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Cyan Fluorescent Protein Carries a Constitutive Mutation That Prevents Its Dimerization†

Agathe Espagne,*,‡ Marie Erard,‡ Karine Madiona,§ Valérie Derrien,‡ Gabriella Jonasson,‡ Bernard Lévy,‡ Hélène Pasquier,‡ Ronald Melki,§ and Fabienne Mérola‡

‡*Laboratoire de Chimie Physique, Université Paris-Sud and CNRS (UMR 8000), 91405 Orsay, France, and* §*Laboratoire d'Enzymologie et Biochimie Structurales, CNRS (UPR 3082), 91198 Gif-sur-Yvette, France*

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ABSTRACT: The tendency of GFP-like fluorescent proteins to dimerize in vitro is a permanent concern as it may lead to artifacts in FRET imaging applications. However, we have found recently that CFP and YFP (the couple of GFP variants mostly used in FRET studies) show no trace of association in the cytosol of living cells up to millimolar concentrations. In this study, we investigated the oligomerization properties of purified CFP, by fluorescence anisotropy and sedimentation velocity. Surprisingly, we found that CFP has a much weaker homoaffinity than other fluorescent proteins ($K_d \geq 3 \times 10^{-3}$ M), and that this is due to the constitutive N146I mutation, originally introduced into CFP to improve its brightness.

Fluorescent proteins of the GFP family are widely used as probes in protein interaction and conformation studies based on FRET (Förster resonance energy transfer). The FRET approach relies on the assumption that the probes do not have any affinity for each other. Unfortunately, it has been a long time since GFPs were suspected of dimerizing. The first indication was the concentration dependence of the absorption spectrum of GFP (*1*). Later, Yang et al. crystallized GFP as a dimer (*2*), where the two monomers are associated in an antiparallel way (Figure 1). The contact region consists of a core of three hydrophobic residues, one of which is alanine 206, and numerous hydrophilic residues. Dissociation constants on the order of 1×10^{-4} M were measured by equilibrium sedimentation for GFP and YFP, one of its spectral variants (*3*, *4*), and Zacharias et al. proposed to introduce the A206K mutation to destabilize the dimer (*3*). Additional evidence of the tendency of YFP to dimerize was revealed recently by time-resolved fluorescence anisotropy measurements (*5*, *6*).

CFP (enhanced cyan fluorescent protein) is the fluorescent protein most often used as the FRET donor, usually in conjunction with YFP as the acceptor. Contrary to that of YFP, oligomerization of CFP has not been the subject of any dedicated study in solution, and the crystallographic structures of CFP do not show any trace of dimer (*7*, *8*). A strong concentration-dependent quenching of CFP fluorescence, frequently observed when it is expressed together with YFP, in living cells, is usually attributed to heterodimerization (*9*, *10*). However, we found recently that cytosolic CFP carrying the A206K mutation presents exactly the same concentration-dependent decreases in fluorescence lifetime. We also showed that this concentration-dependent quenching is well described by nonspecific FRET taking place between nearby proteins at high levels of expression (*11*).

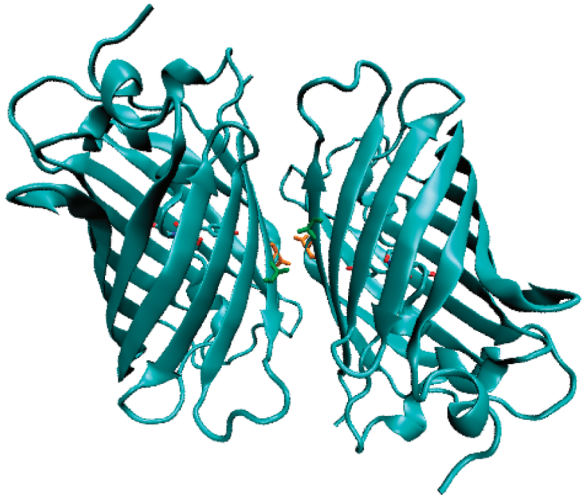

FIGURE 1: Structure of the GFP dimer from Yang et al. (*2*) showing residues 146 (orange) and 206 (green) at the dimer interface.

