Extreme dependence of *Chloroflexus aggregans* LOV domain thermo- and photostability on the bound flavin species

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Abstract

Light-oxygen-voltage (LOV) domains are common photosensory modules that found many applications in fluorescence microscopy and optogenetics. Here, we show that the *Chloroflexus aggregans* LOV domain can bind different flavin species (lumichrome, LC; riboflavin, RF; flavin mononucleotide, FMN; flavin adenine dinucleotide, FAD) during heterologous expression and that its physicochemical properties depend strongly on the nature of the bound flavin. We show that whereas the dissociation constants for different chromophores are similar, the melting temperature of the protein reconstituted with single flavin species varies from ~60 °C for LC to ~81 °C for FMN, and photobleaching half-times vary almost 100-fold. These observations serve as a caution for future studies of LOV domains in non-native conditions yet raise the possibility of fine-tuning various properties of LOV-based fluorescent probes and optogenetic tools by manipulating the chromophore composition.

Graphical abstract

Keywords LOV domain · Flavin-based fluorescent protein · Riboflavin · Flavin mononucleotide · Chromophore · Thermal stability · Protein–ligand interactions

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Abbreviations

CFP	Cyan fluorescent protein
FAD	Flavin adenine dinucleotide
FbFP	Flavin-based fluorescent protein
FMN	Flavin mononucleotide
GFP	Green fluorescent protein
HPLC	High-performance liquid chromatography
IPTG	Isopropyl β -D-1-thiogalactopyranoside
LC	Lumichrome
LOV	Light-oxygen-voltage
PAS	Per-Arnt-Sim
RF	Riboflavin
RFP	Red fluorescent protein
SEC	Size-exclusion chromatography
YFP	Yellow fluorescent protein

1 Introduction

Light-oxygen-voltage (LOV) domains are ubiquitous photosensory modules found in photoreceptor proteins from all three domains of life [1, 2]. They sense blue light and regulate diverse physiological processes such as circadian rhythms [3], phototropism [4, 5], virulence [6] and stress responses [7]. LOV domains are members of the PAS (Per-Arnt-Sim) superfamily and share the common globular α/β fold [8]. In the core, LOV proteins may bind different flavin chromophores (riboflavin, RF; flavin mononucleotide, FMN; flavin adenine dinucleotide, FAD) with dissociation constants around 0.1–0.9 μ M [9, 10]. These flavins are present in all living organisms, and consequently, heterologous expression of LOV domains usually does not require flavin supplementation or genetic introduction of their biosynthesis pathways. Different LOV proteins were also shown to bind modified flavins, such as lumichrome (LC), roseoflavin, 1- or 5-deaza FMN, bromo-, chloro- and fluoro-substituted FMN, and others, in vivo or after renaturation [11–18]. This resulted in shifts of the absorption maxima from – 45 nm to + 92 nm and variation of other optical and photophysical properties [11–18].

LOV domains have found many applications in optogenetics, allowing e.g. for the control of protein interactions and protein localization by light [19, 20]. Due to their photophysical properties, they are also used for design of fluorescent reporters [21–24]. Unlike reporters based on the green fluorescent protein (GFP) and GFP-related β -barrel proteins such as YFP, CFP or RFP, LOV domains do not require oxygen for chromophore maturation [21, 25]. Therefore, they can be used for studying low oxygen phenomena such as cerebral ischemia, tumor hypoxia, pathogenesis, and biofilm development.

