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Orange fluorescent proteins constructed from cyanobacteriochromes chromophorylated with phycoerythrobilin†

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Cyanobacteriochromes are a structurally and spectrally highly diverse class of phytochrome-related photosensory biliproteins. They contain one or more GAF domains that bind phycocyanobilin (PCB) autocatalytically; some of these proteins are also capable of further modifying PCB to phycoviolobilin or rubins. We tested the chromophorylation with the non-photochromic phycoerythrobilin (PEB) of 16 cyanobacteriochrome GAFs from *Nostoc* sp. PCC 7120, of Slr1393 from *Synechocystis* sp. PCC 6803, and of Tlr0911 from *Thermosynechococcus elongatus* BP-1. Nine GAFs could be autocatalytically chromophorylated *in vivo*/in *E. coli* with PEB, resulting in highly fluorescent biliproteins with brightness comparable to that of fluorescent proteins like GFP. In several GAFs, PEB was concomitantly converted to phycourobilin (PUB) during binding. This not only shifted the spectra, but also increased the Stokes shift. The chromophorylated GAFs could be oligomerized further by attaching a GCN4 leucine zipper domain, thereby enhancing the absorbance and fluorescence of the complexes. The presence of both PEB and PUB makes these oligomeric GAF-"bundles" interesting models for energy transfer akin to the antenna complexes found in cyanobacterial phycobilisomes. The thermal and photochemical stability and their strong brightness make these constructs promising orange fluorescent biomarkers.

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Introduction

Cyanobacteriochromes (CBCRs)[1] are cyanobacterial biliproteins acting as sensory photoreceptors. They are related to the red/far red responsive canonical phytochromes (Phys),[2] but absorb light at different wavelengths; the absorptions of the different CBCRs nearly cover the entire visible spectrum.[1,3,4] In contrast to canonical Phys, the sensory modules of CBCRs consist of several GAF domains (GAF, acronym of cGMP phosphodiesterase, adenylyl cyclase and FhlA protein domain), of which one or more bind covalently open-chain tetrapyrroles as photoactive chromophores.

The large extinction coefficient of canonical Phys and of CBCRs, and the possibility of switching these proteins by light in a photochromic manner between two thermally stable states has triggered experiments for generating constructs with modified properties.[5–9] There are several advantages of Phys[10] and CBCRs,[1] or domains thereof, that make them potentially interesting tools in biological analytics: they can be obtained by heterologous co-expression of protein- and chromophore-coding genes; and chromophore attachment is autocatalytic and does not require co-expression of lyases.[11] Their use as fluorescent biomarkers is impaired, however, by the relatively low fluorescence quantum yield. This obstacle can be overcome if phycoerythrobilin (PEB) is used as the chromophore instead of the genuine phycocyanobilin (PCB). PEB is isomeric to PCB but lacks the Δ15,16 double bond that is responsible for the photoisomerization. Such phytofluors were already obtained by chromophorylation of Phy with PEB: these orange fluorescent proteins show remarkably strong brightness (defined as the product of fluorescence quantum yield (Φ_F) and molar extinction coefficients (ε)).[6] Some CBCRs contain doubly linked PCB or phycoviolobilin (PVB) chromophores.[3,4,12–15] Such GAF domains of *Nostoc punctiforme* ATCC 29133 and one of Cph2 of *Synechocystis* sp. PCC 6803 have been tested for PEB chromophorylation.[4,16,17]

The use of CBCRs as fluorescence biomarkers is still somewhat limited. Unlike the single-gene-encoded markers such as the green fluorescent protein, GFP,[18] biliproteins require the

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external supply of suitable chromophores, or the co-transformation with genes providing the enzymes for generating the chromophore. A novel, modular access to fluorescent CBCRs has been reported; it is based on the expression of multi-gene constructs: in one approach, the structural gene is fused to genes coding for chromophore biosynthesis from endogenous heme, thereby requiring the introduction of only a single gene for labeling. This strategy has been demonstrated with a red-green photoreversible biliprotein (RGS, PCB-Slr1393 GAF3) that covalently binds *in vivo* the co-synthesized PCB and fluoresces stronger in its red state compared to the green-absorbing state.[9] The fluorescence yield of this product is larger than those of canonical Phys, but further improvement and spectral tunability of the fluorescence is desirable.