Here, we present the first study of CFP oligomerization in solution. We show by fluorescence anisotropy and sedimentation velocity that CFP has a much weaker homoaffinity than GFP and YFP. In contrast, CFP I146N dimerizes in the 100 μM concentration range, suggesting that the N146I mutation, which is one of the constitutive mutations of CFP, intrinsically prevents CFP dimerization.

Fluorescence anisotropy is an established method for measuring protein−protein and protein−macromolecule interactions, because of its sensitivity to rotational diffusion. It has also been used to detect protein self-association through homo-FRET between fluorophores bound to the protein of interest (*12*−*14*). Here, we make use of the same approach, except that we probe the fluorescence of CFP itself. In the hypothesis for a dimer, the fluorescence of CFP is expected to be depolarized by three mechanisms: rotational diffusion, which is slower for the dimer than for the monomer, homo-FRET between the two CFP units in the dimer, and nonspecific FRET between CFP monomers at high concentrations. We call nonspecific FRET the radiationless energy migration that occurs in concentrated (typically millimolar) solutions of like fluorophores, due to molecular crowding. When the CFP concentration increases, rotational diffusion should increase the anisotropy whereas intradimer FRET and nonspecific FRET should decrease it. In contrast, if no dimer forms, the contribution from rotational diffusion should be constant, and the only concentration-dependent depolarization mechanism should be nonspecific FRET.

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*To whom correspondence should be addressed. E-mail: agathe.espagne@u-psud.fr. Phone: +33(0)169155618. Fax: +33(0)169156188.

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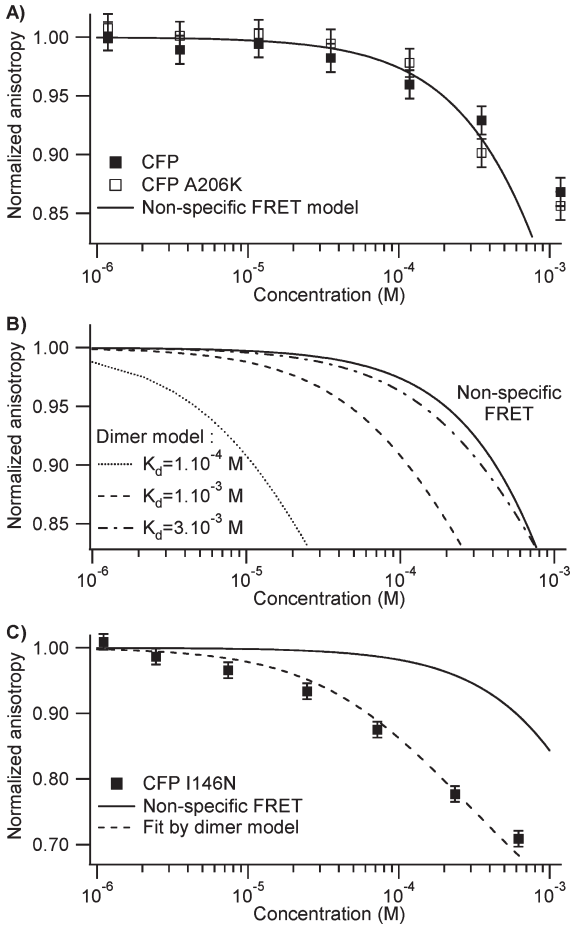

FIGURE 2: Fluorescence anisotropy of CFP and its A206K and I146N variants as a function of concentration. Experimental data are compared with simulations based on the nonspecific FRET model, or on the dimer model (see the Supporting Information). (A) Data for CFP (■) and monomeric variant CFP A206K (□), compared with a simulation based on the nonspecific FRET model (—). (B) CFP simulations based on the dimer model with $K_d = 1 \times 10^{-4}$ to 3×10^{-3} M (···, ---, and -·-·). Comparison with the expected nonspecific FRET contribution (—). (C) CFP I146N data (markers) and simulated nonspecific FRET (—). A fit of the data to the dimer model (---) leads to a K_d of $(5.1 \pm 0.5) \times 10^{-4}$ M.

