Probing Stability and Unfolding of the N-terminal Domains 5-6 of Wilson Protein

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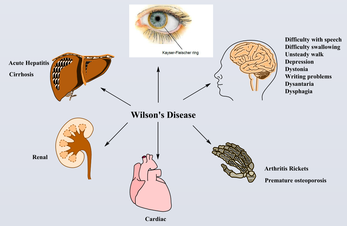
Abstract

The Wilson protein (ATP7B) is a copper transporting P_{1b} type ATPase found in the liver, brain, and other organs. The N-terminal end consists of six copper binding domains which have a ferrodoxin βαββαβ fold with a CxxC motif. Despite similarities in copper binding affinities they interact differently with the HAH1 metallochaperone. Studying the stability of these domains will help understanding the differences in their functions. The stability of WLN5-6 was probed using several different methods: Dynamic light scattering (DLS), Circular dichroism (CD) and Fluorescence spectroscopy methods.

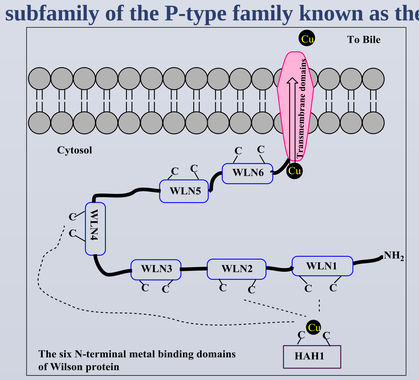
Previous studies show that WLN5-6 has a spherical shape and CD chemical unfolding studies indicate that mutants prepared had the same stability. Variations in Two Photon Absorption (2PA) cross-section are attributed to changes in local electric fields of the protein as confirmed from femtosecond fluorescence anisotropy and fluorescence lifetime. Present results show that the 2PA cross-sections can be used as a tool to probe local environments and unfolding of proteins.

Introduction



Wilson disease is caused by a buildup of copper in the body.

In Wilson disease, copper is not sufficiently excreted into bile due to the absence or malfunction of the Wilson protein copper ATPase in the excretory pathway of hepatocytes. Wilson Protein is a copper ATPase in the family of P-type ATPases which all share common structures and features. This protein belongs to a subfamily of the P-type family known as the P1B-ATPases.



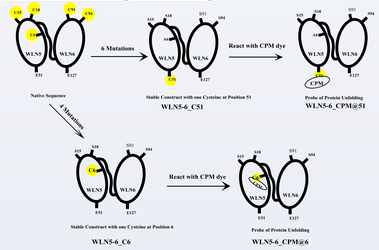
Wilson's disease protein (ATP7B) play important roles in maintaining copper ion homeostasis. Mutations in the ATP7B gene causes the disease

Research Objective:

Probing stability of the last two metal binding domains WLN5-6

Materials and Methods

Plasmid Engineering



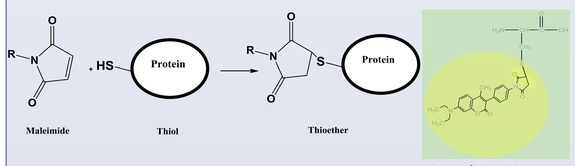
5 original cysteines were mutated, one was produced at position 51

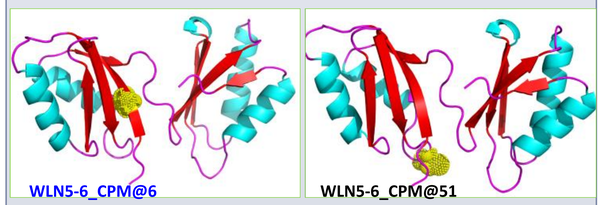
4 cysteines were mutated to serine, cysteine at position 6 remained

Protein expression and purification

The plasmid was transformed into E. Coli-Rosetta 2 cells and grown in LB media until the OD600 reaches 0.7 then the cells induced with 1mM IPTG for 4 hours at 28 ˚C. The cells harvested by centrifugation then the protein extracted by freeze thaw method. Purification of protein was accomplished by using anion exchange column (DEAE) and gel filtration technique.

Protein probing





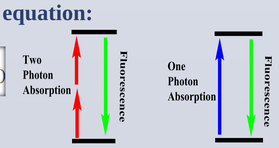
Theory

Why 2PA cross sections for unfolding?