In this work, we show that many GAFs of CBCRs reported as PCB-binding proteins can be chromophorylated autocatalytically with PEB. Furthermore, in some of them, the PEB chromophore is partially converted to phycourobilin (PUB), thereby yielding orange fluorescent proteins with remarkably large Stokes shifts. By fusing the genes required for the biosynthesis of PEB, the orange fluorescent proteins could be engineered as gene-encoded fluorescent tools. As an approach to enhance the properties of phycobiliprotein biomarkers in fluorescence immunoassay, these phycobiliproteins were oligomerized to nano-complexes *via* affinity tags, such as the GCN4 leucine zipper domain: the resulting complexes showed significantly increased brightness.[19]

Materials and methods

Cloning and expression

All genetic manipulations were carried out according to standard protocols.[20] The DNA fragments of respective *gaf* domains were PCR amplified from the genomic DNA of *Nostoc* or *Thermosynechococcus elongatus* BP-1, by using the corresponding primers (Table S1†) and *Taq* DNA polymerase (Thermo Scientific, Beijing). Plasmids pET-all2699 gaf1 and pET-all2699 gaf3 carrying His-tagged *gaf1* or *gaf3* of *all2699*, respectively,[21] plasmid pET-slr1393 gaf3 carrying His-tagged *gaf3 of slr1393* (*rgs*) that encodes a His-tagged red-green switchable fluorescent CBCR (RGS) of *Synechocystis* sp. PCC6803,[9] and dual plasmid pACYC-ho1-pebS carrying *ho1* and *pebS* that yield PEB in *E. coli*[22] were reported before. To construct the plasmids for the fluorescent proteins and oligomers with affinity tags, the respective gene segments were PCR amplified from the aforementioned plasmids with the corresponding primers (Table S1†). The cloned sequences and their fusing locations are shown in Fig. S1.†

All PCR products were ligated into the cloning vector pBluescript (Stratagene). After sequence verification, the gene segments were subcloned into the expression vectors pACYC-Duet or pET30 (Novagen) (Table S2†). For expressions of these fluorescent proteins and oligomers, the pACYC-derived vectors and/or pET30-derived vectors were transformed into *E. coli* Tuner™ (DE3) (Novagen) according to the combinations shown in Table S2.† The multiply transformed cells were cultured at 18 °C in Luria–Bertani (LB) medium supplemented with kanamycin (20 μg ml^{-1}) and/or chloromycetin (17 μg ml^{-1}). After induction with isopropyl β-D-thiogalactoside (1 mM) for 4–12 hours, the cells were centrifuged at 12 000*g* for 3 minutes at 4 °C.

Protein assay

For isolation of the fluorescent proteins, the cell pellet was resuspended in ice-cold potassium phosphate buffer (KPB, 20 mM, pH 7.0) containing 0.5 M NaCl, and disrupted by sonication for 5 min at 200 W (JY92-II, Scientz Biotechnology, Ningbo, China). The suspension was centrifuged at 12 000*g* for 15 min at 4 °C, and the supernatant was purified *via* Ni^{2+}-affinity chromatography on chelating Sepharose (GE Healthcare), developed with KPB containing 0.5 M NaCl. Bound proteins were eluted with the same saline KPB containing, in addition, imidazole (0.5 M). After collection, the samples were dialyzed twice against the saline KPB.[23]

Protein concentrations were determined by the Bradford assay,[24] calibrated with bovine serum albumin. SDS-PAGE was performed with the buffer system of Laemmli.[25] Proteins were stained with Coomassie Brilliant Blue and those containing chromophores were identified by Zn^{2+}-dependent fluorescence of bilin adducts.[26]

