Multiphoton Fluorescence Spectroscopy to Monitor the Folding/Unfolding of the N-terminal Domains 5-6 of Wilson Disease Protein

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Abstract

Denaturation of the N-terminal domains 5-6 of Wilson Disease protein has been monitored through its mutant WLN5-6Cys51 by circular dichroism (CD), one and two-photon fluorescence spectroscopy after binding it to the thiol-reactive dye 7-diethylamino-3-(4'-maleimidylphenyl) -4-methylcoumarin (CPM). Far-UV CD measurements have shown that the mutations and the binding of the dye did not affect the secondary structure of the protein as the unfolding transition remained constant. The fluorescence maximum of the dye changed with the denaturant concentration and followed the trend obtained from CD and shown that one dye labeled protein can be used to monitor the unfolding of protein. Interestingly, the two-photon absorption (TPA) cross-section (ratio of two-photon to one-photon fluorescence intensity) of the dye increased 40% when it is unfolded and no such change was observed for the control dye. The variations in TPA cross-section are attributed to the changes in local electric fields of the protein as confirmed from femtosecond fluorescence anisotropy and fluorescence lifetime. Present results show an evidence that the TPA cross-sections can be used to monitor the local electric fields in proteins and thereby image the folded and unfolded forms of protein or metal binding to the proteins.

Introduction

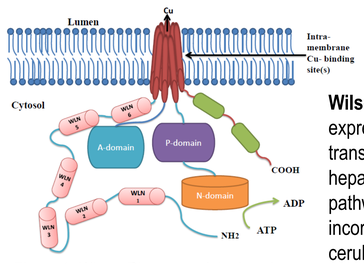
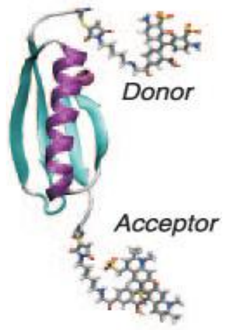


Figure 1: Wilson Disease protein domains

Wilson's protein is expressed in the liver and transports copper into the hepatocyte secretory pathway for the subsequent incorporation into ceruloplasmin and excretion into the bile.



Unfolding of protein is usually monitored by circular dichroism[2] fluorescence resonance energy transfer[3], nuclear magnetic resonance with hydrogen exchange and atomic force microscopy experiments.

We propose to follow the local electrostatic fields in proteins to study protein folding. However, there are few experimental techniques that can follow the local electric field directly.

Figure 2: Donor and acceptor dyes for FRET

Materials and Methods

Plasmid engineering

WLN5-6Cys51 plasmid was produced by several mutations using QuikChange II XL Site Directed Mutagenesis Kit protocol (Strategene).

Protein expression and purification

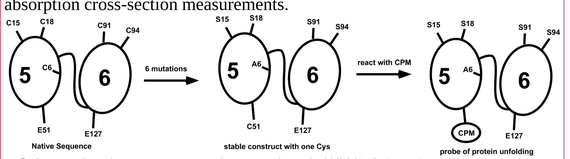
The plasmid was transformed into E. Coli-Rosetta 2 cells and grown in LB media until the OD_{600} reaches 0.7 then the cells induced with 1mM IPTG for 4 hours at 28 ˚C. The cells harvested by centrifugation then the protein extracted by freeze thaw method. Purification of protein was accomplished by using anion exchange column (DEAE) and gel filtration technique.

Protein probing

The CPM dye was used as a fluorophore to monitor the unfolding of the protein, it was bound to the thiol group in the cysteine residue of WLN5-6Cys51 via a coupling reaction with the maleimide group.

Unfolding of the protein

The dye bound protein was unfolded at different concentration of Guan-HCl and the unfolding was monitored by circular dichroism, multiphoton fluorescence, femtosecond fluorescence anisotropy and two-photon absorption cross-section measurements.