We introduced the A206K mutation into CFP to split the dimer and measured the fluorescence anisotropy of wild-type and mutant proteins as a function of concentration, in the range of 1 μM to 1 mM (Figure 2A, markers). If CFP were to dimerize in the probed concentration range, we would expect significant differences between the two data series. Surprisingly, they show the same trend: the anisotropy is constant up to 3×10^{-5} M and then decreases.

We first checked that the anisotropy decrease beyond 3×10^{-5} M may be explained by nonspecific FRET, using an equation derived by Weber (*15*) (see the Supporting Information). The model assumes that no rotational diffusion occurs during the excited-state lifetime. Fluorescent proteins fulfill this condition: the average lifetime of CFP is 2.5 ns (*16*), whereas the rotational correlation time of GFP is 16 ns (*17*). The model curve in Figure 2A (line) suggests that the depolarization observed at high concentrations is indeed compatible with nonspecific FRET.

A more trivial cause of depolarization at high concentrations might have been the reabsorption of emission, or radiative energy migration. However, for a given concentration, fluorescence anisotropy is essentially independent of the emission wavelength (see Figure S1 of the Supporting Information), which points to the negligible contribution of this mechanism (*18*).

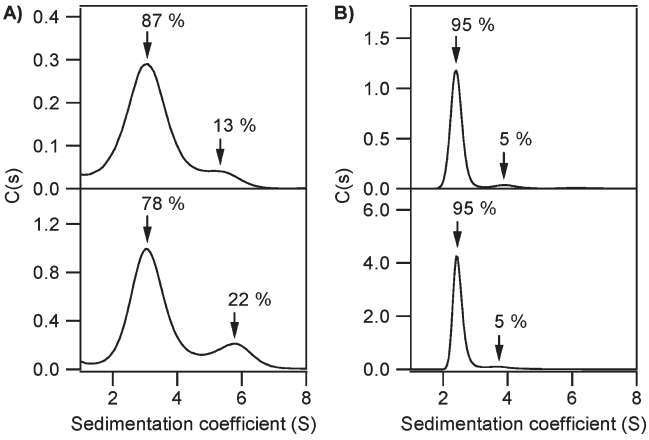

FIGURE 3: Distributions of sedimentation velocity $C(s)$ of CFP and YFP centrifuged at 180000*g* and 15 °C. (A) YFP at concentrations of 30 μM (0.89 mg/mL) (top) and 90 μM (2.67 mg/mL) (bottom). (B) CFP at concentrations of 40 μM (1.18 mg/mL) (top) and 120 μM (3.54 mg/mL) (bottom). Arrows indicate the positions of the peaks of $C(s)$. The proportions of the corresponding species are given.

There may be several explanations for the similarity of the CFP and CFP A206K data in Figure 2A. First, it could be that the CFP dimer does not form under our experimental conditions. We checked that the polyhistidine tag used to purify CFP did not have any influence on the shape or transition point of the fluorescence anisotropy curve (see Figure S2 of the Supporting Information). We also checked the influence of salt concentration, as the YFP dimer has been observed in an isotonic PBS buffer (I = 160 mM) (*3*). We found the same concentration dependence of CFP fluorescence anisotropy in PBS buffer as in the phosphate buffer we use with an ionic strength of 67 mM (see Figure S3 of the Supporting Information).