Spectrophotometric analyses

Photoconversions were carried out with a fiber optic cold-light source (Intralux 5100, Volpi, 150 W) equipped with appropriate filters. Samples were irradiated for 5 min using 500, 570, or 650 nm interference filters (15 nm width (fwhm), light intensity 15 μmol m^{-2} s^{-1}). Reversible photochemistry was tested *via* a 500–570–500 nm or *via* a 570–650–570 nm irradiation cycle. Spectra were recorded before and after the irradiation.

All chromoproteins were investigated by UV-Vis absorbance spectroscopy (Beckman-Coulter DU 800). Covalently bound PCB, PEB and PUB in biliprotein derivatives were quantified after denaturation with acidic urea (8 M, pH 2.0) by their absorbances at 662 nm (ε = 35 500 M^{-1} cm^{-1} (ref. 27)) for PCB, 550 nm (ε = 42 800 M^{-1} cm^{-1} (ref. 28)) for PEB and 495 nm (104 000 M^{-1} cm^{-1} (ref. 29)) for PUB, respectively. Fluorescence spectra were recorded at room temperature with a model LS 55 spectrofluorometer (Perkin-Elmer). Fluorescence quantum yields, Φ_F, were determined in KPB (pH 7.0) using the known Φ_F = 0.27 of C-PC from *Nostoc*[19] and Φ_F = 0.98 of the biosynthetically-obtained PEB-CpcA as standards.[30]

Oligomerization analysis

To determine the oligomerization state of the constructed chromoproteins reconstituted in *E. coli*, they were first purified by Zn^{2+} affinity chromatography. As a second step, 0.2 ml of the concentrated purified sample (0.5 mM) were loaded on a Superdex 75 column for GAFs or Superdex 200 column for GCN4 derived GAFs. Both were developed (0.2 ml min^{-1}) with KPB (20 mM, pH 7.0) containing NaCl (0.2 M). The apparent molecular mass was determined by comparison with a marker

set (12.4–150 kDa) for GAFs or a marker set (12.4–669 kDa) for GCN4 derived GAFs.

Microscopic analysis

To detect the fluorescence of cells expressing fluorescent biliprotein(s), the respective strains were induced and grown for several hours. To let biliproteins be assembled gradually and cells be labeled well, we adjusted the growth temperature (20–30 °C) and expression time (3–8 h) after induction of *E. coli* cells. After harvesting, the cells were deposited on a glass slide, and micrographs taken with a fluorescence microscope (Immersol 518F, Carl Zeiss), fitted with a color CCD camera (SPOT RT3 25.2 2 Mp color Mosaic, SPOT).

