Biophysical & Structure Properties of the UspA712 of *Micrococcus Luteus*

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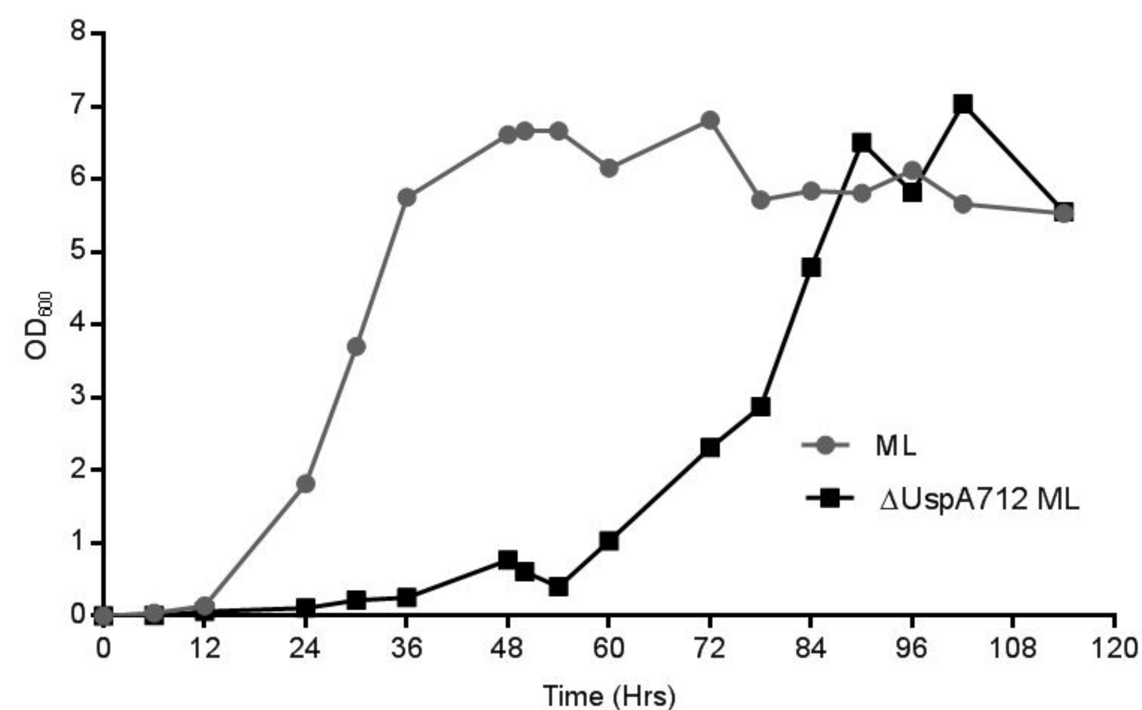
Introduction

UspA712 is a 12kD single domain protein. It is upregulated in exponentially growing wild type strain of Micrococcus luteus, one of the two models used in the study of bacterial dormancy. When UspA712 is knocked out, the mutant Ml strain exhibits an extended lag phase. The mechanism of action of the Universal Stress Protein(USP) in relation to stress response are not currently known. Unlike the two other Usp's in Micrococcus luteus (UspA616 & UspA184), UspA712 protein does not bind nucleotides and so we hypothesize that it might have an allosteric property in modulating its regulatory functions and molecular interactions. UspA712 protein is purified as a dimer and structure of the protein shows an extended α-helix wrapped around the β sheets. UspA712 is quite unstable and degradation occurs after a number of days. We hypothesize that degradation starts at the α helices, which lacks most of its electron density. This is highlighted with HSQC experiment data gathered over a couple of days showing a collapse in the middle of the spectra (α-helices) indicative of degradations while showing much distinct peaks around the edges (β sheets). To study the proteins conformational changes, future experiments are directed towards NMR structural dynamics studies after the first step of assigning the protein backbone structure. To characterize the biophysical properties of the UspA712 protein, melting temperature and thermodynamic parameters were estimated using differential scanning flourimetry experiments. The protein exhibits a one state unfolding mechanism as opposed to two state characteristic of a dimeric protein. At increasing concentrations of the protein, melting temperature and hence enthalpy and free energy changes have roughly the same values. This was also correlated with differential scanning calorimetry experiments showing an almost consistent enthalpy and free energy values with the DSF.