Thermal stability is one of the most important properties of fluorescent reporters, as it may increase the resistance of the

protein against proteolysis [26, 27], expands its usability in extreme conditions and often allows for easier engineering of modified variants [28]. Previously, genome mining approaches were used to discover naturally occurring thermostable proteins [29], including *CagFbFP*, based on the LOV domain from a putative sensory histidine kinase of the thermophilic bacterium *Chloroflexus aggregans* [30]. Interestingly, studying its thermal denaturation, we observed two transitions (Fig. 1a), and overall variation of properties from batch to batch. Moreover, it was not clear whether the two melting transitions were consecutive, observed for all protein molecules in the sample, or represented different fractions of the protein samples. In addition, thermal stability measurements of several variants of *CagFbFP* also revealed two melting temperatures. In proline-substituted *CagFbFP* variants, the temperature of both transitions is raised by up to ~ 2°C [31]. In the variants generated to achieve spectral tuning, both transitions are shifted to lower temperatures [32, 33]. Finally, two or even three different transitions were observed in variants with extended flexible loops of different lengths, which were designed for identification of the best potential split sites during the process of generating a split flavin-based fluorescent reported based on *CagFbFP* [34].

Another important property of fluorescent proteins is photostability. In solution, illuminated flavins are degraded to LC and lumiflavin derivatives [35]. Similarly, in a LOV1 domain of phototropin from *Chlamydomonas reinhardtii* complexed with roseoflavin monophosphate, prolonged illumination caused photodegradation of the cofactor to fully reduced flavin and lumichrome derivatives [18]. In a representative FbFP, mini-SOG, generation of reactive oxygen species leads to photodegradation of the bound FMN to LC, as well as to modification of protein amino acids [36]. While normally LC is not synthesized by the cells directly, it sometimes can be found in LOV variants after heterologous expression and purification [37].

In this work, we developed a protocol for deflavination of heterologously expressed *CagFbFP*, and reconstituted the resulting apoprotein with RF, FMN, FAD, and LC. We studied physicochemical properties of the resulting holoproteins and found that they depend strongly on the chromophore type. We show that the melting transitions observed in the heterologously expressed samples may be ascribed to protein species bound to different flavins. The results highlight the importance of accurate characterization of heterologously expressed and purified protein samples and suggest that the properties of LOV-based proteins used in optogenetics and fluorescence microscopy may be tuned by altering the chromophore composition.

Fig. 1 Thermal stability of *CagFbFP* and other FbFPs. **a** The decrease in the FbFP-specific fluorescence intensity during heating (upper panel) and the first derivative of the data (lower panel). Two transitions are observed. Corresponding melting temperatures (68 and 81 °C) are marked with the dashed lines. **b** Fluorescence melting curves of *EcFbFP* and a set of FbFPs from thermophilic micro-

organisms reported by Wingen et al. in 2017 [29] (upper panel) and the first derivative of the data (lower panel). The proteins are sourced from *Meiothermus ruber* (*MrFbFP*), *Escherichia coli* (*EcFbFP*), Yellowstone National Park Metagenome Project (YNP FbFPs), *Thermosynechococcus elongatus* (*TeFbFP*), *Dinoroseobacter shibae* (*DsFbFP*) and *Pseudomonas putida* (*Pp1FbFP* and *Pp2FbFP*)

2 Results and discussion

2.1 Properties of heterologously expressed LOV domains

Recently, we have identified and characterized a flavin-binding fluorescent protein *CagFbFP*, derived from the LOV domain of a putative sensory histidine kinase Cagg_3753 from the thermophilic bacterium *C. aggregans*. During temperature-induced denaturation, the heterologously expressed protein revealed two melting transitions, at ~68 and 81 °C (Fig. 1a), the reason for which was not clear. Moreover, reanalysis of the data for other FbFPs, including those from thermophilic organisms [29], shows that some other proteins (*EcFbFP*, *MrFbFP* and *YNP3FbFP*, derived from proteins from *Escherichia coli*, *Meiothermus ruber*, and Yellowstone National Park Metagenome Project, respectively) also display melting in at least two steps (Fig. 1b). Interestingly, we observed that while *CagFbFP* elutes in a single peak during size-exclusion chromatography (SEC), the different fractions taken from this peak have different thermal stability, with the heavier fractions being more thermostable (Fig. 2).