Results

Autocatalytic chromophorylation of GAFs with PEB

During initial experiments, PEB was synthesized from heme catalyzed by the enzymes PebA and PebB.[31] However, PEB-chromophorylated GAFs did not form well in *E. coli* cells following this protocol.[9,21] Changing to PebS was more effective,[22] as it generates PEB from biliverdin as a single enzyme.[32] The PebS-based approach allowed many GAFs of CBCRs to be chromophorylated with PEB (Fig. 1, S2, S3;† Table 1). Although PEB is not the native chromophore in the CBCRs studied here, it also binds covalently to the GAFs, as demonstrated by the Zn^{2+}-dependent fluorescence of bilin adducts (Fig. S4†). While Cph1 bearing PEB absorbs at 580 nm and fluoresces at 590 nm,[6,33] the here studied PEB-GAFs of CBCRs show slightly blue shifted maxima of absorbance and fluorescence emission (Fig. 1, S2†). According to the spectra of the formed chromoproteins, the tested GAFs could be divided into three groups. Proteins from group 1, comprising All2699 GAF3 and its derivatives, bind PEB with a relatively large yield (Table 1), and the covalently bound PEB was not further modified (Fig. 1 and 2). GAFs from group 2 bound the chromophore in lower yield (Table 1), and part of the PEB was transformed to other bilins, mainly to PUB (Fig. 1, S2†). In both All3691 and Alr5272 GAF1, more than half of the covalently bound PEB was isomerized to PUB, judged from the relative absorbances at 485–495 nm (PUB) and 530–550 nm (PEB) (Fig. S2†). For chromophorylated All1280 GAF2, the PUB absorbance at 495 nm was about 1.5-fold that of PEB (550 nm) (Fig. 1). Here, the molar ratio between PEB and PUB is close to 1 : 1, in view of the fact that in native biliproteins the molar extinction coefficient of PUB is about 1.5-fold bigger than that of PEB.[28,29,34] The autocatalytic isomerization of PEB to PUB has been reported before in one GAF-domain of CBCR from *Nostoc punctiforme* ATCC 29133.[16] In a R-phycocyanin[34] and phycoerythrin,[35] the isomerization is catalyzed by a lyase. The reaction is similar to the isomerization of PCB to PVB that is lyase-catalyzed in the α-subunit of phycoerythrocyanin,[36,37] and autocatalytic in several CBCRs that contain GAF domains carrying a conserved DXCF motif.[4,13–16] For GAFs of group 3, no PEB (PUB) binding could be determined (Fig. S5†).

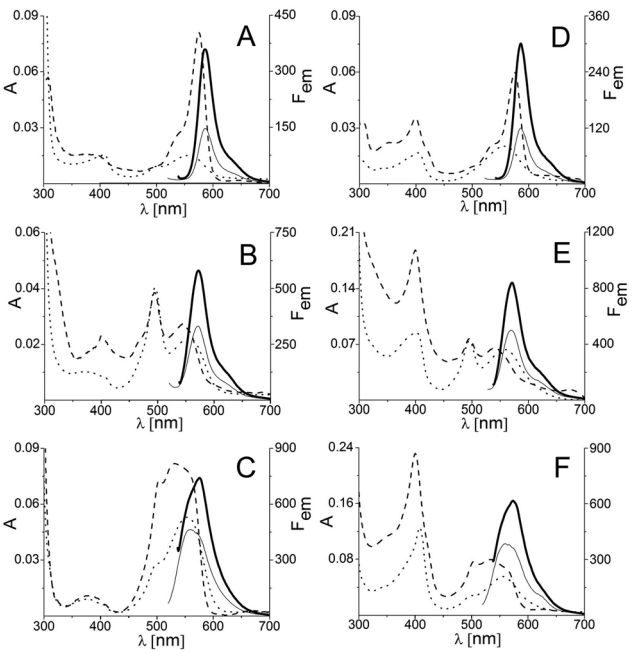

Fig. 1 Absorbance (- - -) and fluorescence emission spectra (——) of PEB-All2699 GAF1 (A), PEB(PUB)-All1280 GAF2 (B), PEB(PUB)-Slr1393 GAF3 (C), All2699 GAF1::HO1:PebS (D), All1280 GAF2::HO1::PebS (E) and Slr1393 GAF3::HO1::PebS (F). Samples were reconstituted in *E. coli*, purified with Ni^{2+} affinity chromatography and then kept in KPB (20 mM, 0.5 M NaCl, pH 7.0). Emission spectra were obtained by excitation at 500 nm (thin lines) or 520 nm (thick lines). The denatured absorbance (···) was measured at 5 min after denaturation with 8 M acidic urea (pH 2.0). Spectra of the other PEB-chromophorylated GAFs are shown in Fig. S2.†

