

One-Photon and Two-Photon Excitation of Fluorescent Proteins

R. Nifosì and V. Tozzini

Abstract Fluorescent proteins (FPs) offer a wide palette of colors for imaging applications. One purpose of this chapter is to review the variety of FP spectral properties, with a focus on their structural basis. Fluorescence in FPs originates from the autocatalytically formed chromophore. Several studies exist on synthetic chromophore analogs in gas phase and in solution. Together with the X-ray structures of many FPs, these studies help to understand how excitation and emission energies are tuned by chromophore structure, protonation state, and interactions with the surrounding environment, either solvent molecules or amino acids residues. The increasing use of FPs in two-photon microscopy also prompted detailed investigations of their two-photon excitation properties. The comparison with one-photon excitation reveals nontrivial features, which are relevant both for their implications in understanding multiphoton properties of fluorophores and for application purposes.

Keywords Fluorescent proteins · Chromophore structures · Computational studies · Isolated chromophores · Multiphoton spectroscopy · Structure-property relationship · Spectral tuning

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1 Introduction

By the term fluorescent proteins (FPs), it is customary to indicate all fluorescent homologues of the original *Aequorea victoria* green fluorescent protein (GFP or *avGFP*). An accessory protein of the bioluminescence system of jellyfish *A. victoria*, *avGFP* was discovered as early as the 1960s [1]. Thirty years later, with the cloning of the gene [2] and the demonstration that its expression in other organisms generates fluorescence [3, 4], interest in GFP began to rise dramatically. Since then, it has triggered a revolution in bioimaging by fluorescence microscopy [5]. Soon, many other fluorescent and nonfluorescent GFP homologues were discovered in a variety of sea organisms, such as reef corals and sea anemones [6]. Further discoveries and mutagenesis engineering have produced a profusion of FPs with optical properties spanning most of the visible spectrum and beyond.

Fluorescence in FPs stems from the presence of a chromophore moiety, formed within the conserved β -barrel fold via a mechanism entailing autocatalytic backbone cyclization at an internal tripeptide sequence. Distinct postcyclization processing leads to different chromophore structures. The multiplicity of optical properties of FPs is surely one of the factors that contribute to their usefulness. It primarily arises from the different chemical structures of the chromophore. A finer tuning originates from the noncovalent interactions of the chromophores with the surrounding molecular matrix.

This chapter focuses on the mechanisms behind this spectral tuning, covering both experimental and theoretical/computational work. The reader is first presented with the more familiar case of *avGFP*. The chromophore structures of other FPs are described in Sect. 3. The following section surveys various studies on synthetic analogs of chromophores of FPs in gas phase and in solution. Section 5 provides a detailed description of spectral modifications due to interactions between chromophore and surrounding protein matrix. Finally, the last section covers two-photon properties of FPs. Several other reviews on FP optical properties are available, some treating more exhaustively the variety of GFP-like fluoro and chromoproteins, and other more focused on application purposes. For recent surveys, see [7–9].

2 *avGFP*: Structure and Optical Properties

The first to be cloned [2] and functionally expressed in other organisms [3, 4], *avGFP* has actually been replaced in most applications by its mutants and homologues. Nonetheless, being one of the best characterized in terms of optical and photophysical properties, it is a suitable starting point to introduce the concepts recurring in this chapter.

2.1 Structure and Chromophore Formation

The 238 amino acid (27 kDa) long sequence of *av*GFP folds in a compact cylindrical form called β -barrel, its lateral wall being an 11-stranded β -sheet (Fig. 1) [10, 11]. Several X-ray structural studies support the notion that also all other FPs share the β -barrel fold. They can differ in quaternary structure, though most natural FPs are tightly bound tetramers and some are dimers, a feature that initially hampered their applications. However, mutagenesis studies were able in most cases to produce viable monomeric variants of the parent proteins.

The β -barrel is capped on both ends by short α -helical sections and traversed by an α -helix segment. This segment contains the chromophore, a 4-(*p*-hydroxybenzylidene) imidazolinone, originating from the posttranslational cyclization of three consecutive amino acids at position 65–67, such as Ser, Tyr, and Gly. As demonstrated by the fact that expression of *av*GFP gene in other organisms leads to fluorescence, the posttranslational synthesis of the chromophore does not require any jellyfish-specific enzyme [3, 4]. It requires, however, exogenous oxygen, in the absence of which GFP does not develop fluorescence [4, 12].