OD vs Time (Hrs) ML vs ΔUspA712 in LB media

Figure 1 – Growth curve analysis of the Usp712 mutant knocked out with a kanamycin insertion sequence and the wildtype *Micrococcus luteus.* The mutant bacteria shows an extended lag time in rich media when compared with the wildtype bacteria strain.

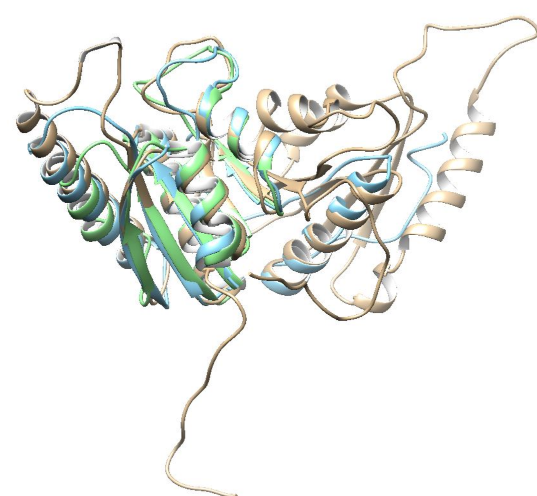


Figure 2 – Structure alignment of the three Usp's in Micrococcus luteus. UspA616 (grey), UspA184 (light blue) & UspA712 (light green). UspA616 and UspA184 have the walker A & B sequences which confers nucleotide binding properties. UspA712 does not have the same sequence similarity and hence does not bind ATP. Figure was produced using chimera.

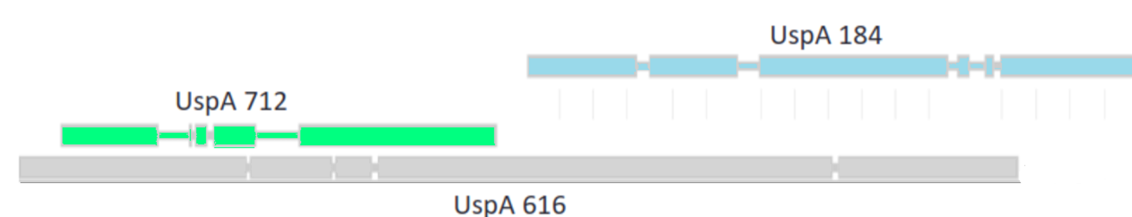


Figure 3 – Domain alignment structure of the Usp616 with Usp712 & Usp184 single domains on either end of the terminal region of the protein.

Protein expression and purification

UspA712 protein was expressed using the amplified UspA712 gene from *Micrococcus luteus* genome and ligated with pET14b expression vector. The ligated plasmid was transformed into BL21 PLYS bacteria cells. Exponentially growing cells were IPTG induced and grown for 4 – 5 hours at 37°C. Growing cells were spun down, homogenized and protein precipitation was done at 60% with ammonium sulphate. Precipitates was resuspended in buffer and ran through the S200 and then S75 column respectively. Confirmation of dimeric protein was done at 2mg/ml and at 0.2mg/ml using the S75 analytical column.

