (Kendrew et al., 1958). Distal His residue from F-helix of Mb has been shown to directly interact with heme. Comparison of the crystal structure of holo and NMR structure of apoMb showed that 78-106 residues (F-helix region) in apoMb were in dynamic equilibrium between partially folded and unfolded state, since resonance corresponding to this region was not detectable (Eliezer and Wright, 1996). It is most likely that this region (Floppy F-helix) is acting as ‘the cis-acting element’ for apoMb degradation. In holoMb this region was buried and we found no ATPases stimulation as well as degradation, but upon heme removal ATPase stimulation and degradation by 26S proteasome was seen. And as mentioned above the F-helix becomes floppy in apoMb suggesting its probable role in ATPase stimulation and degradation of apoMb.

As suggested earlier, an unstructured region might act as recognition element or as initiator of degradation. If this is true, then local stabilization of this helix may result in stabilization of apoMb. Stabilization of local F-helix without affecting the global stability could be challenging as modified residues may rewire the non-covalent interaction network and result in stabilization of overall structure. Under such circumstances, it will be difficult to propose the role the floppy helix. We, therefore,

took expert advice of Prof. P. Balaram (IISc Bangalore) in engineering the floppy F-helix to induce helicity even in the absence of heme. If our hypothesis is correct this will provide us the opportunity to dissect the structure function correlation and understand the several steps in proteasomal degradation where degradation is not guided by Ub or other factors.

## **5.2 MATERIALS and METHODS**

The floppy F-helix (78 to 106) encompasses the EF-loop, F-helix, FG-loop and first few residues from G-helix. We wanted to engineer the helix in such a way that once formed, it remains stable in the absence of heme. The Gly80 in the EF-loop, Pro 88 and Ser 92 within the helix were changed to helix stabilizing residue Ala as well as H97 in the FG-loop to Asn.

**5.2.1 Prediction of helical propensity:** Agadir predicts the helical behavior of monomeric peptides based on helix coil transition theory. For short sequences, it provides residue level helical content.

**Program:** AGADIR (<http://agadir.crg.es>)

**Analysis parameter:** pH-7.5, Temperature- 298K and ionic strength - 0.15

We compared the residue level helix content of 78-106 residues (floppy F-helix) in WtMb, and G80A/P88A/S92A/H97N.

**5.2.2 Molecular dynamics simulation of wt and F-helix mutant:** We wanted to test the effect of the above mentioned replacements on stability of F-helix by molecular dynamic simulation. Based on known physical principles, this computational method provides time dependent detailed information on the fluctuations and conformational changes of proteins.

**Program and tools used:** Amber 10 and Amber tools, modeler 9v7

**PDB:** 2JHO

Molecular dynamic simulation was performed using AMBER 10 and Amber Tools. The initial co-ordinates were obtained from the PDB file 2JHO. The holo Mb was then stripped of all metal ions and the heme moiety to create apoMb. All the hydrogen atoms and anisotropic atoms were removed. A program from Amber tools called LEaP was then used to add the hydrogen atoms. ApoMb was solvated in TIP3P model of water. For energy minimization using Sander, a combination of steepest descent and conjugate gradient methods was used for 2000 steps. Energy minimized apoMb was equilibrated to a temperature of 400K over 10 ps. An all atom force field was used for simulation with the temperature set to 400K for a period of 2.8 ns. The trajectories resulting from the simulation were converted to a PDB file and analyzed using Visual Molecular Dynamics (VMD). The apoMb was then mutated with residues intended to stabilize the F-helix. The mutant was created and modeled using modeler 9v7 with 2JHO as the template. Molecular dynamic simulation of the mutant was performed exactly as described for the wild type (performed by Satish Balakrishnan in Dr Saraswathi's lab in IISc Bangalore).

**5.2.3 Mutagenesis, expression and purification:** To generate the F-helix stabilized mutant, G80A was generated with the help of one set of mutation primer (F-helix 2) (Table 5.1). The PCR product was treated with DpnI to digest parental wtMb containing plasmid. 2 $\mu$ l of this product was further used for second round of PCR using primers harboring P88A, S92A and H97N replacements (Table 5.1). PCR mixture and condition was similar to site directed mutagenesis described earlier. After confirming mutation by Sanger sequencing, F-helix mutant Mb was expressed in DH5 $\alpha$  and purified by cation exchange chromatography as described earlier.

**Table 5.1 Oligonucleotides used to stabilize floppy helix**

<b>Mb mutant</b>	<b>Prime sequence</b>
F-helix1F (P88AS92AH97N)	CTCAAAGCGCTTGCGCAAGCGCATGCTACTAAAAACAAGA TCC
F-helix1R (P88AS92AH97N)	GGATCTTGTTTTTAGTAGCATGCGCTTGCGCAAGCGCTTTG AG
F-helix 2 F (G80A)	CCTTAAGAAAAAAGCCCATCATGAAG
F-helix 2 R (G80A)	CTTCATGATGGGCTTTTTTCTTAAGG

