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Chromophore Packing Leads to Hysteresis in GFP

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Green fluorescent protein (GFP) possesses a unique folding landscape with a dual basin leading to the hysteretic folding behavior observed in experiment. While theoretical data do not have the resolution necessary to observe details of the chromophore during refolding, experimental results point to the chromophore as the cause of the observed hysteresis. With the use of NMR spectroscopy, which probes at the level of the individual residue, the hysteretic intermediate state is further characterized in the context of the loosely folded isomerized native-like state $\{N_{iso}\}$ predicted in simulation. In the present study, several residues located in the lid of GFP indicate heterogeneity of the native states. Some of these residues show chemical shifts when the native-like intermediate $\{N_{iso}\}$ responsible for GFP's hysteretic folding behavior is trapped. Observed changes in the chromophore are consistent with increased flexibility or isomerization in $\{N_{iso}\}$ as predicted in recent theoretical work. Here, we observed that multiple chromophore environments within the native state are averaged in the trapped intermediate, linking chromophore flexibility to mispacking in the trapped intermediate. The present work is experimental evidence for the proposed final "locking" mechanism in GFP folding forming an incorrectly or loosely packed barrel under intermediate (hysteretic) folding conditions.

Keywords: protein folding; energy landscape; proline isomerization; folding intermediate; fluorescent protein

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Introduction

Green fluorescent protein (GFP) is a commonly used reporter both *in vivo* and *in vitro* due to its ability to fold and autocatalytically form a visually fluorescent chromophore.[1] Chromophore formation is linked to folding[2] and is required for reporter applications. However, the time scale of GFP folding limits its usefulness for following time-related phenomena. Thus, improving the rate and efficiency of GFP protein folding could improve its use as a reporter.[3] Recent work on the folding of GFP details large barriers and hysteresis in folding[4] linked to the chromophore[5] (Fig. 1). Proline isomerization in the lid is linked to chromophore formation.[5] The question remains, how does a formed chromophore lead to the hysteresis observed in folding?

The structure of superfolder GFP (sfGFP) is a highly regular 11-stranded β-barrel with a central kinked α-helix running through the center (Fig. 2a and b).[7] The chromophore forms from an autocatalytic reaction of the backbone involving cyclization, oxidation, and dehydration reactions.[8–12] Chromophore formation follows the kinking of the helix; hence, the chromophore does not cause the kinked helix.[2] Chromophore fluorescence depends on segregation from bulk solvent as provided by the barrel[13] and requires rigidity in chromophore structure.[14] However, despite being surrounded by an 11-stranded β-barrel, the chromophore is not always sufficiently rigid for fluorescence and may isomerize in a hula-twist (HT) motion (Fig. 2c).

Chromophore isomerization in GFP was originally linked to the "blinking" observed in single-molecule FP studies.[15] This is similar to the isomerization observed via crystal structures in photoactive yellow protein, another FP structurally distant from GFP,[16,17] and to the photoisomerization observed in DsRed, a red FP structurally analogous

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Abbreviations used: FP, fluorescent protein; GFP, green fluorescent protein; sfGFP, superfolder GFP; $\{N_{iso}\}$, isomerized native state; HSQC, heteronuclear single quantum coherence; HT, hula-twist; TOCSY, total correlation spectroscopy; 2-D, two-dimensional; HDX, hydrogen/deuterium exchange; Gdn–HCl, guanidine hydrochloride.

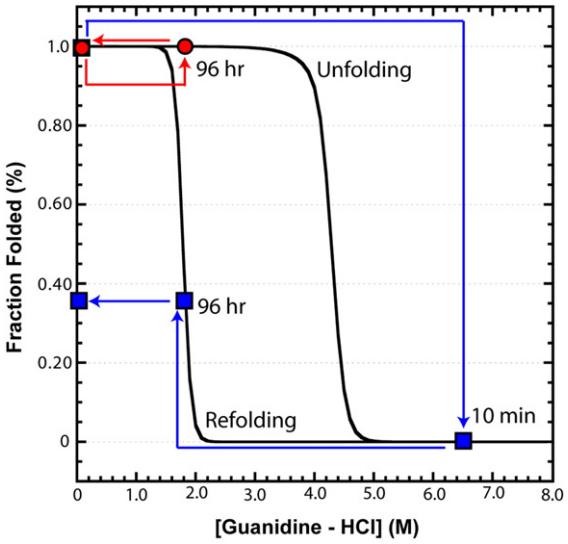

Fig. 1. The folding of sfGFP exhibits hysteresis with a long-lived trapped intermediate. The non-coincidence of the simulated folding equilibrium curves shows hysteresis. The red circles show the preparation of the native sample by equilibration at 1.8 M Gdn–HCl for 96 h. The trapped sample is initially unfolded to 6.5 M Gdn and then equilibrated to 1.8 M Gdn–HCl for 96 h. Both samples are placed under NMR conditions by 2× spin columns into 0 M Gdn–HCl.