To simplify chromophore assembly and the construction of gene-encoded orange biomarkers, fusion proteins were generated from GAF domains and the enzymes synthesizing PEB, using a similar approach as reported for PCB-binding constructs.[9] PEB-All2699 GAF1, PEB(PUB)-All1280 GAF2 and PEB (PUB)-Slr1393 GAF3 are strongly fluorescent (Table 1). The fusion with *ho1* and *pebS* was successful in *all2699 gaf1::ho1::pebS*, *all1280 gaf2::ho1::pebS* and *slr1393 gaf3::ho1::pebS* (Fig. S1, Table S1†), respectively. After expression in *E. coli* cells, PEB-All2699 GAF1::HO1::PebS and PEB(PUB)-Slr1393 GAF3::HO1::PebS could be detected (Fig. 1), which retain the spectral properties of the respective chromophorylated GAF-only chromoproteins (Table 1). The fusion proteins PEB(PUB)-All1280 GAF2::HO1::PebS and PEB(PUB)-Slr1393 GAF3::HO1::PebS exhibited a remarkable absorption at 400 nm (Fig. 1), which affects their fluorescence capacity (Table 1). The absorption band at 400 nm maybe due to bound rubinoid chromophore,[38] heme and/or derivatives.[39,40]

PEB-chromophorylated GAFs are not photochromic but highly fluorescent

With one exception (PEB(PUB)-Alr2279, Φ_F = 0.04), the fluorescence quantum yield of all PEB(PUB)-GAFs was remarkably high: it was generally well above 0.1, and reached 0.8 in All3691 GAF2 (Fig. 1, S2,† Table 1). As phytofluors,[6] PEB(PUB)-

Table 1 Quantitative absorption and fluorescence data of PEB chromophorylated GAFs of cyanobacteriochromes. Spectra were obtained in potassium phosphate buffer (20 mM, pH 7.0). Extinction coefficients and fluorescence yields were averaged from two independent experiments. The proteins beginning with "A" are from *Nostoc* PCC 7120, those beginning with "S" are from *Synechocystis* PCC 6803, and those beginning with "T" are from *Thermosynechococcus elongatus* BP-1. GCN4 zipper domains form tri- or tetrameric coiled coils structure.[42] "w" denotes weak peaks. The chromophorylation yield was determined by absorption spectroscopy of the denatured purified GAFs (see Materials and methods). GAFs of group 3 could not be chromophorylated with PEB (Fig. S5)

			Absorption		Fluorescence		Brightness	
Bilin-chromophorylated GAFs		Chromophore	λ_{max} [nm]	ε_{Vis} [mM^{-1} cm^{-1}]	λ_{max} [nm]	Φ_F	ε_{Vis} Φ_F	Yield [mg l^{-1} culture]
Group1	GCN4::All2699 GAF1	PEB	576	92	586	1.0	92	1.6
	All2699 GAF1	PEB	575	92	586	0.55	51	1.6
	All2699 GAF1::HO1::PebS	PEB	575	78	586	0.21	16	4.7
Group 2	All3691 GAF2	PUB, PEB	488, 530 (w)	125, 30	498, 556	0.80, 0.28	100, 8.4	0.60
	All1280 GAF2	PUB, PEB	495, 546	49, 24	572	0.68	33	0.17
	All1280 GAF2::HO1::PebS	PUB, PEB	495, 540	72, 28	570	0.27	19	0.13
	Slr1393 GAF3	PUB, PEB	530	43	575	0.31	13	1.5
	Slr1393 GAF3::HO1::PebS	PUB, PEB	535	42	574	0.20	8.4	0.80
	Alr5272 GAF1	PUB, PEB	495, 578 (w)	147, 9	610	0.20	29	0.03
	All2699 GAF3	PUB, PEB	502, 562	158, 58	578	0.11	17	0.05
	Tlr0911	PUB, PEB	492, 560	32, 13	574	0.41	13	0.42
	Alr3356	PUB, PEB	570	13	580	0.21	2.7	1.6
	Alr2279	PUB, PEB	492, 562	63, 43	580	0.04	2.5	0.23