**5.2.4 Secondary structure of wt and F-helix mutant:** The Secondary structure of holo as well as apo form of wt and F-helix mutant was compared using far-UV circular dichroism spectroscopy (CD). CD is based on differential absorption of left and right-handed circularly polarized light that arises due to structural asymmetry of a molecule. It is one of the well characterized biophysical technique used to determine secondary (peptide bond below 240 nm), and tertiary structure (aromatic amino acid side chains 260 to 320 nm) of purified proteins, to study the effect of several extrinsic and intrinsic factors on protein structure and stability (effect of pH, temperature, chaotropic agents etc.), to monitor protein folding or unfolding, co-factor binding and protein-protein interactions. Different types of secondary structure ( $\alpha$ -helix,  $\beta$ -sheet, turn and other) found in proteins provide characteristic CD spectra in far-UV range (240-180), for example,  $\alpha$ -helical proteins have negative ellipticity (unit of CD spectrometry) at 222 nm and 208 nm and a positive ellipticity around 190 nm. Based on the high agreement between secondary structures derived from CD and X-ray crystallography several algorithms and databases have been developed to provide an estimation of the secondary structure composition of proteins from accurate CD data. Widely used algorithms include SELCON (self-consistent), VARSLC (variable selection), CDSSTR, K2d and CONTIN (Provencher and Glockner, 1981; Sreerama and Woody, 1993). We have used online server DICHROWEB that provide flexibility of analyzing our data by

various algorithms and databases as well as using several reference sets (Lobley et al., 2002; Whitmore and Wallace, 2004).

**Material:** Quartz cuvette (2mm), Nitric acid

**Instrument:** CD Polarimeter (Jasco, J815)

**Buffers:** 20 mM sodium phosphate buffer, pH 7.5

**5.2.4A Determination of protein concentration:** Concentration of apoMb was determined by UV absorbance at 280 nm using an extinction coefficient  $15470 \text{ M}^{-1}\text{cm}^{-1}$  (as estimated by ExPASy-ProtParam tool). Determination of holoMb concentration by UV absorbance at 280 nm using same extinction coefficient may not be accurate as heme affects the absorbance at 280 nm. For accurate holo protein concentration, estimation using either Bradford assay with BSA as standard or absorbance at 280 nm using extinction coefficient  $34500 \text{ M}^{-1}\text{cm}^{-1}$  was used. Proteins with  $A_{280/260}$  ratio  $\geq 1.5$  were used for CD spectrometry.

**5.2.4B Cleaning quartz cuvette:** The CD cuvette was washed for 1 min with concentrated nitric acid, then several times with MQ water and finally with ethanol and air dried.

*Note: Nitric acid must be handled carefully as it is corrosive and powerful oxidizing agent.*

**5.2.4C Instrument setting and data collection:** To save the optics, instrument was purged with  $\text{N}_2$  gas at 10–15 l/min for 10 to 15 min before turning on the lamp. After turning on instrument and accessories (like peltier cell holder, water bath and computer), lamp was allowed to stabilize (20 to 30 min). Far-UV CD spectrum of holo as well as apo form of wt and F-helix mutant ( $2\mu\text{M}$ ) was collected from 260 to 190 nm (Settings: Scan speed 50 nm/sec, accumulation 5, data pitch 0.1, at  $24^\circ\text{C}$ ) and subtracted

from buffer spectra. After collecting the spectra instrument, accessories were turned off; N<sub>2</sub> gas was purged for another 10 min.

**5.2.4D Data analysis:** CD data from 3 to 5 different experiments with different protein preparations was used for data analysis. CD spectra is plotted as ellipticity ( $\theta$ ) on Y-axis and wavelength (nm) on X-axis. Data is represented in the form of the Mean Residual Ellipticity (MRE or  $[\theta]$ ) since this value is concentration independent and fixed for a protein. Ellipticity was converted to mean residue ellipticity using the formula ( $[\theta] = \theta/\text{number of amino acid in the protein} \times \text{molar concentration} \times \text{path length in mm}$ ) where  $\theta$  is ellipticity at given wavelength. It was then saved in Dichroweb format and subsequently analyzed by SELCON3 and CONTIN by Dichroweb server using reference set 7 (<http://dichroweb.cryst.bbk.ac.uk/html/home.shtml>). While analysis the data using these algorithms, experimental and reconstituted CD spectra overlapped with each other thereby showing that the secondary structure composition provided by the programme was reliable.

Note: *CD is very informative technique provided we take care of some of the following factors: 1. Proteins should be of at least 95% purity on SDS PAGE. 2. Protein should be free of insoluble protein aggregates as due to differential light scattering the shape and ellipticity of CD spectra will be affected. 3. Suitable buffer system like Phosphate, Tris and borate buffer (concentration <100 mM and pH 6-10) should be used for farUV CD, some buffers system like HEPES, MOPS, MES, PIPES absorb strongly below 200 nm and should only be used at low concentrations. 4. Accurate protein concentration is essential for faithful CD data. 5. Multiple scan of CD spectra will improve the signal/noise (S/N) ratio as S/N ratio is proportional to the square root of the number of scans. 6. For reliable data, detector should not saturate (dynode voltage should be less*

than 700V). 7. On the basis of experimental requirement appropriate algorithm and data set should be used for the analysis.

**5.2.5 Global stability of wt and F-helix mutant:** Stabilizing a floppy helix may result in overall stabilization of protein structure. To test overall stability of apo F-helix mutant, we compared the tryptophan environment, thermal stability and urea denaturation of apowt and F-helix mutant.

