Multiphoton Fluorescence Spectroscopy to Monitor the Unfolding Of The Green Fluorescent Protein

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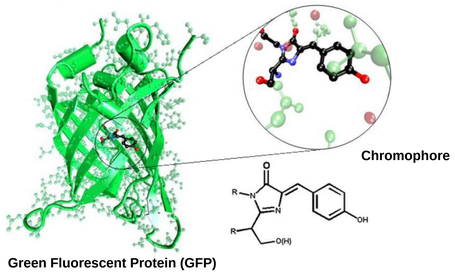
Abstract

Green fluorescent protein (GFP), from *Aequorea coerulescens*, has been expressed as a His-tag fusion protein in *E. coli* cells and purified using metal chelate affinity and gel filtration chromatography. The protein fluoresces at 510 nm due to its natural chromophore that is spontaneously formed after properly folding and remains stable and intact after protein unfolding. The chromophore is buried in a beta barrel and has a permanent dipole moment in both the ground state and the excited state. The difference between permanent dipole moments $\Delta\mu_{ge}$ is highly proportional to the electrostatic environment around the chromophore. Since $\Delta\mu_{ge}$ is related to the two photon absorption (TPA) cross section, δ, and the TPA cross section is related to the electric field, the change of electric field through unfolding of the protein was monitored by the measurement of the two photon absorption cross-section.
The fact that the TPA cross section is sensitive to the electric field makes it a powerful tool to study metal binding, protein-protein interactions, and protein misfolding or aggregation.

Introduction

Green Fluorescent Protein (GFP)

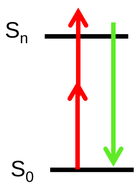
GFP possesses a stable and highly-defined cylindrical polypeptide structure, called a beta barrel, with a mass of 26.9 kDa (238 amino acids). The principle chromophore in GFP is a tripeptide consisting of the residues serine, tyrosine, and glycine at positions 65-67 in the sequence. The chromophore forms spontaneously through a self-catalyzed intramolecular rearrangement of the tripeptide sequence to produce the fluorescent species. It absorbs blue light (480 nm) and fluoresces green light (510 nm).



Two-photon absorption

Two-photon absorption is a process where two photons are absorbed simultaneously, exciting a molecule to a higher state.

Two-photon absorption cross-section, δ, (measured in Goeppert-Mayer units, 1 GM = 10^{-50} cm^4 s) characterizes the probability of the simultaneous absorption of two photons whose energies add up to match the molecular transition energy.



Objectives

- Monitor the unfolding of GFP by multiphoton fluorescence spectroscopy
- Measure the change in the two-photon absorbance (TPA) cross-sections though unfolding
- Demonstrate that the TPA cross section is an effective tool to monitor the electric field of the proteins as TPA cross section (δ) is related to the dipole moment of the chromophore, while the dipole moment is related to the local electric field of the protein.

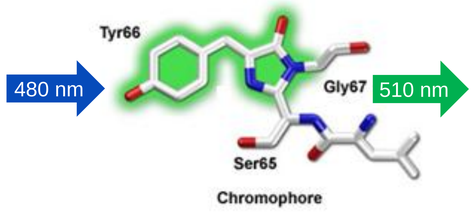
Methods

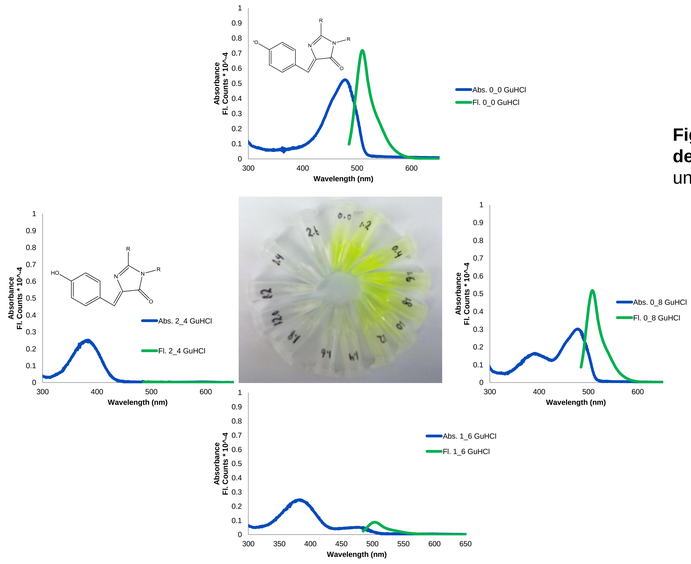
Unfolding of GFP

Guanidine Hydrochloride (GuHCl) has been used to unfold GFP. Samples of 30 μM of GFP were prepared in a GuHCl solution with a concentration range from 0 to 3.0 M. The samples were incubated at 4˚C for 2 days prior to the fluorescence measurement.

Excitation and Fluorescence Emission of GFP

Denatured GFP is not fluorescent and its absorbtion spectrum is significantly different from native GFP, which implies that noncovalent interactions of the chromophore with its local environment have a great influence on the spectral characteristics and that fluorescence is mediated by amino acids close to the chromophore in the tertiary structure of GFP.





