Communication

Deconstructing Green Fluorescent Protein

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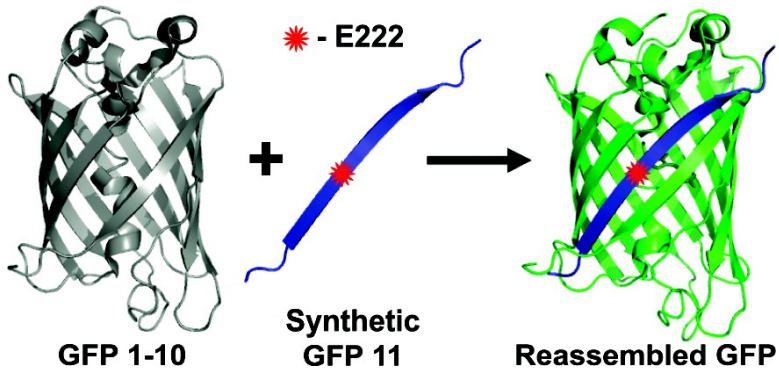

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Deconstructing Green Fluorescent Protein

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Many proteins can be cleaved into two parts, which can then be reassembled noncovalently into a stable and functional holoprotein.[1] Conversely, several split-protein-based reporter systems have been created in which the fragments do not reassemble spontaneously, but require the association of other proteins fused to each part in order to generate function.[2,3] Examples of the latter include split GFPs,[4–9] luciferases,[7,10–12] and several enzymes.[3] Background from spontaneous assembly of the fragments compromises their utility for detecting protein−protein interactions in vivo.[13] In this note, we turn spontaneous assembly to our advantage as in the original examples[1] and reassemble GFP from two pieces in vitro as illustrated in Figure 1: the larger piece denoted GFP 1−10, is produced recombinantly, while the smaller piece, GFP 11, is a synthetic peptide with the same primary sequence as the last stave of the β-barrel. We demonstrate that the reassembled protein exhibits properties indistinguishable from the native protein, and because GFP 11 is prepared synthetically, direct covalent modification to produce new properties is straightforward.

Waldo and co-workers created "Superfolder" GFP,[14] a particularly stable variant that is useful for probing protein folding in vivo, and demonstrated that GFP 1−10 and GFP 11 derived from this sequence assemble in vivo into a fluorescent protein without fused interacting proteins.[15] The gene coding for GFP 1−10 was kindly provided by Waldo; it has 16 mutations from wtGFP, including the S65T mutation that suppresses the protonated state of the chromophore (A-state), enhances the deprotonated state of the chromophore (B-state), and prevents excited-state proton transfer.[16,17] As these are unique functional signatures for intact GFP, residue 65 was mutated back to Ser on GFP 1−10 (hereafter simply called GFP 1−10), and this was expressed in *E. coli*, as was a fully synthetic gene corresponding to the complete sequence (hereafter called whole GFP; see Supporting Information).

GFP 1−10 isolated from inclusion bodies in denaturing solution initially does not exhibit the absorption or fluorescence characteristics of the GFP chromophore. After addition of fully synthetic GFP 11, chromophore maturation is observed[18] and the reassembled protein can be isolated by ion exchange chromatography (see Supporting Information). Reassembled GFP is sufficiently stable that it can be directly observed by electrospray time-of-flight mass spectrometry under mild conditions or broken apart under harsher conditions (see Supporting Information), and in both cases the expected mass and mass change associated with chromophore maturation are observed[19] (Supplementary Table 1 and Figure 1). Circular dichroism spectra of whole and reassembled GFP show identical spectra that are similar to spectra for other GFPs[20] (Supplementary Figure 2). The CD spectrum for GFP 1−10 shows weaker molar ellipticity than reassembled GFP below 200 nm implying more random coil in the structure of GFP 1−10. The lack of structure in GFP 1−10 and the observed formation of the chromophore only after addition of GFP 11 suggest that GFP 11 induces the precyclization structural constraints necessary for chromophore formation.[21]

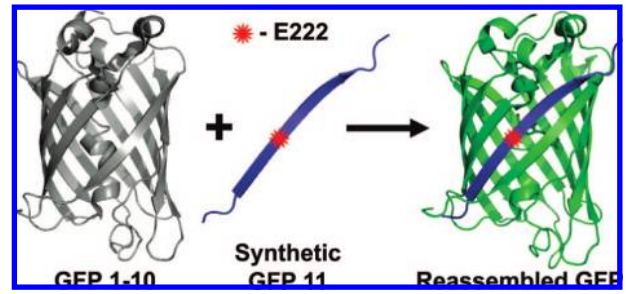

Figure 1. Schematic diagram illustrating reassembly of the eleven-stranded β-barrel of GFP from GFP 1−10 (the first 214 amino acids of GFP, including residues 65−67 that become the chromophore) and a synthetic, 16 amino acid 11th strand, GFP 11. The red star marks residue E222 on GFP 11, which is known to be an excited-state proton acceptor in several GFP variants.[24,25] The chromophore is not formed in GFP 1−10, but does mature upon addition of synthetic GFP 11. The topology of GFP 1−10 in solution is not yet well characterized and is drawn based on the structure of whole GFP minus the 11th strand (see text).