2PA cross sections (δ) used to monitor protein unfolding: it is measured as the ratio between the intensities of the two-photon fluorescence (I2P) and the one-photon fluorescence (I1P) based on the following equation:

$$\delta_{(s)} = \frac{I_{2P\,(s)} \cdot I_{1P\,(folded)}}{I_{1P\,(s)} \cdot I_{2P\,(folded)}} * \delta_{(folded)}$$

$$\delta \alpha \Delta \mu_{ge}^{2} \qquad \overrightarrow{\Delta\mu_{ge}^{0}} = \overrightarrow{\Delta\mu_{ge}} \mp 0.5\,\overrightarrow{\varepsilon}$$



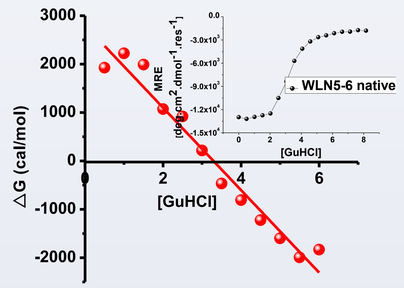
Why ultrafast anisotropy for unfolding?

Anisotropy measurements reveal the average angular displacement of the fluorophore that occurs between absorption and subsequent emission of a photon. It is quite sensitive to micro environments.

$$r = \frac{I_{VV} - G * I_{VH}}{I_{VV} + 2 * G * I_{VH}} \qquad G = \frac{I_{HV}}{I_{HH}}$$

Results

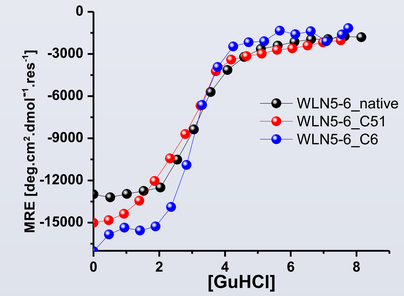
Linear extrapolation method used to determine the free energy (ΔG).



ΔG = 3.33 Kcal/mol

C_m = 3.3 M

Figure 1: Unfolding of WLN5-6 using Guanidine Hydrochloride, assuming a two-state unfolding mechanism.



The mutations did not have significant effect in secondary structure.

Figure 2: Molar residue ellipticity at 222 nm.

Unfolding of WLN5-6 and two different mutants that contain one cysteine residue.

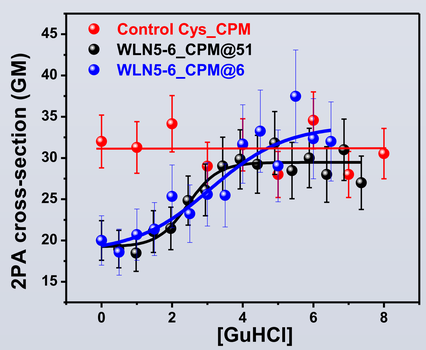


Figure 3: 2PA cross-section of CPM dye bound to WLN5-6 at position 51 and 6. This method can be used to monitor unfolding of proteins at specific positions.

✓The local electric field in the folded form of protein is anti-parallel to the orientation of the dipole and thus the 2PA cross-section is enhanced when the protein is unfolded.

Results

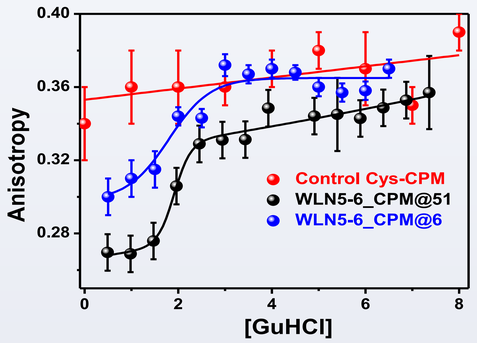


Figure 4: Ultrafast anisotropy at 10 ps time delay. Anisotropy changes are attributed to the changes in the orientation of the molecular dipole in the bound state, probably hydrogen bonding with amino acids adjacent to it.

✓ The dye is bound to protein via hydrogen bonding and the unfolding of the protein alters it.

Conclusion

•WLN5-6 unfolds in a apparent two-state pathway with a free energy of 3.33 Kcal/mol with a denaturation midpoint of 3.3 M. Also, labeling WLN5-6 with CPM dyes did not affect the chemical unfolding pathway.

• The results of 2PA cross-sections of chromophores have shown a novel way to monitor the unfolding of proteins. The increase in 2PA cross-sections is ascribed to the differences in the local electric field around the chromophore during unfolding. This technique paves way for imaging folded and unfolded forms of proteins.

• Femtosecond fluorescence anisotropy technique of the chromophores was successfully used to monitor the unfolding of WLN5-6 protein. This is one of the first reports where ultrafast anisotropy was used to study unfolding. It is important to note that the change in anisotropy is not due to HOMO FRET rather due to the hydrogen bonding of chromophore with protein.

References

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