We hypothesized that the two melting transitions might correspond to protein populations bound to different flavin molecules. It is generally assumed that most LOV domains

predominantly bind FMN as a cofactor, whereas others preferentially bind FAD. Yet, heterologously expressed LOV domains sometimes contain a mixture of all naturally occurring flavins (RF, FMN and FAD) [10, 38]. We, therefore, analyzed the chromophore composition of *CagFbFP* overexpressed in *E. coli* using high-performance liquid chromatography (HPLC) (Fig. S1a). We found that with respect to the total flavin content, the majority (44%) of the protein contained riboflavin, while 29% and 19% of *CagFbFP* bound FMN and FAD, respectively. We also observed the presence of lumichrome (LC), a photodegradation product of flavins (8%). It is not clear, if and how the cofactor identity could affect SEC elution volume of the respective protein species.

To test whether the physicochemical properties of *CagFbFP* depend on the chromophore, we developed a protocol for production of apo-*CagFbFP*, based on denaturation using guanidinium hydrochloride (GuHCl). The approach is similar to those reported previously [11, 14, 15], but requires incubation at 70 °C in a more concentrated GuHCl solution for complete removal of the chromophores because of higher stability of *CagFbFP*. After denaturation and removal of the released flavins, the refolded apo-protein was reconstituted with the natural flavins (FMN, RF, FAD) or with LC (Fig. 3a, b) as described in the Methods section. The flavin load of the resulting *CagFbFP* samples was estimated to be

Fig. 2 Dependence of the thermal stability of size-exclusion chromatography (SEC)-purified *CagFbFP* on fraction number. **a** SEC profile of *CagFbFP* purified using Superdex® 200 Increase 10/300 GL col-

umn. Fraction volume is 0.5 ml. **b** First derivative of the FbFP-specific fluorescence as a function of temperature for different *CagFbFP* SEC fractions (normalized to have equal area under curve)

65–75% as described in the Methods section. Chromophore composition of each sample was verified by HPLC (Fig. S1b–e).

2.2 Thermal stability

We first determined the thermal stability of *CagFbFP* reconstituted with different chromophores by measuring the flavin fluorescence using spectrofluorimetry and the fluorescence of aromatic amino acids by nano-differential scanning fluorimetry (nanoDSF) (Fig. 3c, d). When heated, the protein denatures and releases the flavin cofactor. This results in a decrease of fluorescence emission with the maximum at 495 nm, characteristic for most protein-bound flavins. When *CagFbFP* contained exclusively one type of cofactor, all flavin fluorescence-based melting measurements revealed a single transition (Fig. 3c). Proteins containing FMN exhibited the highest thermal stability ($T_{\text{FMN}} = 81^\circ\text{C}$), which is 14–15 degrees higher than that of the proteins containing RF or FAD ($T_{\text{RF}} = 66^\circ\text{C}$, $T_{\text{FAD}} = 67^\circ\text{C}$). The least thermostable is *CagFbFP* containing LC ($T_{\text{LC}} = 60^\circ\text{C}$).

When bound to a LOV domain, the flavin molecule quenches the fluorescence of the nearby aromatic amino acids. Therefore, release of flavin during protein unfolding results in an increase of fluorescence intensity at 330 nm. NanoDSF-based experiments revealed similar melting transitions for *CagFbFP* reconstituted with different flavins (Fig. 3d, $T_{\text{FMN}} = 81.7^\circ\text{C}$, $T_{\text{RF}} = 70.1^\circ\text{C}$, $T_{\text{FAD}} = 71.0^\circ\text{C}$, $T_{\text{LC}} = 61.5^\circ\text{C}$). Thus, we conclude that FMN-bound *CagFbFP* is more thermostable than the *Bacillus subtilis* YtvA-LOV domain engineered for thermostability [39] and comparable to engineered iLOV [40] and the most thermostable LOV domains from other microorganisms [29] (Fig. 1b).

