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Water diffusion in and out of the β-barrel of GFP and the fast maturing fluorescent protein, TurboGFP

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ABSTRACT

The chromophore of fluorescent proteins is formed by an internal cyclization of the tripeptide 65SYG67 fragment and a subsequent oxidation. The oxidation is slow – the kinetics of this step is presumably improved in fast maturing GFPs. Water molecules can aid in the chromophore formation. We have used 50 ns molecular dynamics simulations of the mature and immature forms of avGFP and TurboGFP to examine the diffusion of water molecules in-and-out of the protein β-barrel. Most crystal structures of GFPs have well-structured waters within hydrogen-bonding distance of Glu222 and Arg96. It has been proposed that they have an important role in chromophore formation. Stable waters are found in similar positions in all simulations conducted. The simulations confirm the existence of a pore that leads to the chromophore in the rapidly maturing TurboGFP; decreased water diffusion upon chromophore formation; and increased water diffusion due to the pore formation.

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1. Introduction

Green fluorescent protein (GFP) and GFP-like proteins are commonly used in scientific research [1–6]. Their utility as genetic tracer molecules, as highlighters in high resolution microscopy, and as a critical component in many modern biotechnological methods has led to an increased effort to understand their photochemistry, and to find and design new GFP-like proteins. Over 150 distinct GFP-like proteins are currently known. GFP-like fluorescent proteins (FPs) have been found in marine organisms ranging from chordates (e.g. amphioxus) to cnidarians (e.g. corals and sea pansies) [7]. Enhanced GFP is still the most commonly used fluorescent protein, despite the fact that it has some major shortcomings such as its slow attainment of fluorescence, especially at 37 °C. Second generation fluorescent proteins, such as Vivid Verde (from the *Cyphastrea microphthalma* coral) [8] and TurboGFP (from the *Pontellina plumata* copepoda) [9] overcome these problems.

In *Aequorea victoria* GFP (*av*GFP), the chromophore is formed by an autocatalytic internal cyclization of the tripeptide 65SYG67 fragment and subsequent oxidation of the intrinsically formed structure, see Fig. 1. GFP fluorescence is not observed until 90 min to 4 h after protein synthesis [10,11]. The protein folds quickly and GFP refolding from an acid-, base-, or guanidine HCl-denatured state (chromophore containing but non-fluorescent) occurs with a half-life of between 24 s [12] and 5 min [13] and the recovered fluorescence is indistinguishable from that of native GFP [14]. The folding of GFP exhibits hysteresis that is due to the decreased flexibility of the chromophore vs. its immature analog [15]. The chromophore formation and oxidation is slow [16], and it is the kinetics of this step that are presumably improved in fast maturing GFP-like proteins.

The two most probable mechanisms [17] for chromophore formation are a cyclization–oxidation–dehydration mechanism proposed by Wachter [18] and the cyclization–dehydration–oxidation mechanism proposed by Getzoff [19], see Fig. 1.

The GFP-maturation kinetics have been separated into pre-oxidation, oxidation and postoxidation events [20]. The preoxidation step, which includes folding and protein backbone condensation, and is thought to lead to intermediate **2** (Fig. 1) is the fastest. The following oxidation steps that lead to intermediate **3** (Fig. 1) are the slowest [20]. Although the final post-oxidation steps are slow and contribute to rate retardation, they are not the rate limiting steps. Numerous roles have been proposed for water molecules in the postcyclization steps, see Fig. 2.

To determine whether the pore in TurboGFP increases water diffusion in and out of the β-barrel, and whether the water molecules might be involved in the chromophore formation we have undertaken 50 ns molecular dynamics simulations of avGFP, TurboGFP and the V197L TurboGFP mutant, as well as the immature (precyclized) forms of avGFP and TurboGFP.

2. Materials and methods

Structural comparisons of the 566 GFP-like chains [23] were done using the protein–ligand database Relibase + v3.01 [24,25]. The coordinates of the *A. victoria* GFP crystal structure (1gfl) [26] and *P. plumata* TurboGFP (2g6x) [9] were obtained from the Protein

