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► **To cite this version:**

Agathe Espagne, Marie Erard, Karine Madiona, Valérie Derrien, Gabriella Jonasson, et al.. Cyan fluorescent protein carries a constitutive mutation that prevents its dimerization.. *Biochemistry*, 2011, 50 (4), pp.437-439. 10.1021/bi1015875 . hal-01183186

**HAL Id: hal-01183186**

**<https://hal.science/hal-01183186v1>**

Submitted on 8 Feb 2024

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# Cyan Fluorescent Protein Carries a Constitutive Mutation that Prevents its Dimerization

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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 homo-affinity than other fluorescent proteins ( $K_d \geq 3.10^{-3}$  M), and that this is due to the constitutive N146I mutation, originally introduced in 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 (F orster resonance energy transfer). The FRET approach relies on the assumption that the probes do not have any affinity for each other. Unfortunately, it has already 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 on, Yang et al. crystallized GFP as a dimer (2), where the two monomers are associated in an antiparallel way (Fig. 1). The contact region is made of a core of three hydrophobic residues, among which alanine 206, and of 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 evidences for the tendency of YFP to dimerize were brought recently by time-resolved fluorescence anisotropy measurements (5, 6).

CFP (enhanced Cyan Fluorescent Protein) is the fluorescent protein mostly used as FRET donor, usually in conjunction with YFP as acceptor. Contrary to YFP, CFP oligomerization 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 accounted for by non-specific FRET taking place between nearby proteins at high expression levels (11).

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 homo-affinity than GFP and YFP. In contrast, CFP I146N dimerizes in the 100  $\mu$ M concentration range, suggesting that the N146I mutation, which is one of the constitutive mutations of CFP, intrinsically prevents CFP dimerization.

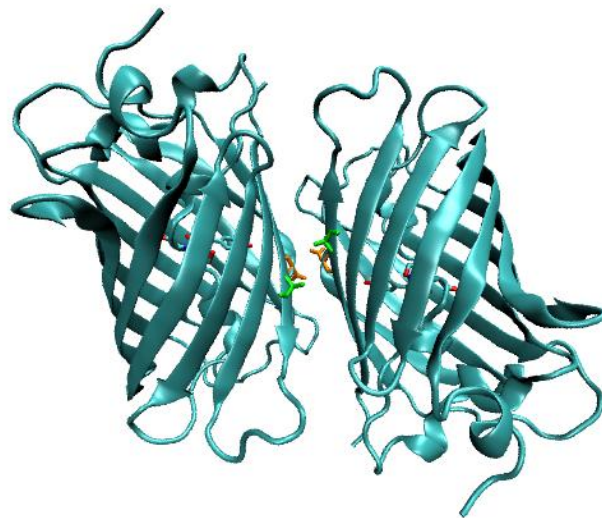
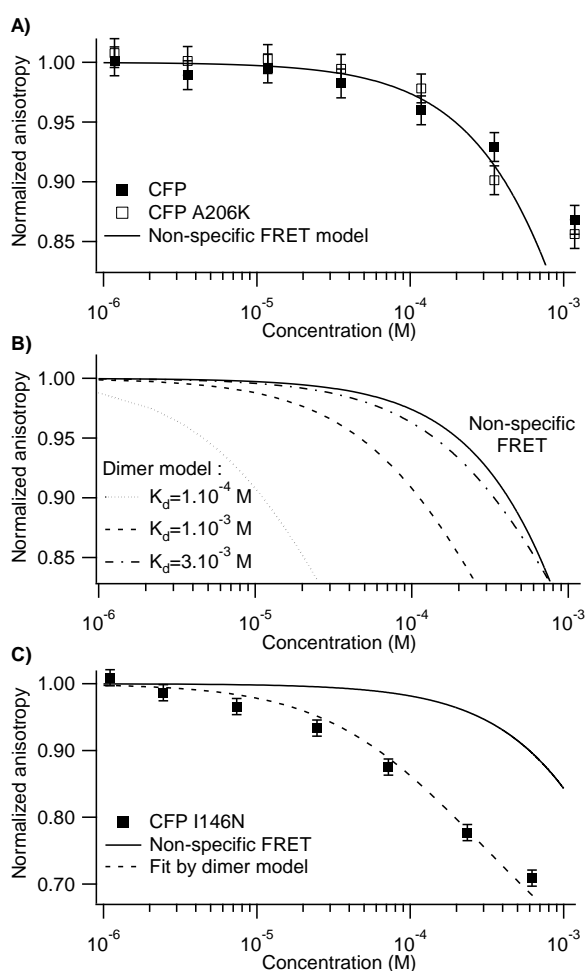


Figure 1. Structure of the GFP dimer from Yang et al. (2) showing residues 146 (orange) and 206 (green) at the dimer interface.

Fluorescence anisotropy is an established method for measuring protein-protein and protein-macromolecules interactions, thanks to 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 of 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 non-specific FRET between CFP monomers at high concentration. We call non-specific FRET the radiationless energy migration that occurs in concentrated (typically mM) solutions of like fluorophores, due to molecular crowding. When the CFP concentration increases, rotational diffusion should increase the anisotropy whereas intra-dimer FRET and non-specific 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 non-specific FRET.