Moreover, the two transitions observed for the heterologously expressed *CagFbFP* may be explained by binding of different chromophores during heterologous expression. Increased stability in the series LC-RF-FMN (Fig. 3a) may be explained by increasing favorable stabilizing interactions. The LC moiety is hydrogen-bonded to Gln89, Asn117, Asn127 and Gln148; the ribityl moiety is additionally bonded to Asn84 and Gln89; the phosphate group is coordinated by Arg86 and Arg102 (Fig. 3b). Yet, decreased stability of the FAD-bound protein is surprising, since FAD contains all of the atoms of FMN, may interact with the protein similarly, and there are no evident unfavorable interactions (Fig. 3a, b). Finally, while we cannot be certain that the previously studied LOV proteins, displaying two-step melting (*EcFbFP*, *MrFbFP* and *YNP3FbFP*, Fig. 1b), also bind different flavins, we note that the temperature difference between their two melting steps is also around 10°C . We also note that the bacterium *C. aggregans*, from which *CagFbFP* was derived, was found in bacterial mats with cyanobacteria in freshwater of hot springs with a temperature of 50 – 67°C [41]. Thus, in natural conditions in vivo, the protein might be binding preferentially FMN, since it would be frequently unfolded when bound to other flavin species, assuming similar melting behavior in an in vivo environment.

We next tested dependence of the melting temperature of *CagFbFP* reconstituted with different flavins on the buffer pH value (Fig. 4). We found that the FMN-bound protein is the most stable in the pH range 7–9, and the melting temperature is reduced to $\sim 62^\circ\text{C}$ at acidic pH. This might be explained by protonation of the phosphate group, which at neutral pH interacts favorably with the nearby positively charged side chains of arginines 86 and 102. Accordingly, stability of RF- and FAD-bound proteins is less dependent

Fig. 3 Thermal stability of *CagFbFP* reconstituted with different flavins. **a** Chemical structures of the flavins used as *CagFbFP* chromophores in this work: LC (7,8-dimethyl-isoalloxazine tautomer is shown), RF, FMN and FAD. **b** Protein–chromophore interactions in *CagFbFP*. Polar and charged amino acids are shown explicitly. The protein model (yellow) is PDB ID 6RHF [30]; the FAD struc-

ture (green) is taken from PDB ID 2PD7 [43]. **c, d** Melting curves of *CagFbFP* variants determined based on flavin (**c**) and aromatic amino acid (**d**) fluorescence. Upper panels show dependence of the fluorescence intensity on temperature and lower panels show the first derivative of the data. Melting temperatures are marked with dashed lines

on pH, since RF lacks the phosphate group, and the pyrophosphate moiety in FAD has higher negative charge. *Avena sativa* phototropin 1 LOV2 (AsLOV2) was recently found to be stable in a broad pH range as well [42].

2.3 Photophysical properties

To further evaluate the photophysical properties of *CagFbFP* reconstituted with different chromophores, we measured fluorescence excitation, fluorescence emission and absorption spectra (Fig. 5), as well as fluorescence quantum

Fig. 4 Dependence of the melting temperature of *CagFbFP* reconstituted with different flavins on the buffer pH value

yield (Φ_F) and extinction coefficients of the protein-bound flavin (Table 1). *CagFbFP* samples containing the natural

flavins FMN, FAD or RF exhibit similar properties that differ drastically from the protein containing LC. The spectra of *CagFbFP*-LC are similar to those of miniSOG after FMN photodegradation to LC [36]. Fluorescence quantum yield of *CagFbFP*-LC (0.5) is 1.4 times higher compared to that of other samples (0.34–0.36). However, due to its low extinction coefficient, *CagFbFP*-LC is almost three times less bright than *CagFbFP* loaded with RF/FMN/FAD (when excited at the respective excitation maximum). The results are consistent with findings for the protein W619_1-LOV [11].