Results

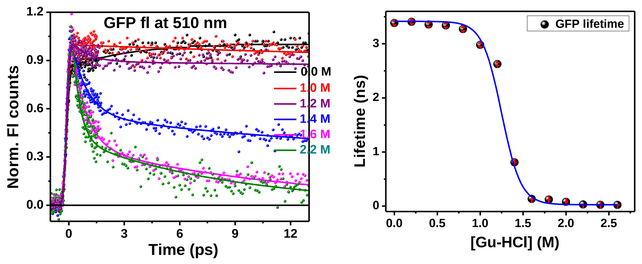


Figure 1: Fluorescence decay traces as a function of increasing Guanidine hydrochloride. Also, shown is the average lifetime with increasing Gu-HCl following the unfolding transition

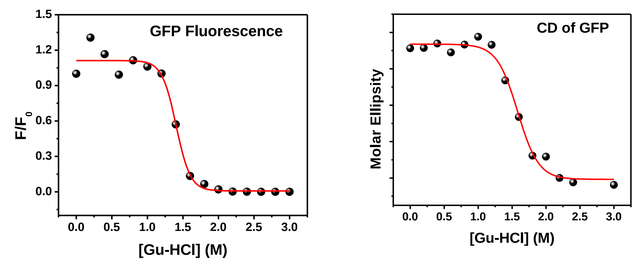


Figure 2. Fluorescence at 510 nm of GFP as a function of unfolding and the CD at 218 nm as a function of unfolding transition

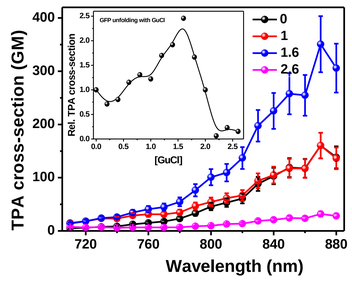


Figure 3:Change of two photon absorption cross-sections with denaturant . TPA cross-sections values increased until the protein totally unfolds, then it decreases.

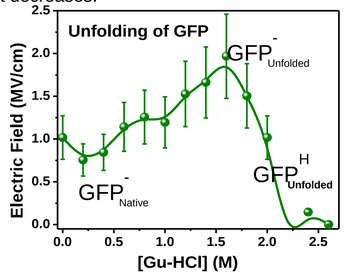


Figure 4:Change of two photon absorption cross-sections with denaturant . TPA cross-sections values increased until the protein totally unfolds, then it decreases.

Discussion

A model for the Denaturation of GFP

$$GFP^-_{NATIVE} \longrightarrow GFP^-_{UNFOLDED} \longrightarrow GFP^H_{UNFOLDED}$$

The native GFP, GFP^-_{Native}, has small but fast structural modification that increase the local electric field.
The modified GFP, $GFP^-_{Unfolded}$ has the maximum value of the local electric field around the anionic form of the chromophore.
The non-fluorescent GFP, $GFP^H_{Unfolded}$, has the lowest electric field which is actually like a chromophore in water.

Discussion

GFP has an absorbance peak at 480 nm and emission at 510 nm. while unfolding the absorbance intensity at 480 nm decrease and another peak with lower intensity appears at 395 nm. This is because the chromophore initially was in its anionic but while unfolding it became neutral. Since the anionic form is the form that able to emit light the protein lost its fluorescence when unfold at 1.6 M GuHCl. At that concentration the fluorescence decay is fast .

As the protein unfolds the electrostatic environment around the chromophore changes and so the dipole moment (Δμ) will change. Because dipole moment is related to TPA cross-sections (δ) and the later related to local electric field we were able to calculate the local electric field (E) through the following equations

$$\delta_{TPA} \propto \Delta\mu_{ge}^2$$

$$\Delta\mu_{ge} = \Delta\mu_{ge}^0 + 0.5 \times \Delta\alpha \times \boldsymbol{E}$$

$$\boldsymbol{E} = 2 \times \frac{\Delta\mu_{ge}^0}{\Delta\alpha} \times \left(\sqrt{\frac{\delta_{protein}}{\delta_0}} - 1\right)$$

Where Δμ for GFP is 6.8 ± 0.3 D and Δα = 21 ± 7 Å

Conclusions

1-The GFP from Aequorea coerulescens has been cloned and expressed in e-coli cells. The purified protein completely unfolds at 1.6 M of Guanidine Hydrochloride.
2-The unfolding of GFP was monitored by the absorbance and the fluorescence of its chromophore. Absorbance peak at 480 nm quenched and a peak at 395 nm appear but with lower extinction coefficient. The fluorescence emission at 510 nm (due to the anionic form of the chromophore) quenches while unfolding.
3-The local electric field was monitored through TPA cross section analysis. It increased by more than a factor of 2 in 1.6 M GuHCl, due the electrostatic environment around the anionic chromophore. The electric field decreases from 1.6-2.6 M GuHCl due to an unfolded protonated chromophore.
4. The local electric fields of 1 MV/cm were determined for GFP in the folded conformation and it increases to 2 MV/cm before it unfolds to give 0 electric field

References

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Acknowledgements
Mr. Jameel Hassan assisted us in upconversion measurement
NSF CAREER Award to DLH
WMU-FRAACA for support and funding