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**INSIGHTS TO CHROMOPHORE INTERACTIONS IN GREEN
FLUORESCENT PROTEIN AND ITS VARIANTS**

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ABSTRACT: Green fluorescent protein (GFP) has wide bio-imaging applications. Chromophore and its surrounding interacting amino acids play an important role in excitation and emission spectra. Amino acid structural architecture and its assembly in fluorescent protein tends to change the fluorescence of chromophore. In this study three variants of GFP derived from *Aequorea victoria* has been compared for residual interaction pattern which may lead to alteration in fluorescent properties. The intermolecular contact and its change has been computed for three variants such as GFPuv, mKalamal and ratiometric pHluorin. Each of these variants are specially tailored for enhanced fluorescent properties based on structural mutations. Important molecular interacting residue which governs the altered spectral characteristics has been studied through molecular dynamics simulations.

KEYWORDS: Green fluorescent proteins, molecular dynamics, molecular interactions. spectral perturbations.

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1.INTRODUCTION

Green Fluorescent proteins (GFP) are proteins exhibiting fluorescence properties[1]. GFP has wide application in bio-imaging and are used for tracking protein expressions in vivo[2–4]. There are wide variety of GFP variants which are specifically engineered to exhibit enhanced fluorescent properties[4–7]. Fluorescent parameter such as excitation, emission, quantum yield, extinction coefficient, brightness, photo-maturation, photo-bleaching and photostability are important parameters widely used to tailor or engineer a new variant[8–12]. Commercially there are many

fluorescent protein variants which are be classified based on the above parameters. GFP has a non-standard fluorescent moiety called chromophore which is responsible for molecular fluorescence. Chromophore and its surrounding amino acids play a critical role in fluorescence spectral emissions[13-15]. Chromophore is formed by three consecutive tripeptides “XZG” where X stands for any amino acid, Z stands for any aromatic acids and G stands for glycine through auto cyclization[16]. The intermolecular interactions between the chromophore residues tends to alter the spectral emissions[17–21]. Here we have taken three similar variants having SYG chromophore such as GFPuv[22], mKalamal[23] and ratiometric pHluorin [24] derived from Aqueous victoria. GFPuv is also called cyclic 3[25] variant which has maximum fluorescence when irradiated in UV region [22]. Similarly, mKalamal has fluorescence emission in blue region and ratiometric pHluorin variant is a variant which is sensitive to pH condition and to its environment. Here all the three variants have similar SYG chromophore and has different spectral characteristic. Hence to probe the spectral difference molecular dynamics based investigations was carried to identify the molecular interactions which can contribute to spectral perturbations.

2. MATERIALS AND METHODS

Material and Methods

PDB ID: 1GFL [26], 4ORN [27] and 5J3N [28] which corresponds to GFPuv, mKalamal and Ratiometric pHluorin respectively. These proteins were structurally optimized using protein preparation wizard of Schrodinger. Hydrogen atoms were fixed along with bond order by standard sampling using ProtAssign module [29]. Protein minimization was done using Impref whereby all the rotatable bonds were minimized with the removal of torsional potential. The minimization is carried until RMSD cut-off of 0.30 Å for non-hydrogen atoms is reached. Altogether the protonation state and hydrogen bonds are re-oriented and fixed with low clash penalty score. The chromophore geometry was subjected to initial ionization and tautomeric state. All the protein and chromophore were parameterized with OPLS-3 force field before subjected to molecular simulation protocol using Desmond. Periodic boundary system containing protein and solvent was constructed as a 10 Å³ orthorhombic box from the center of mass of protein. TIP3P water solvation system was used with charged ions placed isotopically to neutralize the Ewald charge summation of the solvated protein entity. The system was minimized with maximum iterations of 5000 steps with a gradient convergence threshold of 1.0 kcal mol⁻¹ Å⁻¹.

Once the system is minimized, it is subjected to Newtonian dynamics to evaluate energy. 2ps steps were integrated to record the simulation. Six stage relaxation process was carried out before performing molecular dynamics simulation. Initially first state solute restrained Brownian dynamics of the ensemble was carried out by keeping force constant of $50 \text{ kcal mol}^{-1} \text{ \AA}^{-1}$ using NVT condition. In the second stage, using a Berendsen thermostat [NVT (*canonical*)], the ensemble was allowed to relax at 10 K temperature with velocity resampling of every 1ps applied to the non-hydrogen solute sample. Subsequently the resulting NVT resulted ensemble was relaxed with NPT condition with Berendsen barostat (at 1 atm pressure). Restrained equilibration of 12 ps was done on solute atoms followed by system equilibration without restraint for 100 ps. Then the ensemble was subjected to 20 ns Molecular dynamics run. All the simulation was carried using Desmond [30].