to GFP.[18] Fluorescence quenching in yellow protein and that in DsRed are the result of interconversion during a non-adiabatic crossing.[15,19] Several molecular dynamics studies have been performed to predict the details of isomerization within the β-barrel of GFP. These simulation studies consistently observe that the *cis* form found in the crystal structure is a lower-energy form, as the *trans* form appears to lose some of the H-bonds situating it within the barrel.[20–22] Model compound studies also observe the *cis* form to be 2.5 kcal/mol more stable than the *trans* form, with a 13-kcal/mol barrier in the neutral form.[20,22] However, while both *cis* and *trans* chromophore states are fluorescent, interconversion between the two is proposed to quench fluorescence.

Chromophore isomerization has not been experimentally observed within the barrel of GFP, although changes in the chromophore flexibility have been linked with fluorescent and non-fluorescent states.[23–25] Work on sfGFP[7] proposed chromophore isomerization as a potential source of hysteresis in GFP,[5] and recent theoretical work supported that the final β-strand inserting into the barrel is the final step in folding.[26] The theoretically predicted loosely packed native-like intermediate is referred to as $\{N_{iso}\}$, an intermediate dominated by an ensemble of structures likely with non-native proline and chromophore isomerizations. Taken together, these results are consistent with the final strand insertion trapping a poorly packed barrel and chromophore misisomerization. However, until now, there have been no probe to unambiguously follow the chromophore structure or isomerization within the barrel and little experimental evidence to support this conjecture.

Probes of the GFP chromophore exist, but they typically consist of chromophore fluorescence or absorbance, neither of which gives clear structural detail of the chromophore. While fluorescence may change based on the structure of the chromophore,[22] other factors, such as the surrounding environment, may also contribute changes, and one may question

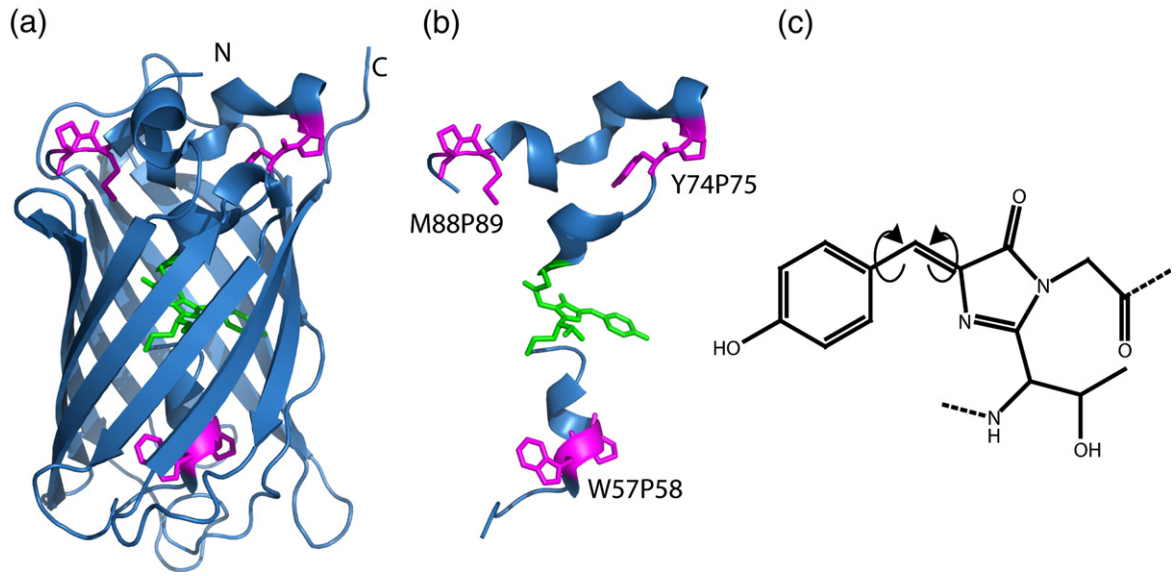

Fig. 2. (a–c) The structure of sfGFP still allows an HT isomerization. While the highly regular barrel (a) holds the rings of the chromophore (green) in a rigid position, proline residues (magenta) are linked to chromophore formation (b). The chromophore is proposed to isomerize in a concerted motion (c) called an HT isomerization. This conserves the volume of the chromophore while potentially leading to a weakly or non-fluorescent state. This figure was rendered using PyMOL[6] with the superfolder crystal structure (2B3P).[7]