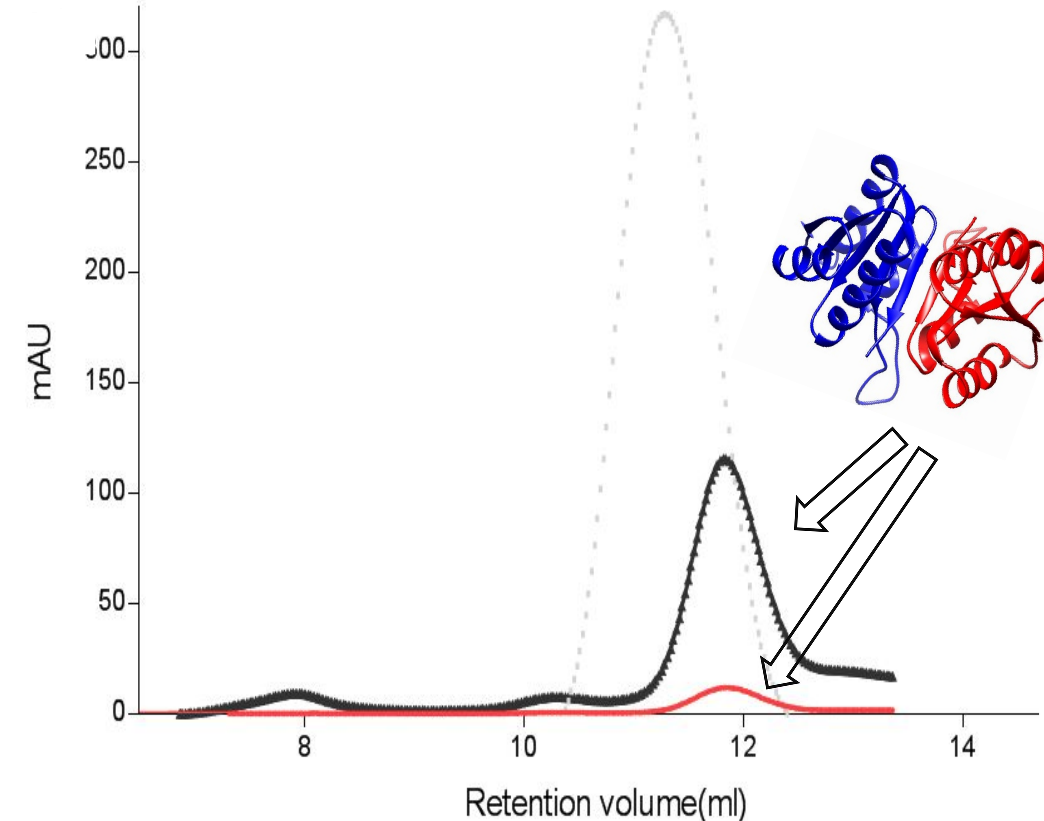


Figure 4 – Size exclusion chromatography purification of the UspA712 with a confirmation of its dimer formation (24kD) at high (black, 2mg/ml) and low (red, 0.2mg/ml) protein concentrations. Transparent line showing 1mg/ml of chymotrypsin standard (25kD)

Structure determination

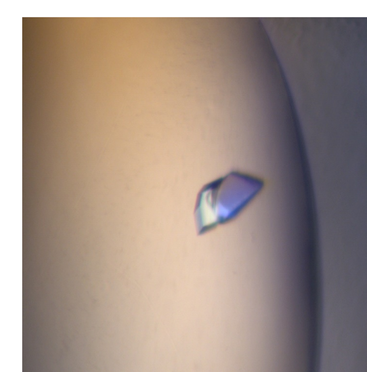


Figure5a – UspA712 crystal. 15mg/ml protein, 20% PEG 3350, 0.1M Bis-tris pH 6.5

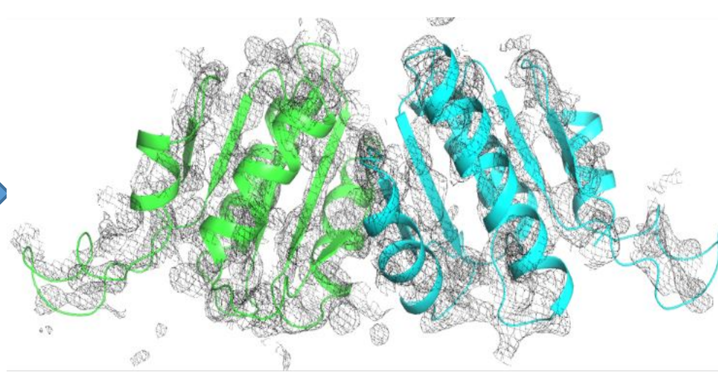


Figure 5b – Electron density map of the UspA712 diffracted at 2.0A resolution

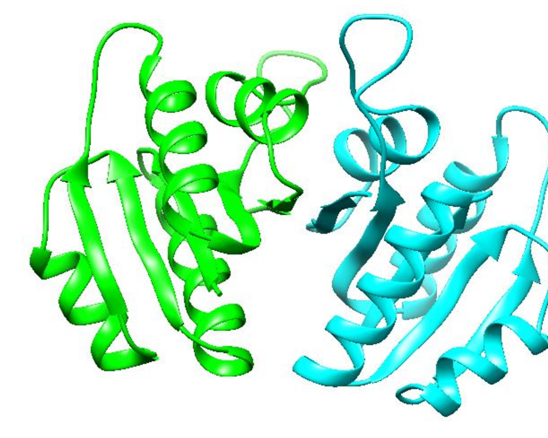


Figure 5c – UspA712 protein predicted using RaptorX structure prediction server, structure view with chimera

Towards NMR backbone assignment

Our future goal is to complete a Sequential Backbone Resonance Assignments using 15N/13C and 15N/13C/2H labeled samples of the Usp712 protein. This will naturally allow for future NMR studies to understand the molecular motions and interactions of the residues of this protein in the presence of small molecules. We have carried out initial HSQC experiments with spectra peaks identified. This was set up over a series of days to identify among other things the rate of degradation of some of the amino acid residues.