Figure 1: SYG chromophore architecture of GFP variant used of molecular dynamics simulations

3. RESULTS AND DISCUSSION

The spectral characteristics of the three GFP variants namely GFPuv, mKalamal and ratiometric pHluorin is shown Table 1. All the above GFP variant are derived from Aequorea victoria having SYG (Figure 1) chromophore. Hence one should expect same excitation and emission spectrum because of the same conserved fluorophore/chromophore. From the observation it is evident that its interacting partners plays a vital role in altering the chromophore topology. To understand the mutations, GFPuv has three mutations namely F99S, M153T and V163A in comparison to wild GFP. The excitation and emission spectra of the GFPuv is in green region and has intense brightness. The sequence conservation of mKalamal and pHluorin and its similarity against GFPuv is as shown in Figure 2.

Table 1: Spectral characteristics of three fluorescent protein studied

Name	Chromophore variant	λ_{ex} (nm)	λ_{em} (nm)	Stoke shift	Extinction coeff. (M ⁻¹ cm ⁻¹)	QY	Brightness
GFPuv	SYG	488	507	19	56000	0.6	33.6
mKalama1	SYG	385	456	71	36000	0.45	16.2
ratiometric pHluorin	SYG	395	475	80	36,000	0.6	21.6

Figure 2. Multisequence analysis of three GFP variants along with their PDB id's. The variants to the corresponding Id's are 1FGL(GFPuv), 4ORN(mKalama1) and 5J3N (pHluorin). The dots show the amino acids which are conserved.

GFP variant 'mKalama1' is mildly acidic blue fluorescent protein, which has 26 mutations in comparison with GFPuv. Similarly, 'pHluorin' is a variant which is sensitive to pH. In comparison to GFPuv, pHluorin has 12 mutations. Molecular dynamic simulation of the proteins can aid in understanding the intermolecular interactions and can track referential amino acid movements. The RMSD profiles of both the proteins and SYG chromophore for all the variants are almost stable. RMSD values of C α for proteins and carbon atoms for chromophore are plotted as shown in Figure 3 for all the variants. The RMSD evolution plot of protein indicates the system is well equilibrated and its residual fluctuations are around the thermal average with limited conformations.

Figure 3: RMSD analysis to understand the conformation displacement of GFP proteins and its SYG chromophores

Figure 4: RMSF plot of GFP proteins to characterize the local changes in protein structures

The RMSF for C α (Figure 4) in the studied variant has limited fluctuations except for the N and C terminal loop regions. Since GFP is composed of beta barrel structure it tends to rigid and stable having very little local fluctuation in the structure.

Figure 5: Chromophore interaction histogram plot with surrounding interacting residue of GFP variants

Figure 6: Torsional profile of SYG chromophore captured during 20 ns simulations. The colour represents the rotational bonds

The inter molecular interactions between SYG chromophore and surrounding residual contact are plotted as histogram (Figure 5). Some of the main contacts of GFPuv preserved during simulations are VAL 67, VAL 74, GLN 100, ARG 102, SER 211 and GLU228. Clearly VAL67,74 has hydrophobic interactions with chromophore and restricts water movement in chromophore cavity which can create the intense brightness accounted in GFPuv. In mKAlama1 main interactions upheld

are VAL 67, VAL 74, GLN 75, TYR 98, GLN 100, ARG 102, ASN 152, VAL 209 and GLU 228 (numbering as per GFPuv). In pHluorin the main intermolecular contacts upheld are VAL 67, VAL 74, GLN 100, ARG 102, TYR 151, ASN 152, HIS 154, VAL 156, PHE 171, THR 209, SER 211, LEU 226, GLU 228. We can see some of the interactions are conserved such as VAL 67, VAL 74, GLN 100, ARG 102 and GLN 228 in all the three variants. The torsional profile of the SYG chromophore (Figure 6) shows the bond torsions captured during 20ns molecular dynamics simulations. The torsional profile shows the probable density of rotatable bond torsions in SYG chromophore. All the three variants have similar bond torsions except for the mKalmal variant having slight variations. From mKalmal's torsional plot we could see the scattered population of bond angle in hydroxyl (OH) position and side chain population of chromophore altered in comparison to other GFP taken. Hence variation in molecular topology, induced by interacting amino acid can alter the ground state geometry of the chromophore with spectral perturbations.

4. CONCLUSION

GFP variants can be classified based on fluorescent spectral parameters. Chromophore composed of same cyclic sequence still can possess different spectral properties. Thus the π conjugated system can be affected by its interacting partner's environment altering the excitation and emission spectrum. It is evident that the interactions tend to alter the topology parameters which may influence spectral characteristics like bond, angle and torsional profiles. Here we have identified similar variant having same SYG chromophore derived from *Aequorea victoria* have altered chromophore interactions which induces spectral perturbations due to alter geometries. This study helps identifying the key amino acid interactions involved with SYG chromophore in GFPuv, mKalmal and ratiometric pHluorin. From the observation molecular topology of the chromophore plays a key role in fluorescence and is altered by molecular interactions which is a dominant factor in designing a new fluorescent protein.

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CONFLICT OF INTEREST

We do not have any conflict of interest for the published data

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