Chromophore formation (Fig. 2) proceeds within the native fold (i.e., no chromophore is formed under denaturing conditions). It first entails backbone cyclization at the Ser65-Tyr66-Gly67 tripeptide through nucleophilic attack of the amide group of Gly67 onto the carbonyl group of Ser65, promoted by Arg96, a conserved residue in all natural FPs (see [13] and references therein). Molecular oxygen is required for the subsequent oxidation reaction. One water molecule is also abstracted from the structure, with most experimental studies supporting the cyclization–oxidation–dehydration sequence of events [13, 14].

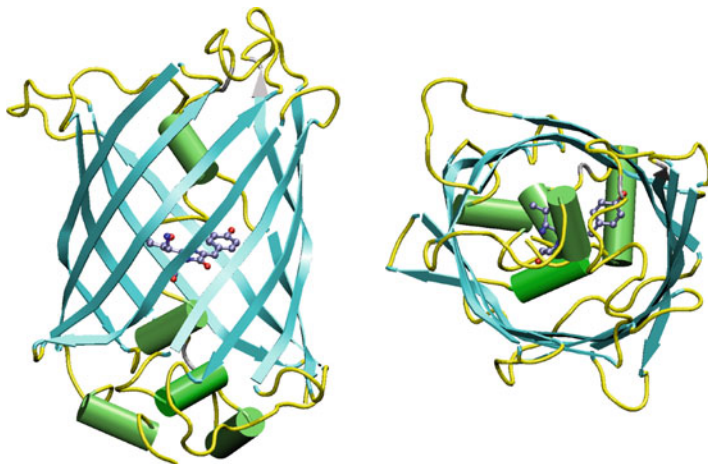


Fig. 1 Tertiary structure of *av*GFP. The usual cartoon representation is used, where α -helices are cylinders and β -sheets arrows. The chromophore is shown in a ball-and-sticks representation with the standard coloration for atom elements (i.e., gray for carbon, blue for nitrogen, and red for oxygen)

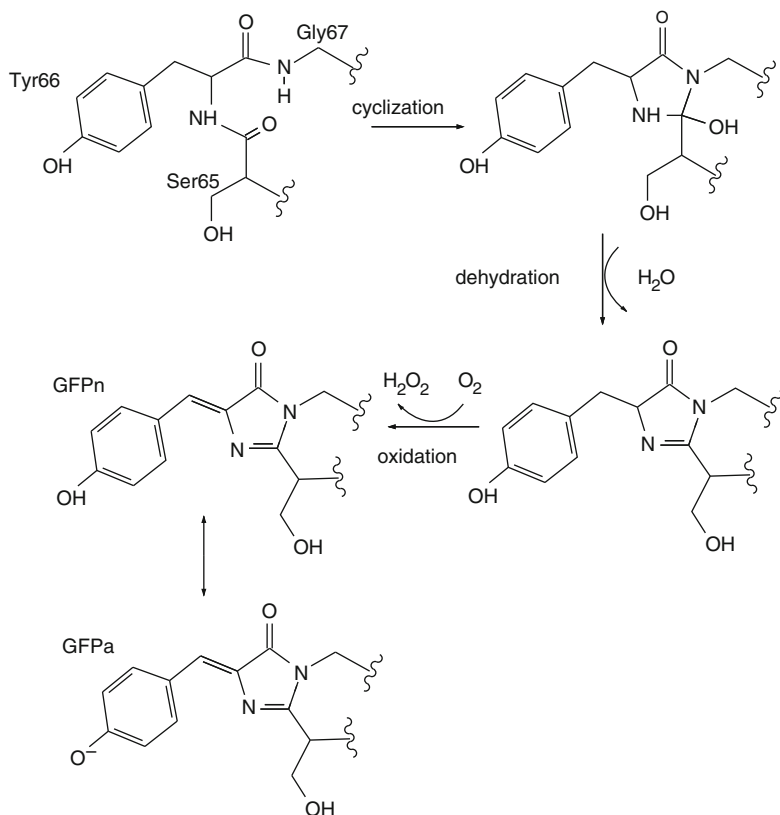


Fig. 2 Proposed mechanisms of biosynthesis of the GFP chromophore. In the last step of the reaction, the two relevant protonation states are shown