**5.2.5A Tryptophan fluorescence:** Tryptophan environment has been used as a measure of global stability. Trp environment of wt and F-helix mutant proteins was analyzed by fluorescence spectroscopy.

**Material:** *Cuvette (2 mm)*

**Instrument:** *Fluorescence spectrometer Fluorolog (Horiba)*

**Buffer:** *20mMHEPES pH 7.5, 1mM ATP and 5mM MgCl<sub>2</sub>*

Excitation was set at 295 nm and emission was monitored from 305 to 400 nm (slit width 5 nm, at 25°C, steps 0.1 sec, average of 2 scan). Fluorescence intensity of protein was subtracted from the buffer and the data was represented as relative fluorescence intensity.

**5.2.5B Thermal denaturation:** Thermal stability of protein is an index of global stability. The co-operative nature of melting curve shows that the protein was well folded.

**Material:** *Quartz cuvette (5mm)*

**Instrument:** *CD Polarimeter (Jasco, J815)*

**Buffers:** *20 mM sodium phosphate buffer, pH 7.5*

Thermal denaturation of apo wt and F-helix mutant was done simultaneously using multi cell cuvette holder. A Far-UV CD spectrum (250 to 190) was collected from

10°C to 90°C with an increment of 1°C/min. At each data point sample was equilibrated for 5 min.

**Data analysis:** Ellipticity at 222 nm at different temperature was collected. It was used to estimate ellipticity of fully folded ( $\theta_f$ ) and unfolded form ( $\theta_u$ ) using nonlinear regression (GraphPad Prism). The  $\theta_f$  and  $\theta_u$  derived from this was used to calculate fraction folded at any temperature ( $\alpha$ ).  $\alpha = [F] / ([F] + [U]) = (\theta_t - \theta_u) / (\theta_f - \theta_u)$ , Where [F] and [U] are concentration of folded and unfolded form respectively and  $\theta_t$  is the observed ellipticity at given temperature. To calculate the  $T_m$ , the fraction folded at given temperature was further analyzed using nonlinear regression (GraphPad Prism).

**5.2.5C Equilibrium unfolding:** Equilibrium unfolding using chemical denaturants provide thermodynamic stability of protein.

**Material:** Quartz cuvette (1mm)

**Instrument:** CD Polarimeter (Jasco, J815).

**Data analysis software:** GraphPad Prism 5.

**Buffers:** 20 mM sodium phosphate buffer pH 7.5.

**10M urea:** 10M urea was prepared in 20mM sodium phosphate buffer pH 7.5.

To determine the thermodynamic stability, apo form of wt or the F-helix mutant proteins (5  $\mu$ M) were incubated overnight with different concentrations of urea (0M to 7M urea) with 0.2 M increment at 25°C and the CD spectra was collected from 240 to 200 nm.

**Data analysis:** Ellipticity at 222nm at varying urea concentration was used for analysis. The data was fitted as described by Shirley using Graph pad (Shirley, 1995). Free energy change was calculated by  $\Delta G_u = -RT \ln K = -RT \ln [(\theta_f - \theta) / (\theta - \theta_u)]$  where R is the gas constant (1.987 calories/deg/mol) and T is the absolute temperature (K),  $\theta$  is ellipticity at given urea concentration,  $\theta_f$  and  $\theta_u$  represent the ellipticity of the folded

and unfolded protein respectively. For a well folded protein  $\Delta G_u$  is generally varies linearly with denaturant concentration (urea). The free energy of protein in absence of denaturant  $\Delta G(H_2O)$  was calculated by the linear extrapolation model. A 'least square' analysis was used to fit the data to equation  $\Delta G_u = \Delta G(H_2O) - m[Urea]$ , where  $m$  measures dependence of free energy on denaturant concentration and amount of polypeptide exposed to solvent on unfolding.

**5.2.6 Limited proteolysis of wt and F-helix mutant:** Well folded proteins are hard to degrade by proteases as recognition sequences are not accessible. When proteolysis experiments are done in suboptimal condition it is referred to as limited proteolysis (Fontana et al., 2004; Picotti et al., 2004). In these conditions protease will be able cleave the most accessible or flexible region in the protein. Limited proteolysis has been used to find flexible local structure, folding intermediate and isolating protein fragments that can fold autonomously (or domains). We planned to use limited proteolysis to find out the floppy region in apoMb and whether it was stabilized in F-helix mutant.

**Material:** *Trypsin (Sigma) and chymotrypsin (Sigma).*

**2X Proteolysis buffer:** *40 mM Tris pH 7.5 (in case of chymotrypsin supplemented with 1 mM CaCl<sub>2</sub>).*

Apowt and F-helix mutant Mb were incubated with trypsin or chymotrypsin at 1/25 (w/w) enzyme to substrate ratio in 1X proteolysis buffer. Just after addition of protease an aliquot was withdrawn (0 min) and reaction was incubated at 25°C. Aliquots were withdrawn at 15, 30 and 60 min and reaction was stopped by adding 3Xsample buffer (containing SDS) or 1% TFA. Samples were resolved on Tricine SDS PAGE and stained by CBB. Reaction stopped by TFA was stored at -20°C for MALDI MS analysis.