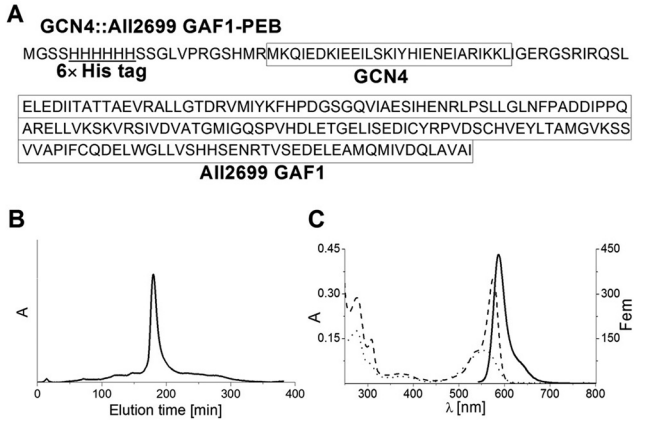

Fig. 2 GCN4::All2699 GAF1 hexamers. A: construction scheme of GCN4::All2699 GAF1, B: gel filtration (Superdex 200) of PEB-chromophorylated GCN4::All2699 GAF1. By comparison with a marker set (12.4–669 kDa), the peak of eluting at 180 min corresponds to a mw of 130 kDa, corresponding to a hexamer $(GCN4::All2699\ GAF1\text{-}PEB)_6$ (calculated 152 kDa); C: absorbance (- - -) and fluorescence (——) spectra of $(GCN4::All2699\ GAF1\text{-}PEB)_6$ with conditions as in Fig. 1. The denatured absorbance (···) was measured 5 min after denaturation with 8 M acidic urea (pH 2.0).

GAFs are not photochromic (Fig. S6†). Unlike photochromic biliproteins containing PCB, PVB, biliverdin, or phytochromobilin, the isomerizing Δ15,16 double-bond is lacking in PEB and PUB; instead these chromophores carry single bonds between rings C and D. Therefore, the photochemical channel is blocked and these proteins show significantly increased fluorescence quantum yields (Table 1). Other factors, including the differential interactions of the apo-proteins with the modified chromophores may also contribute to the higher fluorescence quantum yield. It had been similarly reported that Cph1 bearing PCB has low fluorescence capacity but Cph1 bearing PEB fluoresces highly at 590 nm (Φ_F = 0.72).[6,33]

Based on the successful generation of orange fluorescent GAFs, it is expected that the constructs could well serve for labeling living cells. As an example, fluorescent *E. coli* used in this work for expression is shown in Fig. 3. PEB was attached covalently to All2699 GAF1, which does not further modify the chromophore. The PEB-chromophorylated product, PEB-All2699 GAF1, shows both a high extinction coefficient ($\varepsilon = 92 \times 10^3$) and a high fluorescence yield (Φ_F = 0.55) (Fig. 1, Table 1). This results in brightness as high as 50 mM^{-1} cm^{-1} that compares favorably with green fluorescent proteins (GFPs).[41]

PEB-Slr1393 GAF3 shows the absorption splitting into three peaks at 504, 530 and 560 (shoulder) nm (Fig. 1). By excitation

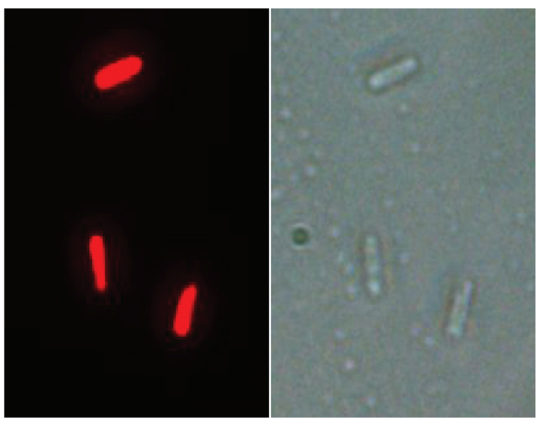

Fig. 3 Fluorescence (left panel) and bright-field (right panel) micrographs of *E. coli* cells expressing PEB-All2699 GAF1. *E. coli* cells yielding only PEB did not fluoresce (data not shown). Excitation was done with a band-pass filter (540–555 nm), and emission collected through a low-pass filter (>590 nm) (for details see Material and methods).