We introduced the A206K mutation in 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  $\mu$ M to 1 mM (Fig. 2A, markers). If CFP would 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  $3 \cdot 10^{-5}$  M, and then decreases.



**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 non-specific FRET model, or on the dimer model (see Supporting Information (SI)). A) Data for CFP (filled squares) and the monomeric variant CFP A206K (empty squares), compared with a simulation based on the non-specific FRET model (line). B) CFP, simulations based on the dimer model for  $K_d = 1 \times 10^{-4}$  M to  $3 \times 10^{-3}$  M (dashed and dotted lines). Comparison with the expected non-specific FRET contribution (plain line). C) CFP I146N, data (markers) and simulated non-specific FRET (plain line). A fit of the data to the dimer model (dashed line) leads to  $K_d = (5.1 \pm 0.5) \times 10^{-4}$  M.

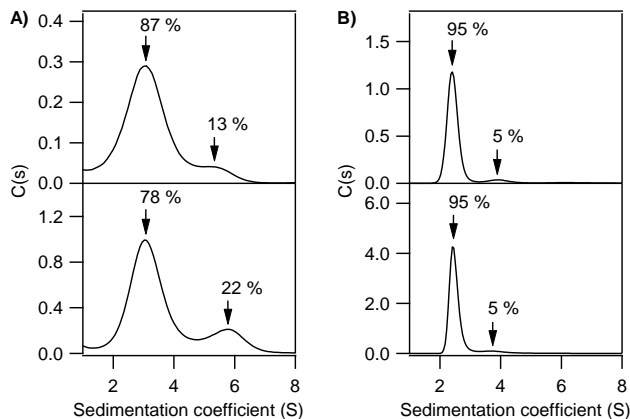
We first checked that the anisotropy decrease beyond  $3 \cdot 10^{-5}$  M may be explained by non-specific FRET, using an equation derived by Weber (15) (see Supporting Information (SI)). The model assumes that no rotational diffusion occurs during the excited-state lifetime. Fluorescent proteins fulfill 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 Fig. 2A (line) suggests that the depolarization observed at high concentration is indeed compatible with non-specific FRET.

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

There may be several explanations to the similarity of the CFP and CFP A206K data in Fig. 2A. First, it could be that the CFP dimer does not form in our experimental conditions. We checked that the poly-histidine tag used to purify CFP did not have any influence on the shape and transition point of the fluorescence anisotropy curve (see SI, Fig. S2). We also checked the influence of salt concentration, as 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 which ionic strength is 67 mM (see SI, Fig. S3).

Alternatively, the similarity of the data in Fig. 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 intra-dimer 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 SI. 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  $K_d$  lower than  $3 \times 10^{-3}$  M should be detectable by fluorescence anisotropy (Fig. 2B), whereas weaker dimers are expected to be hidden by non-specific FRET. As a consequence, the data in Fig. 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 SI).

To double-check this unexpected result, we conducted sedimentation velocity measurements on CFP and YFP at concentrations ranging from 30  $\mu$ M to 120  $\mu$ M (Fig. 3). The sedimentation coefficient distributions of YFP (Fig. 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 (Fig. 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 ( $\approx 5\%$ ), implying a  $K_d$  in the range of 3 mM, in accord with fluorescence anisotropy measurements.



**Figure 3.** Distributions of sedimentation velocity  $C(s)$  of CFP and YFP centrifuged at 180000 g at 15°C. A) YFP. Concentration, top : 30  $\mu\text{M}$  (0.89 mg/mL) ; bottom : 90  $\mu\text{M}$  (2.67 mg/mL). B) CFP. Concentration, top : 40  $\mu\text{M}$  (1.18 mg/mL) ; bottom : 120  $\mu\text{M}$  (3.54 mg/mL). Arrows indicate the positions of the peaks of  $C(s)$ . The proportions of the corresponding species are given.

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 (Fig. 1). At this position, a hydrophilic asparagine (N) in GFP and YFP is replaced by 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 homo-affinity. Contrary to the A206K mutation, the I146N mutation significantly changes the photophysics of CFP (see SI, Table S1), which leads also to changes in FRET efficiency. Fig. 2C shows the concentration dependence of CFP I146N fluorescence anisotropy (markers) together with the estimated non-specific FRET contribution (plain line). Remarkably, the fluorescence of CFP I146N starts to depolarize at concentrations well below those where non-specific FRET is expected, suggesting that the mutant indeed has a higher homo-affinity. This result was confirmed by sedimentation velocity measurements (see SI, Fig. S4), and is also supported by time-resolved anisotropy data (see SI). A fit of the steady-state anisotropy data to the dimer model gives  $K_d = (5.1 \pm 0.5) \times 10^{-4}$  M (Fig. 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 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 conformational 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 SI, Fig. S7), 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 homo-affinity 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. The present 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.

#### ACKNOWLEDGMENT

This work was financially supported by CNRS and Université Paris-Sud. A. E. thanks M. Martin, P. Plaza and P. Changenet-Barret for access to their fluorimeter and T. Bizouarn, L. Baciou and F. Lederer for biochemical advice.

#### SUPPORTING INFORMATION AVAILABLE

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

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