**5.2.6A Tricin SDS PAGE:** Routinely used glycine-SDS PAGE cannot resolve protein <10kDa. For the separation of small protein and peptides, Tricine-SDS PAGE is a popular choice. The different separation characteristics of the two techniques are directly related to the difference in pKa values of glycine and Tricine functional groups. The limited proteolysis products were expected to be of <10kDa, we therefore adopted Tricine SDS PAGE for the separation of cleaved products.

**Material:** *Glycerol, 10% APS, TEMED.*

**Essential buffers:**

*10X Anode buffer: 1M Tris pH 8.9 (with HCl)*

*Cathode buffer: 1M Tris, 1M Tricine and 1% SDS, pH ~8.9 (pH adjustment is not required).*

*Gel buffer: 3M Tris pH 8.45(with HCl) and 0.3% SDS*

*48% Acrylamide stock: 46.5 g acrylamide and 3 g of bisacrylamide was dissolved in 70 ml water and later volume was adjusted to 100 ml.*

*3X SDS sample buffer: 150 mM Tris/HCl (pH 7.0), 12% SDS, 6%  $\beta$ -mercaptoethanol, 30% glycerol and 0.05% Coomassie blue G-250.*

*Mini SDS PAGE apparatus (Bio-Rad)*

16% Tricine separating gel was prepared using 1X gel buffer, 0.033% APS and 33% TEMED. Using separate cathode and anode buffer, limited proteolysis product was resolved initially at 50V for 30 min then at 120V for 3 to 4 h.

**5.2.6B MALDI MS of cleaved products:** From mass spectrometry accurate mass of macromolecule can be measured. To find out site of trypsin and chymotrypsin cleavage, we excised the band from polyacrylamide gel and mass of the fragment was analyzed by MALDI MS. Aliquots withdrawn during limited proteolysis reaction were also analyzed directly by MALDI MS. For extraction of protein from polyacrylamide gel, coomassie

stained gel pieces were first washed, cysteine crosslinking reduction and alkylation of protein was done before extraction.

**Material:** TFA, Acetonitrile, DTT (Sigma), Iodoacetamide,  $\alpha$ -Cyano-4-hydroxycinnamic acid (HCCA).

**Essential buffers:**

*Wash buffer: 1:1 mixture of 100 mM  $\text{NH}_4\text{HCO}_3$  and acetonitrile (50mM  $\text{NH}_4\text{HCO}_3$  and 50% acetonitrile mixture).*

*Reducing buffer: 10 mM dithiothreitol / 50 mM  $\text{NH}_4\text{HCO}_3$  (freshly prepared).*

*Alkylation buffer: 55 mM iodoacetamide in 50 mM  $\text{NH}_4\text{HCO}_3$  (freshly prepared, protect from light).*

*Extraction buffer: 50% Acetonitrile and water mixture containing 5%TFA.*

*Reconstitution buffer: 10% acetonitrile in water containing 0.1% TFA*

*Instrument: Bruker Ultraflex II MALDI TOF-TOF*

Protein gel corresponding to full length and cleaved products were excised, wash several times with water (4 changes, 5 min each). Gel pieces were further washed for 15 min with wash buffer followed by treated with acetonitrile. Gel was rehydrated with 50 mM  $\text{NH}_4\text{HCO}_3$  and after 5 min equal volume of acetonitrile was added and incubated for 15 min. Gel pieces were again treated with acetonitrile to dehydrate it and incubated in reducing buffer for 45 min at 56°C. After cooling to room temperature, reduced protein was alkylated using freshly prepared 55 mM iodoacetamide (in 50 mM  $\text{NH}_4\text{HCO}_3$ ) for 30 min in the dark. Gel pieces were again washed with washing buffer and dehydrated using acetonitrile as described earlier and dried using vacuum centrifuge (~30min). The full length or cleaved products were extracted by incubating corresponding gel pieces with extraction buffer for 30 min. This step was repeated twice. Extracted polypeptides were dried and reconstituted in 20  $\mu\text{l}$  of reconstitution

buffer. Reconstituted peptides or reaction mixture were mixed with matrix (HCCA) (v/v-1:1) and crystalized on MALDI plate by 'dry droplet' method. Peptides were analyzed by mass spectrometry on the Bruker Ultraflex IIMALDI TOF-TOF. The resulting MS data was analyzed using Flex analysis 3.0 software (Bruker Daltonik).

**5.2.7 Proteasomal degradation of wt and F-helix mutant:** Apo wt and F-helix mutant Mb were incubated with 26S proteasome and degradation was monitored by quantifying substrate remaining as described earlier.