at >520 nm, a strong emission was observed at 575 nm, corresponding to a Stokes shift of ~60 nm that would facilitate its application in fluorescence labeling. Excitation at 500 nm causes a 30% lower emission at 560 and 575 nm, but it is even further separated from the absorption peak (Fig. 1). PEB(PUB)-All1280 shows an exceptionally large Stokes shift of almost 80 nm. As mentioned above, this singly chromophorylated construct contains a chromophore mixture of PEB absorbing at 546 nm, and PUB absorbing at 496 nm due to partial conversion of the former into the latter (Fig. 1, Table 1). When PEB(PUB)-All1280 was excited at 450–500 nm into the PUB absorption band, a fluorescence peaking at 572 nm was observed that originates from PEB (Fig. 1), indicating that the absorbed energy is transferred from PUB to PEB (Fig. S3†). This energy transfer requires the two chromophores in close proximity and indicates that the holo-GAFs aggregate in solution.[14] In agreement, the solutions contain exclusively dimers as determined by gel filtration (Fig. S7†).

Another favorable aspect of aggregation is an increased brightness of individual chromoprotein complexes, because the sensitivity increases with the number of chromophores. We were able to enhance the brightness of the chromophorylated GAF by constructs forming stable oligomers (Fig. 2), which facilitates the applications of biliprotein biomarkers in fluorescence immunoassay. When isolated from *E. coli*, PEB-All2699 GAF1 is already dimeric (Fig. S7†), resulting in doubling of the molar extinction coefficient of a single particle to $\varepsilon = 18.4 \times 10^4$ M^{-1} cm^{-1} (Table 1), and a brightness for the dimer of 101×10^3 M^{-1} cm^{-1}. For further increase of fluorescence and brightness, we employed coiled coil structures such as GCN4 leucine zipper domains which are known to oligomerize into trimers or tetramers.[42] Oligomerization of GCN4 fusion products have been reported; they form nano-complexes *via* the GCN4-pII or GCN4-pLI affinity tag.[19] When All2699 GAF1 was linked with GCN4-pII, the resulting GCN4::All2699 GAF1 formed hexamers (Fig. 2). The molar extinction coefficient of the monomer is unchanged by this fusion but, remarkably, the fluorescence yield is increased virtually to 1 (Table 1), possibly as a result of increased rigidity in the aggregated fusion product. Together with the increased number of chromophores per particle, this results in an extraordinary brightness of 552×10^3 M^{-1} cm^{-1}, corresponding to a 4-fold increase over the dimer.

Discussion

The chromophorylation of GAFs of CBCRs with PEB demonstrated by this work is advantageous in three aspects: (1) The GAFs are of comparable size of the phycobiliproteins, but bind bilins autocatalytically and the chromophorylation does not depend on lyases; (2) PEB-chromophorylated GAFs show high fluorescence quantum yields which, in combination with their large extinction coefficients, result in great brightness; (3) certain GAFs having the DXCF motif can isomerize PEB to PUB, thereby extending the Stokes shifts and spectral variation of PEB(PUB)-GAFs. The properties of the here presented fluorescent CBCR-GAF domains compare favorably with GFP derivatives with respect to both wavelengths of excitation and emission, and brightness.