Next, we analyzed photostability—the ability of the protein to remain fluorescent under constant illumination (Fig. 6). *CagFbFP*-FAD and *CagFbFP*-FMN exhibit moderate photostability with photobleaching half-times of 10.1 and 8.6 min, respectively. Decay of *CagFbFP*-FMN fluorescence is more

Fig. 5 Absorption and fluorescence spectra of *CagFbFP* reconstituted with different flavins. **a** Absorption spectra of *CagFbFP* reconstituted with LC, RF, FMN and FAD. **b** Fluorescence excitation (solid) and

emission (dashed lines) spectra of *CagFbFP* reconstituted with LC, RF, FMN and FAD

Table 1 Spectral properties and thermal stability of *CagFbFP* reconstituted with different flavins

	<i>CagFbFP</i> -LC	<i>CagFbFP</i> -RF	<i>CagFbFP</i> -FMN	<i>CagFbFP</i> -FAD
Absorbance $\lambda_{\max}$ (nm)	335, 390	447	447	447
Excitation $\lambda_{\max}$ (nm)	435	450	450	450
Emission $\lambda_{\max}$ (nm)	508	498	499	498
ϵ ($M^{-1} cm^{-1}$)	3937	16,444	15,812	15,401
Φ_F	0.50	0.36	0.34	0.34
Brightness ($M^{-1} cm^{-1}$)	1969	5920	5376	5236
$t_{bl, 50\%}$ (min)	240	0.9 (20.5)	8.6	10.1
T_{Fluor} ($^{\circ}C$)	59.8 ± 1.9	65.7 ± 1.9	80.6 ± 0.8	66.9 ± 1.4
$T_{nanoDSF}$ ($^{\circ}C$)	61.5 ± 0.1	70.1 ± 0.2	81.7 ± 0.2	71.0 ± 0.1

Errors associated with the melting temperatures (thermal stability measurements) are expressed as standard deviation derived from three independent measurements

Fig. 6 Photobleaching kinetics of *CagFbFP* bound to LC, RF, FMN and FAD. Samples were continuously illuminated using a high-power LED. During illumination, fluorescence was excited at 450 nm (435 nm for *CagFbFP*-LC) and recorded at 495 nm (1 data point per second). *CagFbFP*-LC photobleaching kinetics was recorded for 4 h (see also Figure S2)

rapid in approximately the first 5 min and slows down noticeably thereafter. Fluorescence of *CagFbFP*-RF decays with the fastest rate and reaches the half of its initial value within 0.9 min. Unexpectedly, after reaching 45%, the signal from *CagFbFP*-RF rises back to 55%, before decaying further. Finally, *CagFbFP*-LC shows an outstanding photostability with photobleaching half-time of 4 h under the same conditions (Fig. S2). Overall, these results are consistent with the model where the chromophore gets continuously degraded, until it fully converts to lumichrome, which has lower brightness (Table 1) but much higher photostability. Photodegradation of FMN to LC was previously observed for miniSOG [36].

2.4 Chromophore affinity

We have also measured the binding properties of different chromophores. Standard incubation experiments of apo-*CagFbFP* with different concentrations of chromophores were unsuccessful, presumably due to only partial refolding of the protein using our protocol and also due to considerable aggregation of apoprotein during storage and incubation. Consequently, to determine the dissociation constants (K_d), we followed the fluorescence of the samples after dilution (Fig. 7) as described in the Methods section.

As expected, *CagFbFP* has the highest affinity for FMN ($K_d = 0.35 \pm 0.02 \mu\text{M}$) and RF ($K_d = 0.34 \pm 0.04 \mu\text{M}$), which is approximately two times higher than the affinity for FAD ($K_d = 0.71 \pm 0.21 \mu\text{M}$). The weakest binding is observed for LC ($K_d = 2.3 \pm 0.3 \mu\text{M}$). Lacking the ribityl side chain, LC cannot form as many favorable interactions as other flavins.