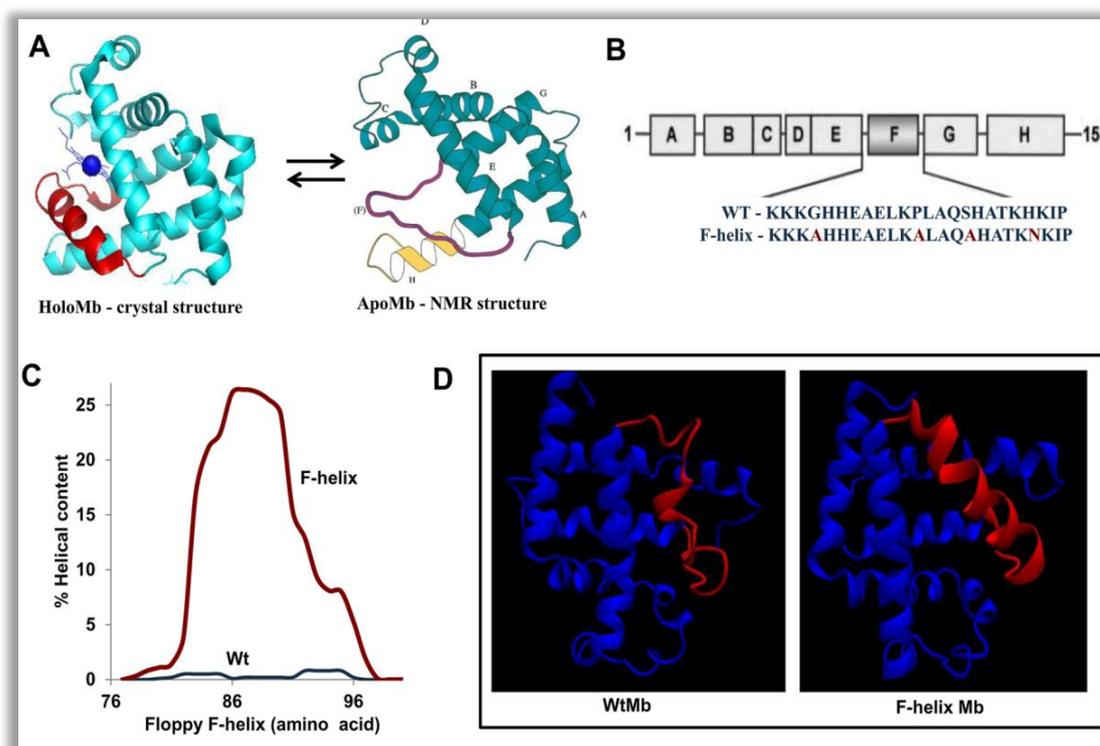
### 5.3 RESULTS and DISCUSSION

**5.3.1 Strategy for F-helix stabilization:** HoloMb was stable for proteasomal degradation. Upon heme removal, dramatic structural change was observed in the F-helix, none of the other helices involved in heme binding were affected. The NMR structure of apoMb showed the residues from 78 to 106 were in dynamic equilibrium between partially folded and unfolded state (**Fig. 5.1A**) (Eliezer and Wright, 1996). We hypothesized that this fluctuating helix, which was buried in holo protein, might be responsible for apoMb degradation. Since apoMb gets degraded without the assistance of 'trans-acting element' this fluctuating structure in protein may act as recognition element or act as initiator of degradation. Stabilization of F-helix may affect any of these steps and therefore should affect the proteasome mediate degradation of apoMb.

We wanted to engineer the helix in such a way that helix once formed remains stable in the absence of heme. Residues 78 to 106 (KKGHHEAELKPLAQSHATKHKIPIKYLEF) encompass the EF-loop, F-helix, FG-loop and first few residues from G-helix. The Gly 80 in the EF-loop, Pro 88 and Ser 92 within the helix were planned to convert in to helix stabilizing residue Ala. The FG-loop is formed by KHKI (96-99) residues (**Fig 5.1B**). The H97N substitution was tested in

FG-loop. The helical propensity of 78-106 residues in wt and the replacement G80A/P88A/S92A/H97N was analyzed using Agadir. Agadir works on helix coil transition theory and for short peptide provides residue level helical propensity. The G80A/P88A/S92A/H97N substitutions were substantially more helical than wt sequence in 78-106 regions (**Fig. 5.1C**). We used this mutant for further analysis and referred this as ‘F-helix mutant’.

**5.3.2 F-helix was stable during MD simulation:** MD simulation of heme free form of Wt and mutant was done at 400K for 2.8 ns. The f-helix in wt melted at the beginning of the simulation while in mutant it was stable even until end (**Fig. 5.1D**). This observation indicates that floppy F-helix was less dynamic in heme free form of F-helix mutant.