The mature chromophore consists of two rings, the phenol ring, coming from the side chain of Tyr66, and the five-member heterocyclic ring (imidazolinone), resulting from the cyclization of the backbone. The imidazolinone core is a common feature of all known FP chromophores. The alternating single and double bonds in the bridge region extend the electron delocalization from the phenolate to the carbonyl of the imidazolinone. Efficient visible-light absorption is ultimately determined by this π -conjugated system, i.e., the single–double bond alternation with connected atomic p-orbitals.

2.2 Absorption, Excitation, and Emission

The room temperature absorption, excitation, and emission spectra of *av*GFP are shown in Fig. 3. Apart from the 278-nm band, which is common to proteins containing aromatic amino acids, *av*GFP exhibits a major absorption band at

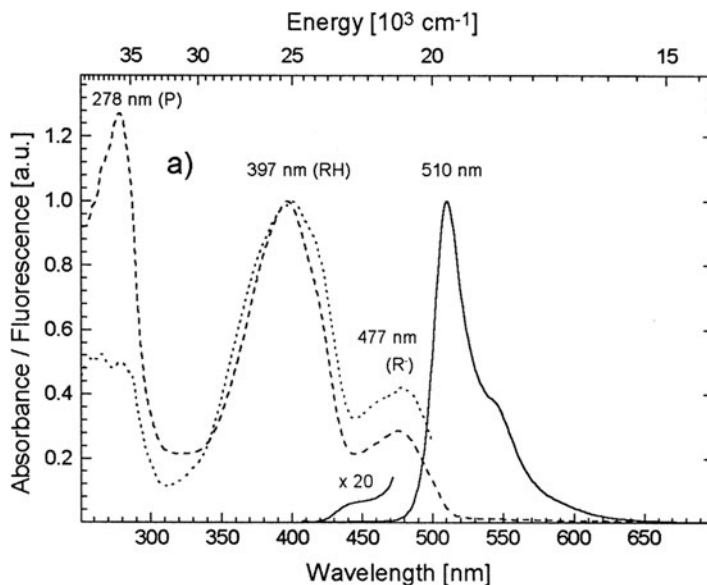


Fig. 3 Room temperature absorption (*dashed line*), fluorescence excitation (*dotted line*) and emission spectra (*solid line*) of avGFP, at room temperature and pH 8.0 (adapted from Kummer et al. [15])

397 nm and a minor band at 477 nm, due to absorption of the chromophore. Their relative height depends on proton concentration: increasing pH above 11, the minor lower-energy band increases at the expense of the higher-energy band. Vice versa, at pH below 4 the minor energy band is completely depleted [16]. The relative population in Fig. 3 is rather constant between pH 6 and 11. This behavior arises from the ground-state equilibrium between two states of the chromophore, differing in protonation of the phenolic group from Tyr66. The phenolic oxygen of the chromophore is protonated in the state absorbing at 397 nm (RH in the following) and deprotonated in that absorbing at 477 nm (state R⁻). It is commonly accepted that the two other possible protonation sites in the chromophore, i.e., the nitrogen and the carbonyl oxygen of the imidazolinone, are deprotonated in both the absorbing states, thereby giving an overall neutral chromophore in state RH (GFPn in Fig. 2) and anionic in state R⁻ (GFPa) [17].

Excitation of state RH leads to a fluorescence spectrum peaking at 510 nm (Fig. 3), with a rather high quantum yield of 0.79. State R⁻ yields a similar fluorescence spectrum, slightly blueshifted and peaking at 503 nm (not shown). In both cases, fluorescence comes from emission of the singlet excited state of the anionic chromophore. Excitation of the neutral chromophore results in ultrafast (4 ps) excited state proton transfer (ESPT) and subsequent emission of the anionic form [18]. The ESPT acceptor has been identified in (deprotonated) Glu222 [18, 19].

Although the anionic chromophore is the emitting species in both states, the configuration of the surrounding residues is different and the decay time of the

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