Dissociation constants of *CagFbFP* for RF, FMN and FAD determined here are similar to the data for other LOV proteins. Dissociation constants of YtvA-LOV from *B. subtilis*

Fig. 7 Determination of *CagFbFP* dissociation constants for different flavins. Titration curves are plotted for the fraction of the bound flavins $[PF]/([PF] + [F])$ versus the concentration of the free protein $[P]$, where $[PF]$ is the concentration of the protein–flavin complex and $[F]$ is the concentration of the free flavin. Shown are the means of three independent replicates (six for *CagFbFP* reconstituted with FAD), and error bars correspond to the standard deviations

determined using ITC experiments were $K_d = 0.351$, 0.715 and $0.224 \mu\text{M}$ [10]. Dissociation constants of *EcFbFP* (variant of YtvA codon-optimized for *E. coli*) determined using fluorescence titration are $K_d = 0.76$, 0.18 and $0.13 \mu\text{M}$ and of iLOV (derived from *A. thaliana* protein) are $K_d = 0.89$, 0.23 and $0.18 \mu\text{M}$ [9], respectively. We note that some LOV domains, such as Vivid [43] and the LOV domain of WC-1 [44], bind exclusively FAD in their native host. In contrast to *CagFbFP*, the latter proteins possess an extended loop between the helices E α and F α that interacts with the phosphate and ribose of the adenosine moiety of FAD.

2.5 Chromophore composition and photostability in vivo

Different flavins are present naturally in the living cells. Since their LOV domain binding constants are comparable to each other, the chromophore composition of a particular protein may vary depending on different factors (the protein itself, the expression system, the expression conditions). To determine whether the chromophore composition of *CagFbFP* is affected by its expression level in *E. coli* strain C41 (DE3), we conducted a series of experiments where the expression was controlled by a *lac* promoter (Fig. S3). Then, expression was induced with 0.1 or $100 \mu\text{M}$ of IPTG (Fig. S4) and the protein-bound flavins were analyzed using HPLC (Fig. 8). When expressed at low levels (using $0.1 \mu\text{M}$ IPTG), the protein contains predominantly FMN (67%), and when it is overexpressed (using $100 \mu\text{M}$ IPTG), more than half of it with respect to the total flavin content contains RF (59%).

Since the affinities of the protein for FMN and RF are similar, these results suggest that initially the cytoplasmic FMN

Fig. 8 Chromophore composition of *CagFbFP* expressed under different conditions. HPLC elution profiles of flavins extracted from *CagFbFP* produced upon high- (a, 0.1 mM IPTG added) and low-level (b, 0.1 μM IPTG added) expression. Absorbance was measured at 263 nm

pool exceeds the RF pool. When FMN is depleted, given that FMN is synthesized from RF in *E. coli*, the protein starts binding more and more RF. Similar to FMN, the amount of protein-bound FAD negatively correlates with the expression level. Given that FAD is synthesized from FMN, this may also be explained by depletion of cellular FMN. Thus, in *E. coli* strain C41 (DE3), overexpression of *CagFbFP* with native flavin profile is limited by the rate of FMN biosynthesis.

Finally, we have analyzed photostability of *CagFbFP* in vivo (Fig. 9). Under overexpression conditions, illumination results in strong decay of fluorescence, which then slightly recovers before decreasing further. Under low-level expression conditions, fluorescence decays monotonically and at a slower rate (Fig. 9). These observations are consistent with the findings that overexpressed protein binds more RF than FMN, and RF-bound protein is less photostable compared to FMN-bound protein.

3 Conclusions

In this work, we developed a protocol for deflavination of *C. aggregans* LOV domain-based protein (*CagFbFP*) and successfully reconstituted it with RF, FMN, FAD and LC. We found that the physicochemical properties of *CagFbFP* strongly depend on the nature of the bound flavin. In particular, *CagFbFP* reconstituted with FMN exhibits remarkably high thermal stability, and its melting temperature exceeds the temperature of other variants tested here by ~14 °C or more. Given that the fluorescence reporter proteins are usually not overexpressed, this hints that the protein may be useful in studies conducted in harsh environments with temperatures reaching ~75 °C. On the other hand, we show that LC-bound protein is extremely

