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A role for molecular compression in the post-translational formation of the Green Fluorescent Protein chromophore

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ABSTRACT

Spontaneous chromophore formation is probably the key feature for the remarkable success of GFPs (Green Fluorescent Proteins) and related proteins in fluorescence microscopy. Though a quantitative analysis of the involved energetics still remains elusive, substantial progress has been made in identifying the steps of chromophore biosynthesis and the contribution of individual residues and surrounding protein matrix. The latter clearly enforces a peculiar configuration of the pre-cyclized chromophore-forming tripeptide. However, it is debated whether a mechanical compression is also at play in triggering backbone cyclization. Here, by molecular dynamics and potential of mean force calculations, we estimate the contribution of the protein scaffold in promoting the proximity of reacting atoms- and hence backbone cyclization - by a sort of compression mechanism. Comparing several mutants we highlight the role of some surrounding residues. Finally, we analyze the case of HAL (Histidine Ammonia-Lyase) active site, which undergoes an analogous cyclization reaction.

1. Introduction

One outstanding example of protein post-translational modifications is the autocatalytic formation of the chromophore in the family of Fluorescent Proteins (FPs), of which the Green Fluorescent Protein (GFP) was the first member to be discovered. Thanks to the spontaneous chromophore biosynthesis, FPs can be functionally expressed in host cells and used to create fluorescent chimeras by molecular biology techniques, allowing noninvasive fluorescent labeling of proteins in living cells [1,2].

Several studies have helped to identify the steps involved in chromophore synthesis, and to reach at least a partial agreement on their sequence [3,4]. Chromophore formation (Fig. 1, upper panel) proceeds within the native GFP structure (i.e. no chromophore is formed under denaturing conditions), and 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. Molecular oxygen is required for the subsequent oxidation reaction, leading to the mature chromophore with the characteristic delocalized electronic π-system. Oxidation is the rate limiting step with a time constant of several minutes (120 min in wild type GFP [5], 27 min in the S65T variant [6], from measurements in living cells), whereas backbone cyclization time constant is estimated to be of few minutes [7,8].

One water molecule is also abstracted from the structure, and both the (i) cyclization-dehydration-oxidation [9,3] and the (ii) cyclization-oxidation-dehydration [8,10] mechanisms have been proposed based on various experimental findings. Evidence for mechanism (i) comes from X-ray studies of chemically reduced GFPs, revealing that reduction of the mature chromophore yields an enolate moiety where the imidazolone ring remains cyclic and dehydrated, but the structure is no longer oxidized [3]. Hence, oxidation seems not to be essential for imidazolone ring stability, whereas dehydration is required for kinetically trapping the unstable cyclic structure. Support for (ii) comes instead from the kinetic trace of hydrogen peroxide (H_2O_2) preceding the evolution in fluorescence acquisition in the GFP-trix variant (F64L/F99S/M153T/V163A/A206K) [8]. X-ray structure of the Y66L mutant [11] also supports mechanism (ii), in that the tripeptide undergoes oxidation while maintaining the hydroxyl group (i.e. it is unable to dehydrate).

Within the β-barrel fold of GFP, the chromophore is positioned between two alpha-helix portions (see Fig. 2), the axes of which form an angle of about 80° with one another. The chromophore-bearing site is surrounded by several water molecules and by two residues Arg96 and Glu222, which are highly conserved among FPs. Mutation of Arg96 to Ala [9], or that of Glu222 to Gln [12], dramatically slows chromophore maturation, the latter only at acidic and physiological pH. Comprehensive targeted mutagenesis [13] points to a role for Arg96 in acidifying N67 (Fig. 1), and hence facilitating nucleophilic attack on the carbonyl at 65.

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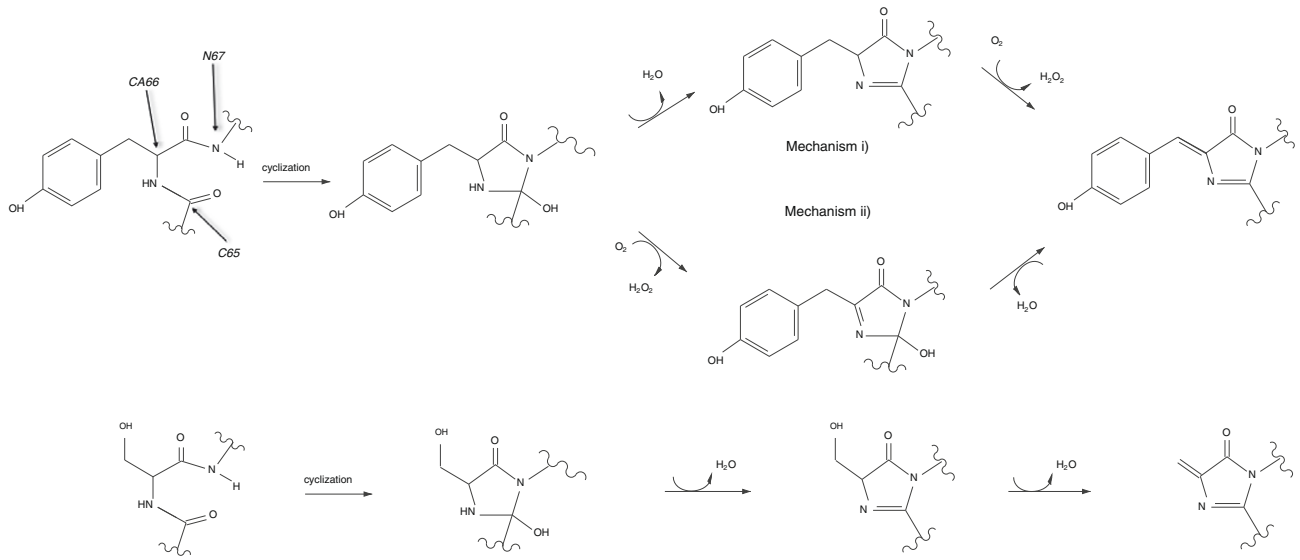

Fig. 1. Proposed mechanisms of biosynthesis of the GFP chromophore (upper panel) and of HAL MIO (lower panel).