Our screening of 18 GAFs with PEB yielded three particularly interesting fluorescent proteins: PEB-All2699 GAF1, PUB-(PEB)-All3691 GAF2, and PEB(PUB)-All1280 GAF2 (Table 1). PEB-All2699 GAF1 fluoresces maximally at 586 nm, emitting orange light, PUB(PEB)-All3691 GAF2 emits maximally at 498 nm with a bright green fluorescence, and PEB(PUB)-Slr1393 GAF3 (as well as PEB(PUB)-All1280 GAF2) show a bright orange fluorescence around 575 nm with a large Stokes shift of 60–80 nm. Together with red fluorescent GAFs,[9,14] these spectral variations can provide, alone or in combination, flexible applications as fluorescent biomarkers. Similar to PCB, PEB could be generated intracellularly from heme by the introduction of only two enzymes, *viz.* heme oxygenase (HO1) and PEB synthase (PebS). Introducing a single plasmid generating their fusion product, HO1::PebS, was similarly efficient in yielding PEB as co-expression of both enzymes, thus facilitating the application of GAFs in fluorescence labeling. Similar to the genuine photoactive chromophore, PCB, the photochemically inactive PEB is autocatalytically attached to GAFs. Moreover, GAFs containing the DXCF motif also accept PEB as the substrate for isomerization to PUB during the autocatalytic attachment.[4,12–16] The isomerization of PEB to PUB extends the Stokes shift of holo-GAFs and facilitates applications based on energy transfer. A certain disadvantage of PEB containing chromoproteins in tissue applications is the blue-shifted absorption and fluorescence, compared to PCB-containing ones. In practice, this needs to be judged against their brightness fluorescence and spectral coverage.

Isomerization of PEB to PUB is determined by the apoproteins. It generally parallels the isomerization of PCB to PVB. All2699 GAF1 binds PCB[21] as well as PEB (Fig. 1) without isomerizing the chromophore. All3691 GAF2, All1280-GAF2 and Alr2279 all bind PCB[15] as well as PEB (Fig. S2†), with concomitant isomerization of comparable fractions of the chromophores to PVB and PUB, respectively (Fig. 1, S2†). There are, however, exceptions: during the chromophorylation of Slr1393 GAF3[21] and Alr3356,[15] PCB does not isomerize, while part of PEB can isomerize to PUB (Fig. 1, S2†). In this case, isomerization efficiency is higher for PEB than for PCB.

The extent of isomerization is particularly relevant with regard to the oligomerization state of the construct *in vivo*. The larger the oligomer with coupled chromophores, the higher the amount of PUB that results in optimum brightness and spectral separation of excitation and emission. Constructs containing the GCN4 leucine zipper domain contain 6 chromophores. Assuming a 1 : 1 ratio of PEB and PUB, only 1.5% of hexamers would lack a long-wavelength fluorescing PEB chromophore, while 98.5% would have at least one such chromophore and an accordingly large Stokes shift. Under such a scenario, higher PUB/PEB ratios would even be more favorable, as the PUB would act as a light-harvesting antenna for the few fluorescent PEB. Assuming an energy transfer

efficiency of 100%, the increase of the PUB : PEB ratio to 10 : 1 would duplicate the brightness: the absorption around 500 nm due to PUB is increased 4.5 fold, while nearly 50% of the particles still contain at least one emitting PEB. Bi-chromophoric aggregates can also serve as model systems for natural antenna systems like R-phycoerythrin that show efficient energy transfer from PUB to PEB.

Abbreviations

CBCR	Cyanobacteriochrome
GAF	Acronym of cGMP phosphodiesterase, adenylyl cyclase and FhlA protein domain (SMART acc. no. SM00065)
GFP	Green fluorescent protein
HO1	Heme oxygenase 1
KPB	Potassium phosphate buffer
Nostoc	*Anabaena* (*Nostoc*) sp. PCC 7120
PCB	Phycocyanobilin
PEB	Phycoerythrobilin
PebS	PEB synthase
PUB	Phycourobilin
PVB	Phycoviolobilin
Phy	Phytochrome
RGS	Red-green switchable protein encoded by *rgs* = *slr1393*.

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