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Ultrafast electronic and vibrational dynamics of stabilized A state mutants of the green fluorescent protein (GFP): Snipping the proton wire

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Abstract

Two blue absorbing and emitting mutants (S65G/T203V/E222Q and S65T at pH 5.5) of the green fluorescent protein (GFP) have been investigated through ultrafast time resolved infra-red (TRIR) and fluorescence spectroscopy. In these mutants, in which the excited state proton transfer reaction observed in wild-type GFP has been blocked, the photophysics are dominated by the neutral A state. It was found that the A^* excited state lifetime is short, indicating that it is relatively less stabilised in the protein matrix than the anionic form. However, the lifetime of the A state can be increased through modifications to the protein structure. The TRIR spectra show that a large shifts in protein vibrational modes on excitation of the A state occurs in both these GFP mutants. This is ascribed to a change in H-bonding interactions between the protein matrix and the excited state.

Keywords: Ultrafast; Transient infra-red; Green fluorescent protein; Proton transfer; Fluorescence; Protein dynamics; Fluorescent protein

1. Introduction

The green fluorescent protein (GFP) family is widely used in cellular and molecular imaging, owing to the presence of an intrinsically fluorescent chromophore formed autocatalytically from residues S65, Y66 and G67 [1]. The cloning and in vivo expression of GFP has made it one of the most powerful tools in cell biology [2]. In the wild-type GFP (wtGFP) two bands are observed in the electronic absorption spectrum which have been assigned to neutral (A form) and deprotonated (B form) states of the chromophore [3–5]. Excitation into either the dominant A form (λ_{max} 395 nm) or the anionic B form (λ_{max} 475 nm) results in very efficient green (λ_{em} 508 and 504 nm, respectively) fluorescence. The 508 nm emission following excitation of A arises from an anionic species formed in the excited state (I^*) by deprotonation of the chromophore and concomitant protonation of the E222 carboxylate group via a proton relay chain (Fig. 1), a rare example of excited state proton transfer (ESPT) in living systems [3,6–8]. The directly excited B^* and indirectly excited I^* states are thought to differ in that the former has a chromophore environment optimized for the anionic form while the latter retains the unrelaxed environment of the neutral form [6].

Numerous mutants of GFP have been produced, many of which are designed to modify the relative population of the A and B forms. For example, the yellow fluorescent

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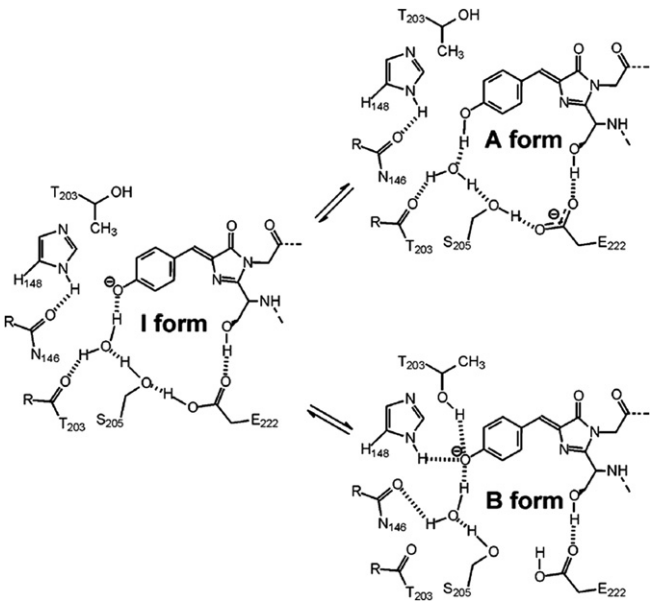

Fig. 1. Model for the interconversion of the A and B forms of the chromophore via the I form (adapted from Brejc et al. [3]). Structures were based on X-ray data from wt (A) or anionic mutants (B), whereas I is a proposed intermediate.

proteins (often containing the S65G mutation) yield mutants in which the B form dominates [9], while the commonly utilised S65T mutation results in fluorescent proteins that are often highly pH sensitive, allowing for either A or B forms to be selected, and conferring on GFP the ability to act as a pH sensor. Remington, Boxer and their co-workers have characterised the structure and photodynamics of a series of such pH sensitive mutants, all containing the S65T mutation [10–12]. They found that at low pH the A^* emission was favoured due to a suppression of the proton transfer reaction arising both from disruption of the proton relay network and from protonation of the E222 acceptor. The excited state lifetime for A^* was generally short, but the A^* fluorescence yield increased with decreasing temperature. At higher pH the B state was formed and some A^* to I^* ESPT was observed [12]. Other mutants also retain the ability of wt GFP to undergo ESPT, but at varying rates (T203V [13]) while yet others undergo ESPT but apparently by a different route to wtGFP (S65T/H148D [14–16]).

In this work, we investigate through both time resolved fluorescence (TRF) and time resolved infra-red (TRIR) spectroscopy the photodynamics of S65G/T203V/E222Q GFP (blGFP), a mutant in which the chromophore is trapped in the neutral, blue (b1) emitting, A form. Although not of major interest for imaging applications, due to its blue shifted absorption and relatively weak emission, this mutant affords the opportunity of investigating interactions between the A state and the protein in the absence of proton transfer. These measurements are extended to S65T GFP at low pH, where both A and B forms exist but the ESPT reaction has been blocked, permitting direct excitation and study of the isolated A form. These studies yield a more detailed view on the factors determining the photophysics of GFP, in particular the mechanism which enables the chromophore to emit strongly in the protein even though it is non fluorescent in the denatured form and in aqueous solution [17]. The mechanism of the radiationless decay of the chromophore in solution has been considered in detail elsewhere, both experimentally [18–24] and in theoretical treatments [25–30]. The results described below will provide new data against which to test theoretical calculations of excited state properties in the protein, examples of which are beginning to appear [25,31].

The remainder of the paper is organised as follows. In the next section mutagenesis and sample preparation will be described, along with the experimental methods used and some additional experimental details. In the third section the TRF and TRIR experimental results for these mutants will be presented and discussed. Conclusions are presented in the final section.

2. Experimental

Plasmid carrying blGFP (S65G/T203V/E222Q GFP) was generated through three rounds of mutagenesis (QuikChange mutagenesis kit, Stratagene) using the following primers and the pRSETb plasmid encoding His-tagged wtGFP.

Thr203Val:

Forward: 5′ CCATTACCTGTCCGTACAATCTGCCCTTTCG 3′
Reverse: 5′ CGAAAGGGCAGATTGTACGGACAGGTAATGG 3′

Glu222Gln:

Forward: 5′ CCACATGGTCCTTCTTCAGTTTGTAACAGCTGC 3′
Reverse: 5′ GCAGCTGTTACAAACTGAAGAAGGACCATGTGG 3′

Ser65Gly:

Forward: 5′ CTTGTCACTACTTTCGGTTATGGTGTTCAATGC 3′
Reverse: 5′ GCATTGAACACCATAACCGAAAGTAGTGACAAG 3′

Both the plasmid for wtGFP and for S65T GFP were obtained from Prof. Rebekka Wachter (Arizona State University). Proteins were expressed in BL21-DE3/pLysS cells (Stratagene) and using an overnight, 25 °C incubation period after addition of 0.8 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Fisher). Following cell lysis (French press) and centrifugation (45 min at 33,000 rpm), the His-tagged protein was purified using Ni–NTA resin (Novagen). Fractions containing pure blGFP were combined and dialyzed