Fig. 9 In vivo photobleaching kinetics of *CagFbFP* expressed at different levels in *E. coli* under continuous laser illumination. When expressed at low level (induction using 0.1 μM IPTG), *CagFbFP* predominantly binds FMN and its fluorescence decays monotonously. Under overexpression conditions (induction using 100 μM IPTG), *CagFbFP* predominantly binds RF and its fluorescence decays more rapidly, displaying complex decay behavior, similar to that of the RF-loaded *CagFbFP* protein in vitro (Fig. 6). Fluorescence is normalized to the initial pre-photobleaching values. The spike in the 0.1 μM IPTG trace at ~18 s is an artifact

photostable. Because the chromophores have different interactions with the peptide chain, the protein can likely be mutated to alter its flavin-binding preferences, opening the possibility for fine-tuning the optical properties. Finally, our observations serve as a caution for future studies of LOV domains that are overexpressed and purified in non-native conditions, as the determined properties may be different from those observed in applications in vivo.

4 Methods

4.1 Preparation of apoprotein

Expression and purification of *CagFbFP* were conducted as described previously [30]. Pure protein sample in buffer A (300 mM NaCl, 50 mM Tris-HCl, pH 8.0) was added to 8 M GuHCl in the ratio 1:7. The solution was incubated at 70 °C for 15 min, then cooled to 40 °C and diluted to a final concentration of 6 M GuHCl (in accordance with Ni-NTA Agarose specification). Then, the solution was incubated with Ni-NTA Agarose for 1 h and loaded onto a gravity flow column. Bound protein was washed with 5 volumes of buffer A supplemented with 6 M GuHCl to remove the chromophores. The protein was refolded by consecutively washing it with 5 volumes of buffer A supplemented with 4 M, 2 M or 0 M GuHCl, respectively, and eluted in the apo form with buffer A supplemented with 200 mM imidazole. The removal of bound flavins was determined by the absence of absorbance at 450 nm.

4.2 Reconstitution of apoprotein with flavins

Refolded *CagFbFP* in apo form was mixed with RF, FMN, FAD or LC in molar ratio 1:3 and incubated for 1 h at room temperature. Reconstituted protein was concentrated with 10 K MWCO Macrosep® Advance Centrifugal Device. Unbound flavin molecules were removed during buffer exchange to 10 mM NaCl, 10 mM sodium phosphate, pH 8 through PD10 columns (GE healthcare, Freiburg, Germany). The flavin load of *CagFbFP* samples was determined from the absorbance at 450 and 280 nm as described previously [30].

4.3 Thermal and pH-stability

Thermal stability was measured as described previously [34]. pH dependence of the thermal stability was measured in a series of buffers composed of 10 mM citrate, 10 mM MES, 10 mM HEPES, 10 mM MOPS, 10 mM CHES or 10 mM CAPS with pH values between 3 and 11. The pH of each solution was adjusted by addition of concentrated HCl or NaOH.

4.4 Spectroscopy

Fluorescence measurements of *CagFbFP* containing exclusively LC, RF, FMN or FAD were conducted using the Synergy H4 Hybrid Microplate Reader (BioTek, USA). Emission was measured between 470 and 700 nm (455 and 700 nm for LC) in increments of 1 nm, with excitation fixed at 450 nm (435 nm for LC). Excitation was measured between 250 and 510 nm in increments of 1 nm, the signal was detected at 530 nm. Photobleaching, fluorescence quantum yield (Φ_F) and extinction coefficient (ϵ) were measured

as described previously [30] except for the following details. For Φ_F determinations, fluorescence of all samples was excited at 440 nm. Extinction coefficient of *CagFbFP*-LC was measured using the extinction coefficient of free LC in aqueous solution at 385 nm ($7\,600\text{ M}^{-1}\text{ cm}^{-1}$) [45].