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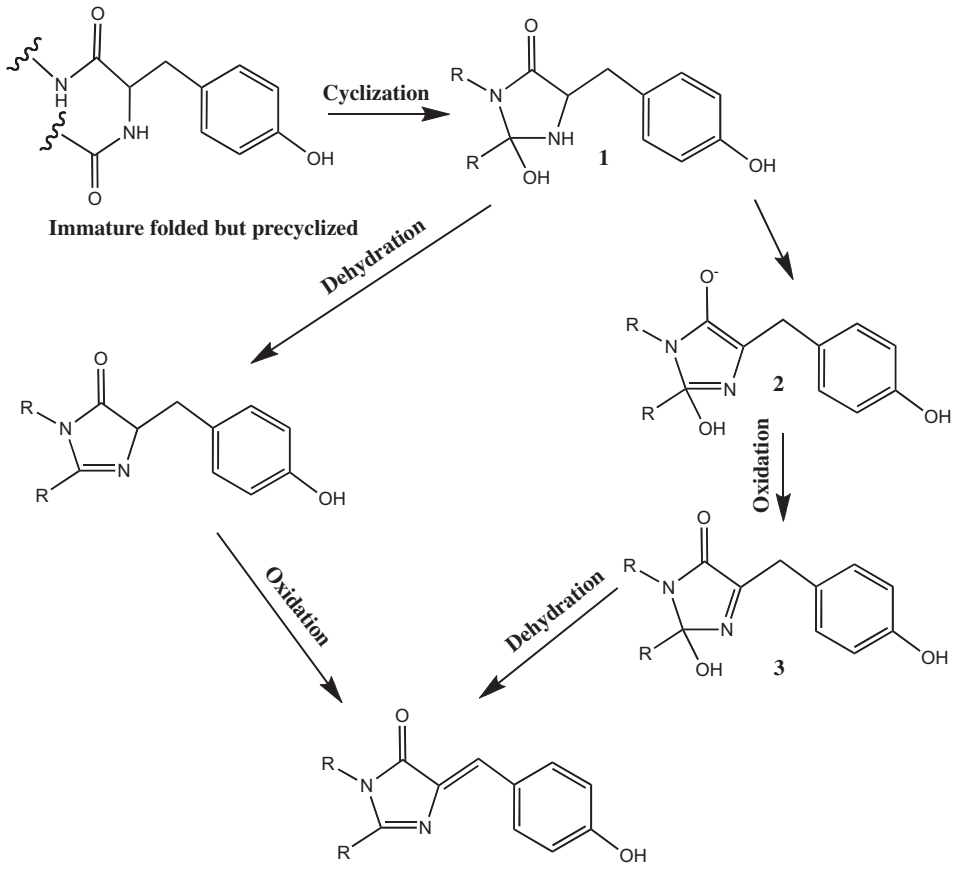

Fig. 1. The cyclization–oxidation–dehydration mechanism proposed by Wachter [18] (right) and the cyclization–dehydration–oxidation mechanism proposed by Getzoff [19] (left).

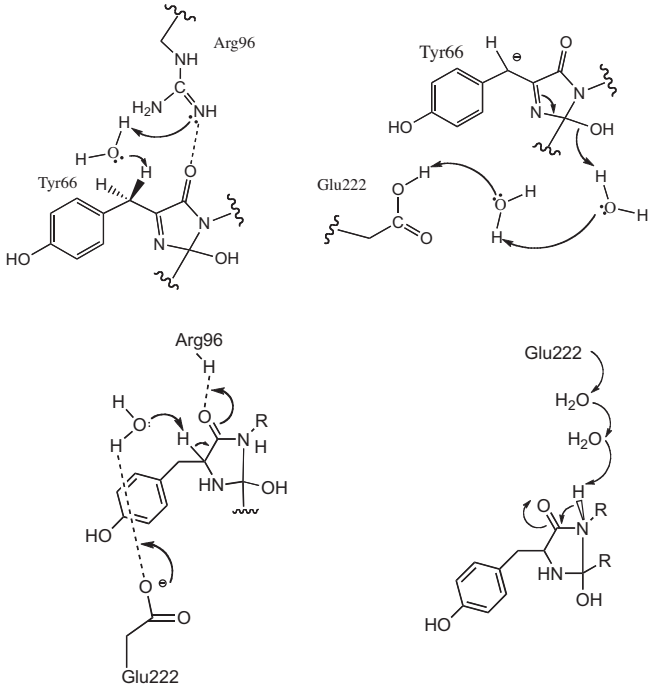

Fig. 2. Possible roles played by the structurally conserved waters in the chromophore formation [1,21]. The cyclization–oxidation–dehydration mechanism proposed by Wachter [18,22] (top and bottom left) and the cyclization–dehydration–oxidation mechanism proposed by Getzoff [19] (bottom right).

Data Bank (PDB) [27]. The protein preparation workflow and Epik v2.0109 [28] were used with hydrogen-bond optimization to add hydrogen atoms to protein and solvent atoms as required. The OPLS_2005 force field of MacroModel v9.8107 [29] was used.

The starting structure for the immature form of avGFP and TurboGFP, for which no crystal structure has been determined, were calculated by graphically mutating the *A. victoria* GFP (1gfl) [26] and *P. plumata* TurboGFP (2g6x) [9] crystal structures so that the chromophore forming tripeptide sequences were in the original precyclized form and undertaking a conformational search. Conformational searches were conducted using the combined Monte Carlo torsional variation and low mode method [30,31]. The flexible dihedral angles of all the side-chains of residues 64, 65, 66, 67 and 68 (1GFL numbering) were randomly rotated by between 0° and 180° and all solvent molecules in an 8.00 Å sphere from residues 64–68 were randomly rotated and translated by between 0° and 1.00Å in each Monte Carlo (MC) step [32]. 15,000 MC steps were taken in each search. Structures within 50 kJ/mol of the lowest energy minimum were kept, and a usage directed method [31] was used to select structures for subsequent MC steps. Structures found in the conformational search were considered unique if the least squared superimposition of equivalent non-hydrogen atoms found one or more pairs separated by 0.25 Å or more. The lowest energy structure obtained in the search was further subjected to a 5000 step large scale low mode conformational search [33,34]. TurboGFP (2g6x) was graphically mutated to get the V197L mutant and conformational searches as described above were used to find low energy conformations.

The final structures obtained from the fully minimized pdb structures and the conformational searches were used to initiate

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