Alternatively, the similarity of the data in Figure 2A could be due to a compensation between the anisotropy increase due to the slower rotational diffusion of the dimer and the anisotropy decrease due to intradimer FRET. To evaluate this possibility, we modeled the effect of dimerization on anisotropy, taking both contributions into account. The step-by-step derivation of our model is available as Supporting Information. We described the contribution of homo-FRET within the CFP dimer using an equation derived by Runnels and Scarlata for FRET between identical clustered molecules (*12*). We assumed that the CFP dimer was structurally similar to the GFP dimer (*2*). The model indicates that a CFP dimer with a K_d of $< 3 \times 10^{-3}$ M should be detectable by fluorescence anisotropy (Figure 2B), whereas weaker dimers are expected to be hidden by nonspecific FRET. As a consequence, the data in Figure 2A strongly suggest that CFP is much less prone to dimerize than GFP and YFP. Time-resolved fluorescence anisotropy data are also consistent with this conclusion (see the Supporting Information).

To double-check this unexpected result, we conducted sedimentation velocity measurements on CFP and YFP at concentrations ranging from 30 to 120 μM (Figure 3). The sedimentation coefficient distributions of YFP (Figure 3A) reveal two species with a concentration-dependent oligomerization, suggesting an equilibrium between monomeric YFP ($s_{20,w}$ of 3.5 S) and oligomeric forms, in agreement with the literature (*3*, *4*). In contrast, CFP solutions (Figure 3B) contain mainly one species

with a sedimentation coefficient $s_{20,w}$ of 2.9 S that can be attributed to the monomer. A second species compatible with a CFP dimer ($s_{20,w}$ of 4.7 S) is at the limit of detection (≈5%), implying a K_d in the range of 3 mM, in accord with fluorescence anisotropy measurements.

We examined the amino acid sequence of CFP in search of a rationale. CFP results from mutations at six positions in GFP (64, 65, 66, 146, 153, and 163). Amino acid residue 146 is the only substitution located within the dimer interface (Figure 1). At this position, a hydrophilic asparagine (N) in GFP and YFP is replaced with a hydrophobic isoleucine (I) in CFP. This N146I mutation was introduced by Heim and Tsien, together with mutations at positions 153 and 163, in an effort to improve the brightness of the blue variant GFP Y66W that later became CFP (*19*). We mutated residue 146 back to asparagine in CFP (CFP I146N), to check the influence of the N146I mutation on homoaffinity. As opposed to the A206K mutation, the I146N mutation significantly changes the photophysics of CFP (see Table S1 of the Supporting Information), which leads also to changes in FRET efficiency. Figure 2C shows the concentration dependence of CFP I146N fluorescence anisotropy (markers) together with the estimated nonspecific FRET contribution (solid line). Remarkably, the fluorescence of CFP I146N starts to depolarize at concentrations well below those at which nonspecific FRET is expected, suggesting that the mutant indeed has a higher homoaffinity. This result was confirmed by sedimentation velocity measurements (see Figure S4 of the Supporting Information) and is also supported by time-resolved anisotropy data (Supporting Information). A fit of the steady-state anisotropy data to the dimer model gives a K_d of $(5.1 \pm 0.5) \times 10^{-4}$ M (Figure 2C, dashed line), a value comparable to literature values for GFP and YFP (*3*, *4*). From a structural point of view, the N146I mutation seems to be associated to the appearance of a new conformation of the [143−150] strand in CFP, in which residue 146 points toward the interior of the protein instead of pointing toward the solvent (*7*, *8*). Such a conformation change and/or disruption of the hydrophilic interactions involving the asparagine residues could be responsible for destabilizing the dimer. The N146I mutation does, however, not prevent YFP dimerization (see Figure S7 of the Supporting Information), suggesting that this effect might be specific to CFP.

In conclusion, we have shown that CFP, the most widely used blue variant of GFP, has a much weaker homoaffinity than other GFP-like fluorescent proteins. This is due to the constitutive N146I mutation that intrinsically destabilizes the dimer, as evidenced by the restored dimerization ability of CFP I146N. These results question the existence of the widely mentioned but never directly observed CFP−YFP heterodimer. This issue has important implications given the intensive use of the CFP−YFP pair in FRET. We are currently working on the characterization of the CFP−YFP interaction in solution.

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SUPPORTING INFORMATION AVAILABLE

Materials and methods, models, time-resolved fluorescence anisotropy data, and supplementary tables and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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