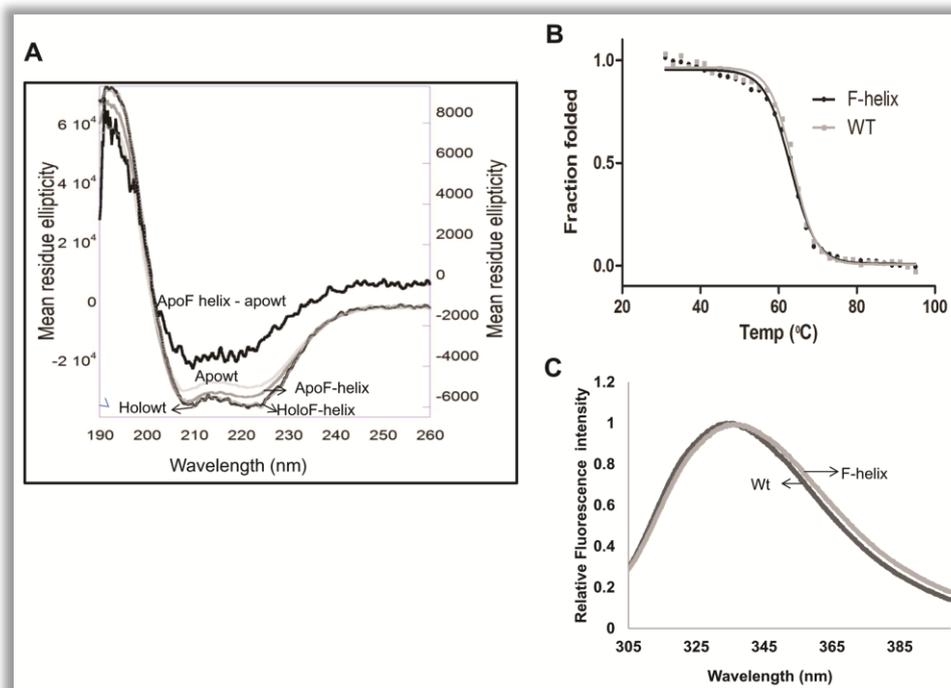


**Figure 5.1 Stabilization F-helix of Mb:** The comparison of X-ray crystal structure of holo (PDB=2JHO) and NMR structure of apoMb (Eliezer and Wright, 1996) showed that 78-106 region was in dynamic equilibrium between partially folded and unfolded stage (A). The proposed modification for stabilizing floppy helix is highlighted in red (B). Prediction of the helical propensity of 78-106 region in Wt and

*G80AP88AS92AH97N (brown) using AGADIR (C). MD simulation of wt apoMb and the F-helix mutant was done at 400 K, wt melts immediately while, F-helix mutant remains stable even at the end of 2.8 ns simulation (D).*

**5.3.3 F-helix mutant Mb was more helical than wt:** When a protein is destabilized or stabilized it is reflected in its secondary structure. We first compared the secondary structures of holo and apo form of Mb by Far-UV CD spectrometry. Mb is all helical (A to H-8 helices) protein, we found signature negative ellipticity at 222, 209 nm and positive ellipticity around 190 nm. The Mean Residue Ellipticity (MRE) at 222 and 209nm of holoMb was more than apoMb showing that heme removal resulted in loss of helicity (**Fig. 5.2A**). Using SELCON, CONTIN (from Dichroweb) and MRE at 222 nm for calculating secondary structural component, we found that heme removal resulted in 18% loss in helicity (pH 7.5 and 24°C). A 20% change in helicity has been reported at near neutral pH (Griko et al., 1988). If we observe the secondary structural components of holo ( $\alpha$ -helix-94%,  $\beta$ -sheet-0% and other- 6%) and apoMb ( $\alpha$ -helix-79%,  $\beta$ -sheet-0%, turn 6% and other- 15%), loss of helicity resulted in increase in unordered conformation (from 6% to 15%).

From the AGADIR prediction and MD simulation it was clear that F-helix was stabilized in F-helix mutant. These changes were incorporated in Mb cDNA by site directed mutagenesis, protein was purified and heme was removed. The secondary structure of the holo and the apo forms of F-helix mutant were compared with their wt counterpart. The secondary structure of holo form of wt and F-helix mutant was comparable (**Fig. 5.2A**). The apoF-helix mutant was 9% ( $\pm 2\%$ ) more helical than that of apowtMb (**Fig. 5.2A**). As compared to holo, apoMb was 18% less helical, half of the loss in helicity (9%) was recovered due to helix stabilizing mutations. Therefore the designed mutations were able to stabilize the heme free form of Mb making it 'holo like' structure without heme.



**Figure 5.2 Effect of mutation on secondary structure and overall stability of F-helix mutant:** To verify the role of floppy F-helix exposed upon removal of heme, helix stabilizing mutations were introduced. Far-UV CD spectrum shows that the apo F-helix mutant has enhanced secondary structure as compared to the wt apoMb. The difference in spectra was obtained by subtracting the spectra of apowt from apoF-helix (MRE values are on Y2) (A). Thermal denaturation of apo wt and F-helix mutant was monitored by secondary structural changes with increasing temperature. Ellipticity at 222 nm was used to calculate the fraction folded which is plotted against the incubation temperature (B). Trp fluorescence of wt apoMb and the F-helix mutant was analyzed under native conditions. Trp environment, an indicator of tertiary fold was similar in the wt and the mutant proteins (C).