4.5 Variation of expression conditions

CagFbFP was expressed in *E. coli* strain C41 (DE3). Cells were cultured in shaking baffled flasks in LB medium containing 100 mg l^{-1} ampicillin. Protein expression was induced by the addition of isopropyl β -D-1-thiogalactopyranoside (IPTG) when the culture reached an optical density of 0.6–0.7 at 600 nm. Two final IPTG concentrations were tested: 0.1 and 100 μM . Incubation continued for 4 h at 37 °C. The cells were harvested by centrifugation at 4000g for 10 min. For photostability measurements, small fractions of the pellets were resuspended in the phosphate-buffered saline buffer and diluted to the optical density of 0.1. For HPLC analysis of chromophore composition, the remaining cells were disrupted and the proteins were purified as described previously [30]. All the manipulations were performed under dim red light.

4.6 Chromophore composition

The protein-bound chromophore was released by denaturation in 7 M GuHCl dissolved in 50 mM ammonium acetate, pH 6. The solution was incubated at 70 °C for 15 min, then diluted to final concentration of 6 M GuHCl and centrifuged to remove denatured protein (14 000 rpm, 20 min). The supernatant was passed through Amicon Ultra-0.5 mL filters (Merck (Millipore)) with pore size of 3 MWC (13 000 rpm, 60 min) and directly used for high-performance liquid chromatography (HPLC).

For HPLC, we used the system from Shimadzu (LC10Ai), employing a XSelect HSS T3 XP Column, 100 Å, 2.5 μm , $4.6 \times 75\text{ mm}$; and from Thermo Scientific (UltiMate 3000 HPLC and UHPLC Systems), employing a Ascentis® Express C18 HPLC Column, 90 Å, 2.7 μm , $2.1 \times 150\text{ mm}$. 50 mM ammonium acetate, pH 6 (eluate B) and 70% acetonitrile in B (eluate A) were used as solvents. Authentic LC, RF, FMN and FAD (Sigma-Aldrich) were used as reference compounds. During the separation, absorbance of the eluted samples at 263 nm was recorded. The following solvent gradient was applied: $t=0$, 10:90 A:B, $t=7\text{ min}$, 35:65 A:B, $t=7.1\text{ min}$, 40:60 A:B, $t=9\text{ min}$, 40:60 A:B, $t=9.1\text{ min}$, 10:90 A:B, $t=11\text{ min}$, 10:90 A:B.

4.7 Fluorescence microscopy

Measurements were performed using LSM780 ELYRA PS.1 system (Carl Zeiss, Germany). *E. coli* cells were placed on a thin glass-bottom eight-well chambered coverslip (Ibidi, Germany), and fluorescence was excited by 488 nm laser in widefield using 100x (NA = 1.46) oil immersion objective. Emission was detected with iXon997 camera (Andor, UK) in 512 × 512 px mode, using low gain and exposure time of 100 ms. Background was masked and mean intensity across all cells was measured in each frame. Image processing was performed in FIJI [46].

4.8 Determination of dissociation constants

Dissociation constants were estimated by measuring the fluorescence of the protein upon controlled dilution. Free and bound flavin molecules have different emission spectra, which allowed us to decompose the spectra of mixtures of both into a sum of two spectra, and, thus, to estimate the concentration of each component. Titration curves were plotted for the fraction of the bound flavins versus the concentration of the free protein (apoprotein). We fitted the model to experimental data and estimated K_d and Hill-like coefficient using nonlinear least squares regression.

The protein–flavin complex was sequentially twofold diluted in buffer A (300 mM NaCl, 50 mM Tris–HCl, pH 8.0), to make a total of eight solutions. After each dilution, the solution was gently mixed using a pipette and incubated at 37 °C for 1 h to ensure complete equilibration [47]. Spectra were measured as described previously. For each protein–flavin pair, the experiment was performed three times to follow statistical errors. Free FAD is nearly nonfluorescent (23 times less bright than bound FAD as determined by authors), which results in large errors in the determination of concentrations. Therefore, six repeats were done for CagFbFP–FAD.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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