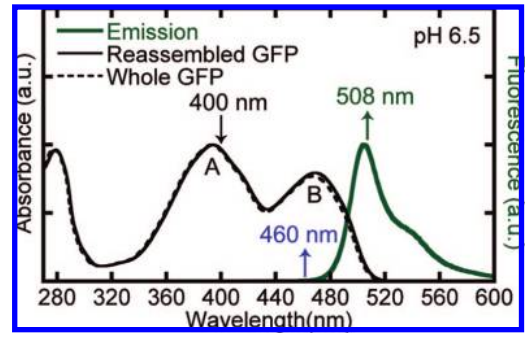

Figure 2. The absorption and steady-state emission (λ_{ex} = 400 nm) of reassembled and whole GFP are indistinguishable. Both the protonated (A) and deprotonated (B) forms of the chromophore are present in whole GFP because we introduced the native Ser at position 65, but the ratio of A to B is quite different from wtGFP because of the collective effect of many amino acid differences.[15] The arrows indicate the excitation (400 nm) and emission (460 and 508 nm) wavelengths for the time-resolved fluorescence data in Figure 3.

As seen in Figure 2, the absorption and fluorescence spectra of reassembled and whole GFP are indistinguishable. Likewise, the time-resolved fluorescence profiles probing excited-state dynamics of the A* or I* states[22,23] shown in Figure 3 are indistinguishable, as is the large kinetic isotope effect observed when deuterated buffer is used.[23] Excited-state proton transfer has been studied extensively in GFP variants, and the glutamic acid at position 222, which is on strand 11, is known to be an excited-state proton acceptor.[24,25] Thus, reconstitution of native absorption, fluorescence, and excited-state dynamics, including the deuterium isotope effect, suggest that strand 11 is in the correct orientation and a fully functional protein has been reassembled.

Once reassembled and following chromophore maturation, the pieces can be separated by denaturation, where now the isolated

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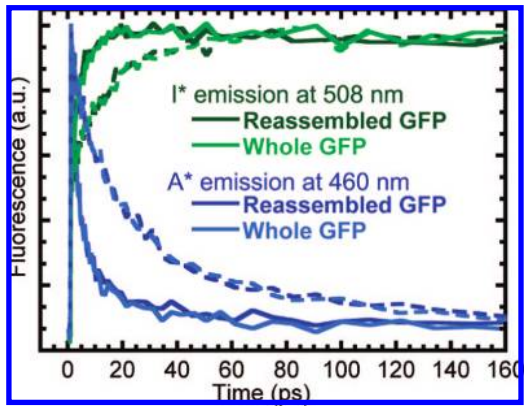

Figure 3. The time-resolved fluorescence of whole and reassembled GFP detected at 460 nm (A* emission) and 508 nm (I* emission)[22,23] upon excitation at 400 nm (cf. Figure 2) are indistinguishable. The dotted lines show the time-resolved emission upon exchange of both proteins into deuterated buffer, demonstrating that both proteins exhibit excited-state proton transfer.

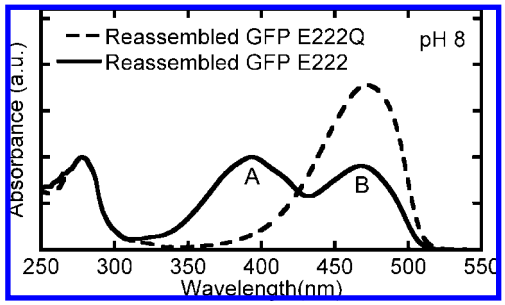

Figure 4. Reassembly of GFP 1−10^{mat} with a synthetic GFP 11 peptide containing glutamine in place of the native glutamic acid at position 222 creates a fluorescent protein with a drastically modified absorption. E222 interacts strongly with the chromophore.[19,24,25,27] Note the slight decrease in A/B ratio for reassembled GFP E222 upon raising the pH from 6.5 (Figure 2) to pH 8; identical behavior is observed in whole GFP and is due to the well-characterized protonation/deprotonation titration of the chromophore.[16,17]

GFP 1−10^{mat} has the mature chromophore. Only weak fluorescence is observed for GFP 1−10^{mat}, consistent with weak fluorescence when GFP is denatured or in the synthetic chromophore.[26] If synthetic GFP 11 is reintroduced to GFP 1−10^{mat}, the absorption and fluorescence seen in Figure 2 are recovered, thus GFP 1−10^{mat} is a substrate for reassembly with synthetic GFP 11 strands of designed sequence, containing natural as well as unnatural amino acids. This is shown for the case of GFP 11 E222Q in Figure 4. The enhancement of the deprotonated B-state is consistent with the results of previous mutations to E222 in wtGFP.[17] In crystal structures of wtGFP E222 interacts with the protonated A-state through hydrogen bonds, but not the B-state,[27] suggesting that disruption of the hydrogen-bond network by mutating E222 would lead to destabilization of the A-state and enhancement of the B-state.

These results demonstrate that the GFP barrel can be reassembled from pieces where one piece is readily prepared on a peptide synthesizer, thus any natural or unnatural amino acids can be introduced. This system is ideally suited for studying the reassembly of β-barrel structures with a built-in fluorescence reporter, and, by using circular permutation,[28,14] it may prove possible to apply the same strategy to any strand of the β-barrel.

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Supporting Information Available: Protein preparation, experimental methods, mass spec data, mass spectra, CD spectra of GFP 1−10, reassembled GFP, and whole GFP and amino acid sequences of all peptide chains. This material is available free of charge via the Internet at http://pubs.acs.org.

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