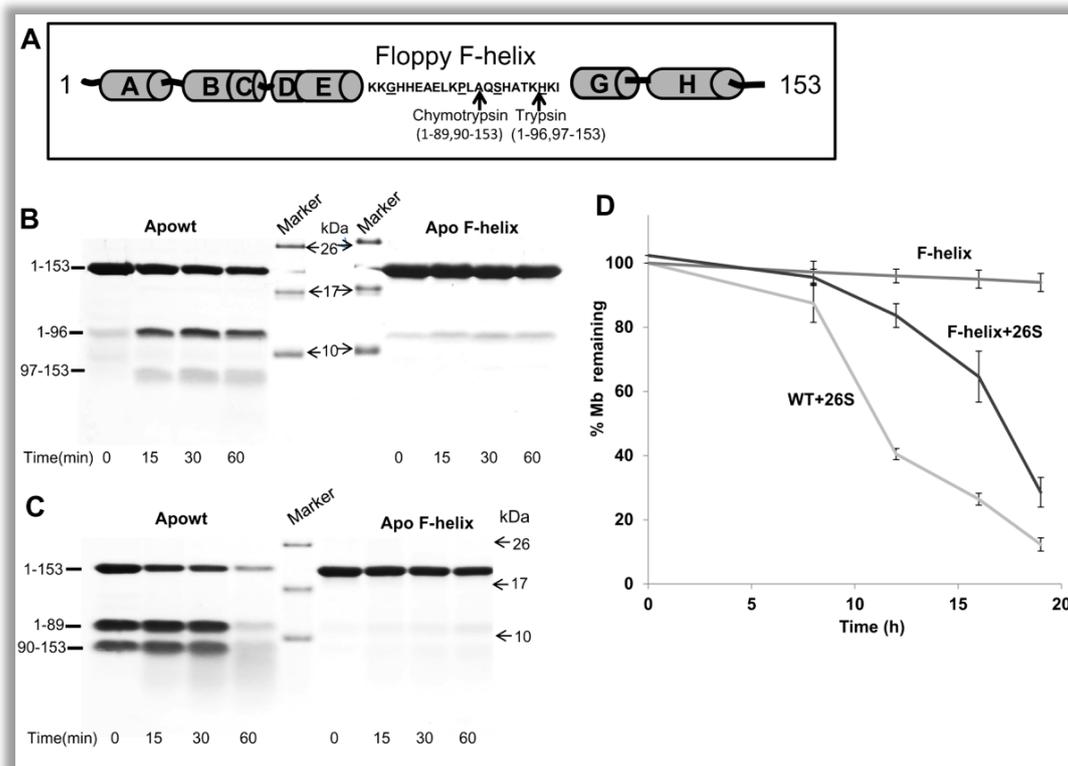
**5.3.4 F-helix was stabilized in apo F-helix mutant:** We engineered the floppy helix to stable helix as can be seen by Far-UV CD data. Although, these mutations were confined to F-helix region of Mb, most probably this region should be stabilized in the protein. However, since CD data provides an average estimate of secondary structure, it may be difficult to pin point the region that have been stabilized. To further prove that the mutations indeed stabilize the floppy F-helix, we performed limited proteolysis experiment. We hypothesize that since in apoMb F-helix was floppy, limited proteolysis experiment will show initial nick in this region while apo F-helix mutant should be

relatively stable towards proteolysis provided it has been stabilized in mutant protein. We choose trypsin and chymotrypsin for our experiment. Trypsin or chymotrypsin cleaved the apoMb in F-helix region. Along with the full length protein (17.3kDa) two fragments corresponding to 1-96, 97-153 (trypsin) and 1-89, 90-150 (chymotrypsin) were observed in Tricin SDS PAGE (**Fig. 5.3 B and C**). Rate of cleavage of chymotrypsin was faster than trypsin, at 60 min the intensity of full length as well as the cleaved product decreased showing further cleavage of the nicked products (**Fig. 5.3 C**). To identify site of cleavage, direct reaction mixture or gel extracted cleaved fragments were analyzed by Matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS). Trypsin and chymotrypsin cleave apoMb after Lys 96 and Leu 89 respectively (**Fig. 5.3A and table. 5.2**) (Picotti et al., 2004). Apo F-helix mutant was very stable towards protease cleavage, minimal amount of products could be seen upon prolonged incubation when most of the wt apoMb was cleaved.

Previous reports, AGADIR prediction, MD simulation, far-UV CD and limited proteolysis showed that upon heme removal major structural changes were confined to F-helix region (78-106) of Mb. We engineered the F-helix region of Mb to induce helicity even in the absence of heme. Taking together above experiments provide enough evidence to prove that designed mutations in F-helix region were able to stabilize the F-helix in absence of heme.

**Table 5.2 Calculated and mass obtained from MS of limited proteolysis fragments**

Protease	Fragments	Calculated mass (Da)	m/z (Da)
Uncut		17330	17341
Trypsin	1-96	10911.6	10924
	97-154	6437.4	6448
Chymotrypsin	1-89	10187.8	10199
	90-154	7161.2	7176



**Figure 5.3 Stabilization of F-helix rendered apoMb more resistant to degradation by the proteasome:** Cleavage sites of trypsin and chymotrypsin on Mb are diagrammatically represented (in F-helix underlined amino acids are mutated) (A). Trypsin (B) and chymotrypsin (C) were added to wt and F-helix mutant. Aliquots at various time intervals were analyzed by Tricine-SDS Page. Cleaved of apoMb by chymotrypsin was almost instantaneous (0 min). The F-helix mutant was stable for cleavage by trypsin and chymotrypsin (B and C). Apo wt and apo F-helix proteins were incubated with proteasome. The rate of degradation was followed by SDS-PAGE. Data represent the mean values  $\pm$ S.D of at least three independent experiments for wt apoMb and five independent experiments for F-helix mutant (D).