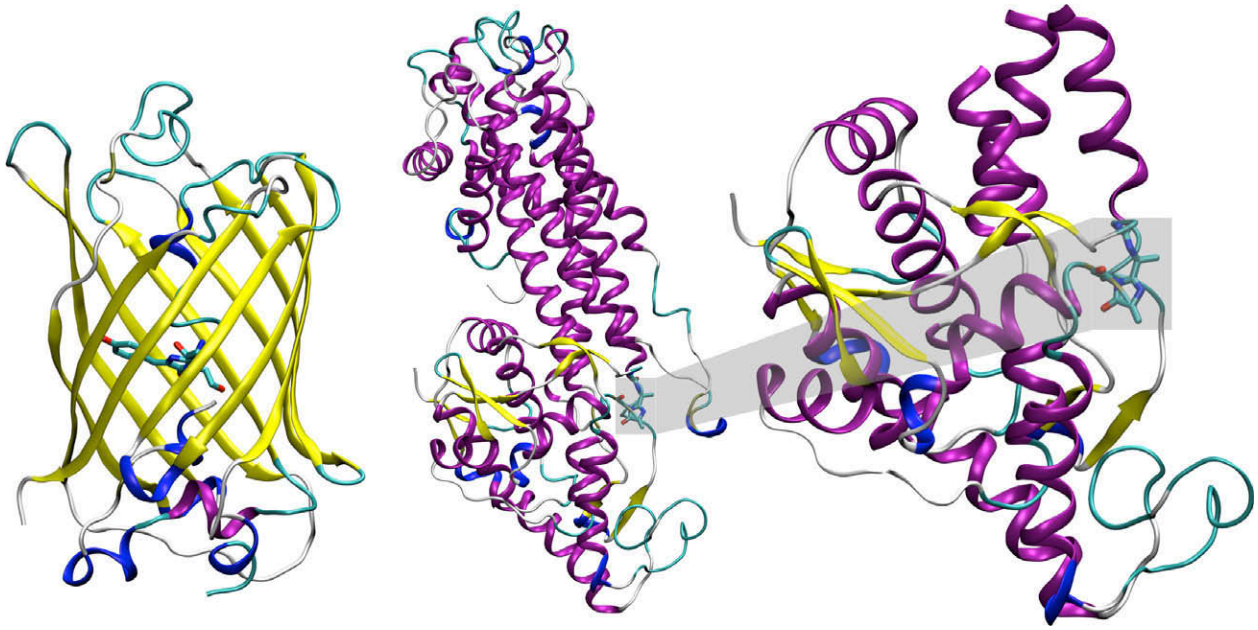

Fig. 2. Leftmost panel: fold of GFP in cartoon representation, with the chromophore explicitly shown. Center: HAL monomer. Rightmost: Portion of HAL used in the molecular dynamics simulations (see Methods section). The shaded area includes the structure of MIO.

Glu222 presumably acts as a base in this proton abstraction mechanisms, and/or it may mediate dehydrogenation by deprotonating CA66 [4,14].

Any aromatic residue (Phe, His and Trp) at position 66 preserves fluorescence [5], and several other non-aromatic residues were tried at position 66, some, such as Gly and Ser [15,16] and Leu [17], leading to different reaction products. By contrast, all mutants not including a Glycine at position 67 resulted in complete loss of fluorescence [18]. The crystal structure of a mutant containing Alanine at position 67 (S65A/Y66S/G67A) reveals a 3_{10}-helix configuration that presumably inhibits backbone cyclization by stabilizing an alternative conformation [15].

By a force field-based computational analysis, Zimmer and coworkers showed that the low-energy pre-cyclization conformations of the chromophore-forming residues are in a unique "tight-turn" conformation (the mechanical compression hypothesis), in which the distance between C65 and N67 is shorter than the sum of the Van der Waals radii of the two atoms, and the nitrogen lone pair of residue 67 is oriented for nucleophilic attack on the carbonyl of Ser65 [19,14]. They suggested that the remainder of the protein may exert a strain between the two atoms, enforcing the tight-turn conformation. The short C65 and N67 distance was confirmed by X-ray studies on the pre-cyclized M96R mutant, (Protein Data Bank (PDB) [20] code 2AWJ [13]).

The fact that the Gly-Gly-Gly tripeptide undergoes cyclization in the S65G/Y66G mutant (GGG mutant hereafter) [9] argues, to some extent, against the mechanical compression hypothesis. Indeed the permanence of cyclization upon substitution with the least bulky amino acid (i.e. Gly) would imply that no steric compression is imposed by the protein scaffold. Moreover, the GGG mutant cyclizes exclusively in aerobic conditions, indicating that cyclization is coupled to oxidation, at least in this mutant.