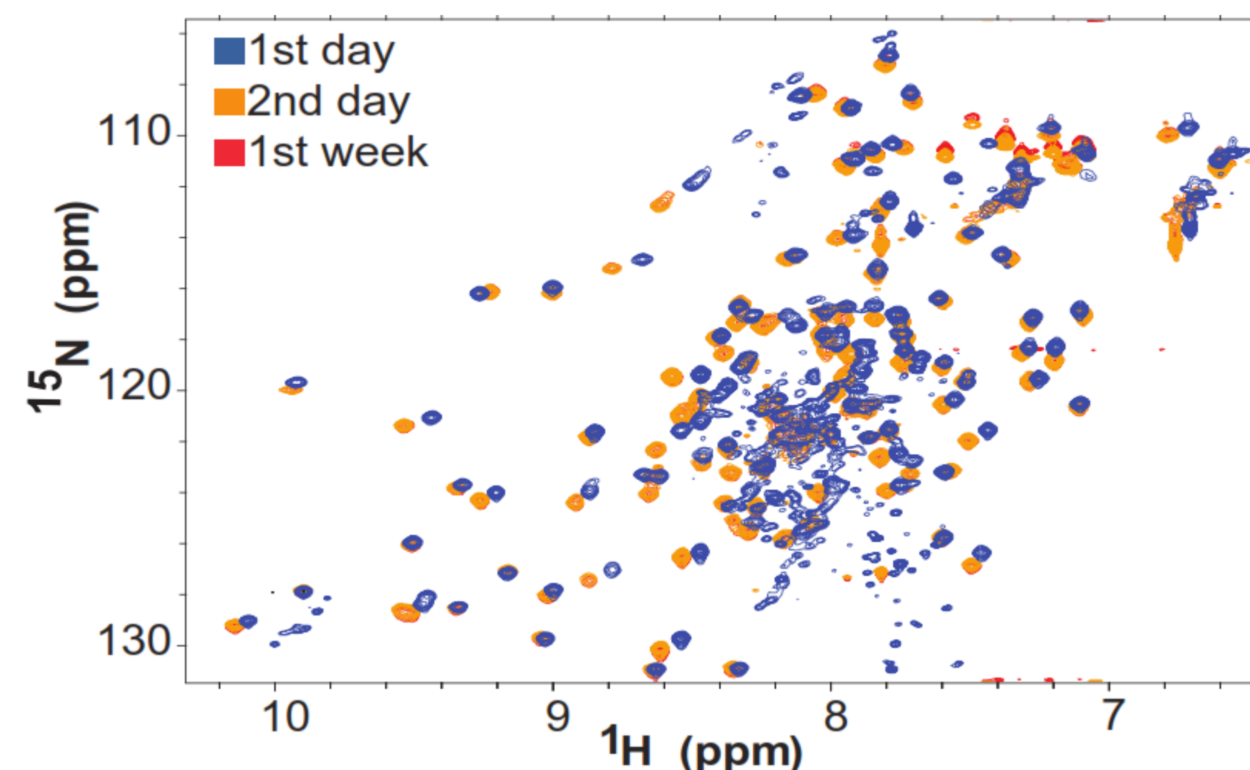


Figure 6 – HSQC of 1H/15N labeled UspA712 (15mg/mL) collected over a weeks long experiment