**5.3.5 Overall stability of F-helix mutant was not affected:** Stabilization of local region in a protein might results in building several new long range interactions resulting in global stabilization of the protein. Stability of apo form of wt and F-helix mutant protein was deciphered using several biophysical techniques like tryptophan environment, thermal denaturation and equilibrium unfolding using urea. Tryptophan fluorescence of apo wt and F-helix mutant Mb was measured to check whether mutations have affected the tertiary structure of the protein. The emission maximum and relative fluorescence intensity of both the protein were similar indicating, tryptophan

environment of both the proteins was comparable (**Fig. 5.2C**). Thermal stability of protein has been used to determine the thermodynamic stability of the protein. The secondary structural changes (ellipticity at 222 nm) of wt and mutant protein were measured as a function of temperature. Both the proteins showed two state transitions of thermal denaturation illustrating co-operative nature of unfolding, the characteristic of well folded protein. The melting temperature (temperature at which protein is 50% unfolded) of wt and mutant protein was 63°C indicating overall fold of the mutant protein was not affected by mutation (**Fig. 5.2B**). To further prove that mutations had a major effect on the secondary structure and that the global stability was unaffected, we compared the thermodynamic stability of wt apoMb and the F-helix mutant by urea denaturation at pH 7.5. Secondary structure of both the proteins was measured as a function of urea concentration. Free energy of stabilization was calculated using the equation  $\Delta G = \Delta G(\text{H}_2\text{O}) - m[\text{urea}]$  where  $\Delta G(\text{H}_2\text{O})$  is  $\Delta G$  in the absence of urea and  $m$ , the dependence of  $\Delta G$  on urea. The  $\Delta G$ , midpoint transition (urea concentration required to unfold 50%), and  $m$  of both apo wt and F-helix mutant protein were similar (**Table 5.3**).

**Table 5.3 Thermodynamic parameters from the urea equilibrium unfolding**

	<b>Wt</b>	<b>F-helix</b>
$\Delta G(\text{H}_2\text{O})$ (kcal mol <sup>-1</sup> )	6.14± 0.25	6.12±0.25
$M$ (kcal mol <sup>-1</sup> M <sup>-1</sup> )	-1.41±0.05	-1.49±0.06
$C_m$ (M)	4.3	4.09

The above experiments provide clear evidence that mutations in the F-helix primarily stabilize the secondary structure in the floppy F-helix and global stability was not affected.

**5.3.6 Exposure of F-helix was essential for apoMb degradation:** It is clear by now that the F-helix was stabilized in mutant protein. We checked the effect of these mutations on proteasomal degradation, apo F-helix mutant was found to be more stable

than wt (**Fig. 5.3D**). We observed substantial increase (4h) in the half-life of proteasomal degradation in F-helix stabilized mutant. The proteasomal stability in F-helix mutant could be due to compromised affinity with proteasome. To rule out this possibility, proteasome binding assay was done with apo wt and F-helix mutant. The affinity of mutant protein with proteasome was not compromised ( $K_d=0.57\pm 1\text{nM}$ ) (**Table 5.4**). This result also indicates that the residues mutated may not be directly involved in interaction

While holoMb was not recognized and degraded by proteasome, heme removal resulted in exposure of a buried helix. ApoMb on the other hand bound tightly, elicited response from proteasome and was degraded by it. Stabilization of exposed helix made it difficult for the 26S proteasome to degrade the mutant apoMb. Long unstructured sequences when attached in trans has been shown to enhance the proteasomal degradation of protein, but how such unstructured regions may originate in the substrate remains unclear. The above results not only strongly support the requirement of floppy region for degradation but provide the elegant example how ‘cis-acting elements’ may get exposed and regulate the half-life of protein. ApoMb is a natural intermediate in biosynthesis of holoMb. Although, *in vivo* how Mb turnover is regulated has not been investigated, it seems that nature protects the protein from proteasome and other proteases by stabilizing the floppy helix due to heme binding.

**Table 5.4 Comparison of wt holo, apo and apo F-helix mutants**

Protein	% helix (SELCON 3)	F-helix	Affinity (Kd nM)	Tm (°C)	Average half-life (h)
Apowt	79	Floppy	3.5±1	63	12
HoloMb	94	Buried	ND	80	ND
Apo F-helix	85	Stabilized	0.57±0.1	63	16

## 5.4 SUMMARY

The clues obtained from structural comparison of both the form of Mb led us to engineer helix stabilizing amino acids in floppy F-helix. We found that F-helix was thermodynamically stable in mutant protein by MD simulation. The Far-UV CD data suggested that apoMb was 18% less helical than its holo form and helix stabilizing mutations does not affect the secondary structure of holo form while apo form of mutant was about 9% more stable than wt. From limited proteolysis experiment we proved that F-helix of apoMb was indeed floppy in nature and it is stabilized in mutant. The tryptophan environment, melting temperature and free energy of stabilization of apowt and mutant Mb were comparable, establishing that overall stability of mutant was similar to wt. The affinity of mutant protein was not compromised while mutant protein was relatively stable for proteasomal degradation.

Taken together these results provide direct proof that the disordered F-helix is stabilized by mutation and therefore conformational changes involving this helix must be responsible for the effect on degradation. These results also reflect the fact that the proteasome is sensitive to local secondary structural alterations. Some of the important findings are -

- a) Conformational change such as exposing a previously buried region ('Cis-acting element') was necessary for recognition.
- b) Localization and high affinity binding was necessary but not sufficient for efficient degradation.
- c) Local changes in the secondary structure alone can determine the half-life of a protein. Similar types of alterations have been reported when a protein undergoes post translational modification or ligand/binding partner is removed.

Several heavily ubiquitinated proteins have been found to be interacting with proteasome but escape degradation. The process of presentation of 'cis-acting element' might be a key switch in decision making to degrade or not to degrade.