Scheme 1: scheme represent the mutations in WLN5-6 domains

Results

Figure 3: Cartoon structure of WLN5-6. PDB, 2EW9

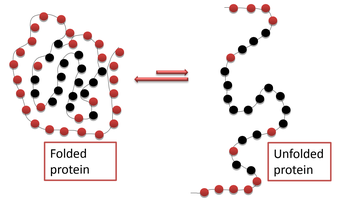


Figure 4: Illustration of the change of the structure by unfolding.

WLN5-6 mutations and binding to the CPM dye did not have significant change in the secondary structure of the native protein.

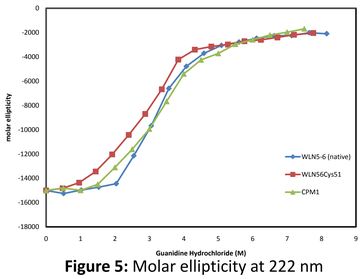
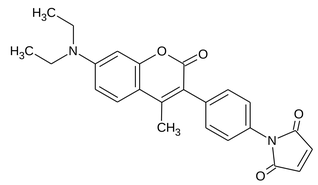


Figure 5: Molar ellipticity at 222 nm



CPM dye

Figure 6: Fluorescence maximum shift with denaturant. The maximum emission wavelength of the bound CPM dye has shifted toward longer wavelength and the transitions of the emission maxima matched with the unfolding transition.

Figure 7: Ultrafast anisotropy at 10 ps time delay. Anisotropy changes are attributed to the changes in the orientation of the molecular dipole in the presence of local electric fields in the protein. Interestingly, the transition matched with the protein unfolding transitions suggesting variations in the local electric field.

Figure 8: Change in the TPA cross-sections of CPM dye with denaturant. TPA cross-section increase as unfolding due to the change in the dipole moment of the dye induced by the local electrostatic fields in proteins.

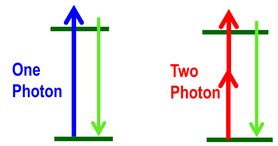
Figure 9: change of the lifetime of CPM dye with unfolding of the protein. lifetime decrease through unfolding because the environments around the CPM dye changed from being rigid to be more flexible. Here again, the changes are attributed to variations in the electric field environment around the probe.

Discussions

TPA cross section to monitor local electric fields

Measurement of the two-photon absorption cross-sections were done by the relative method developed by Rebane and coworkers[4].In this method the TPA cross sections (**δ**) is measured as the ratio between the intensities of the two-photon fluorescence (I_{2P}) and the one-photon fluorescence (I_{1P}) based on the following equation:

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



TPA cross section (δ) is related to the dipole moment of the fluorophore, while dipole moment is related to the local electric field of the protein.

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

Our results showed an increase in the cross section with unfolding which means that the local electric field is working against the dipole moment of the dye.

"TPA cross section is a tool to image the local electric field in proteins"

Conclusions

1. The CD data of the unfolding of WLN5-6 and its mutant showed two states and a transition at ~2.5 M guanidine hydrochloride. This means that the mutations have not changed the secondary structure of the native protein.
2. One-photon and two-photon fluorescence showed a red shift in a trend similar to the CD results which indicates the change in the environment around the fluorophore and the exposure of the dye to the polar solvent while unfolding.
3. Two-photon absorption cross-section have changed with the unfolding. This is an interesting result indicates the change in the dipole moment which can be used to measure the change in the local electric field of the protein.
4. Femtosecond anisotropy increased through unfolding from 0.28 to 0.34 indicates the change of the orientation of the dye when it is unfolded.

Future Work

Since we prove that TPA cross section is a method to monitor the local electric field in proteins, we are planning to apply this method to study the effect of binding WLN5-6 to copper on the local electric field comparing to the mutant that cause disease (WLN5-6Y532H).

References

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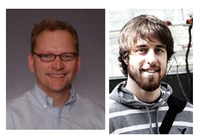

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