Protein unfolding and extracting thermodynamic parameters

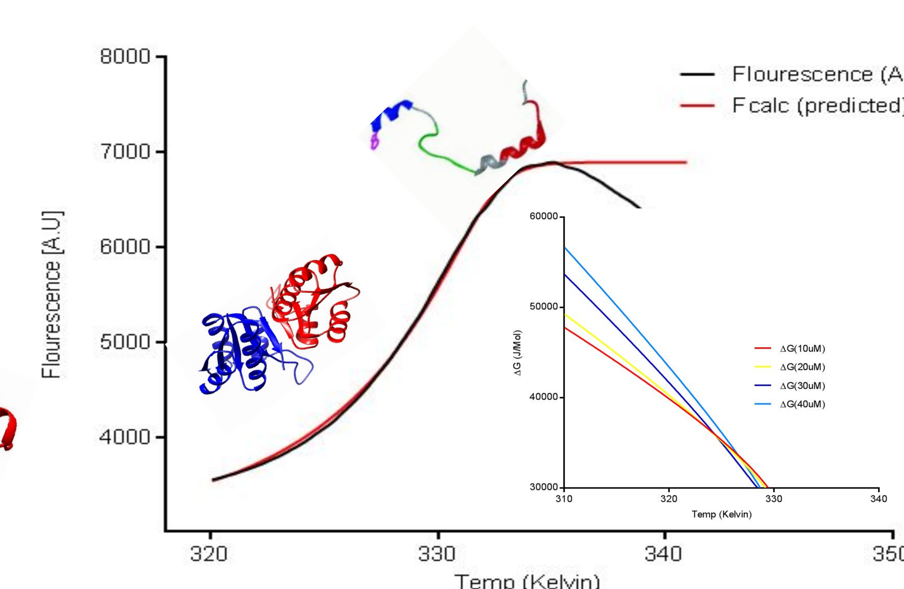


Figure 7 – Differential scanning flourimetry of UspA712 from *Micrococcus luteus*. A Boltzmann-fit enabled determination (red) of Tm and spreadsheet calculations used to estimate thermodynamic parameters.

[Usp712]	Tm	ΔH	ΔG
10uM	55.4	149.6	16.2
20uM	56.3	156.4	17.3
30uM	56.5	162.2	18.1
40uM	56.7	175.1	19.6

Table 1. Thermal Stability and Thermodynamic Data for UspA712 protein at different concentrations using Differential Scanning Flourimetry

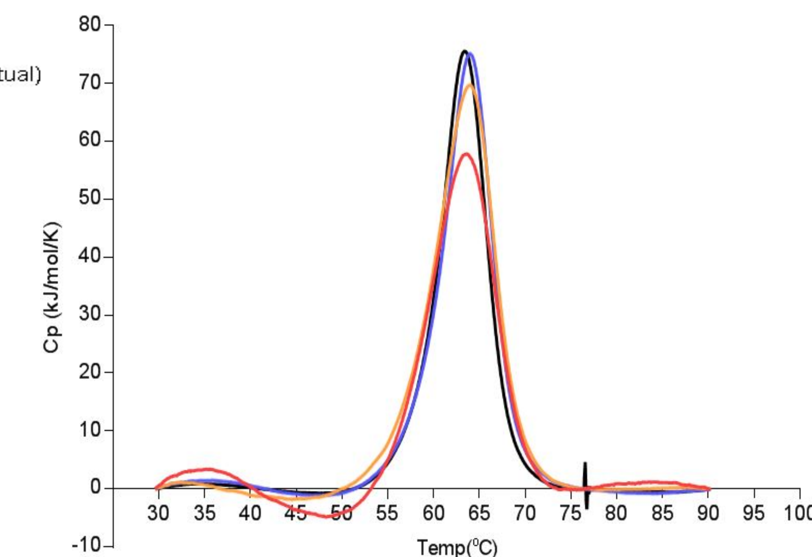


Figure 8 – Differential scanning calorimetry of UspA712 from *Micrococcus luteus*. The area under the curve showing the change in enthalpy and peaks showing the melting temperature.

[Usp712]	Tm	ΔH
10uM	63.14	124
20uM	63.42	147
30uM	63.53	124
40uM	63.23	122

Table 2. Thermal stability and thermodynamic data for UspA712 protein at different concentrations using Differential Scanning Calorimetry

Conclusions

- UspA712 is critical for immediate growth of Micrococcus luteus. Inactivation of this gene leads to an extended lag phase in liquid media
- UspA712 is purified as a dimeric protein and unfolds in a one-step process. Melting temperature of the protein is around 56°C with enthalpy and Gibbs free energy across the different concentration at 161kcal/mol and 18kcal/mol respectively
- UspA712 is unstable and degrades over time. This could be traced to α-helix in the c-terminal region of the protein. This was confirmed with HSQC spectra data collected over couple of days
- UspA712 does not bind or interact with nucleotides. We propose an allosteric mechanism of regulation or interaction is used propagate its functions in vivo

References

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Acknowledgements

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