VIP Photoswitchable Proteins

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Fused-Gene Approach to Photoswitchable and Fluorescent Biliproteins**

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Fluorescent and photoswitchable proteins are invaluable in life sciences and considered for applications in data storage. Of particular interest for in vivo studies are fluorescent proteins whose chromophores are generated autocatalytically from the amino acid chain;[1] some of them can also be switched between two states.[2,3] Alternatively, apoproteins can be used that spontaneously incorporate endogenous chromophores like retinal.[4,5]

The open-chain tetrapyrrole chromophore of biliproteins is subject to remarkable excited-state control of the chromophore by the apoprotein.[6–8] Absorption and fluorescence of free bilins like the phycocyanobilin (PCB) is strongly increased in native biliproteins: the maximum can be shifted by over 100 nm, and a photochemical reaction path is opened in photochromic biliproteins like phytochromes[9] and cyano-(bacterio)chromes.[7] These natural variations and the possibility to modulate the photophysical properties render biliproteins, in principle, excellent biomarkers and photonic materials.

Applications have been limited, however, because the bilin chromophores must be provided separately and then attached covalently to the apoproteins. Previously, genes of the apoprotein were co-expressed with genes whose products generate the bilin chromophore from endogenous heme and then attach it covalently to the apoprotein.[10–12] We now report an alternative approach that generates various biliproteins in situ from a single, multifunctional gene and endogenous heme. This approach is demonstrated by the synthesis of two persistently red-fluorescent biliproteins based on allophycocyanins, and by photochromic biliproteins derived from a novel cyanobacteriochrome that can be reversibly switched from a state absorbing and strongly fluorescing in the red, to a spectroscopically well-separated, less fluorescent state absorbing in the green spectral region.

Gene *slr1393* of the cyanobacterium *Synechocystis* sp. PCC6803 encodes a red–green photoreversible cyanobacteriochrome. The full-length protein contains three GAF domains, but GAF3 (aa 441–596) alone is capable of autocatalytically binding PCB to cysteine-528.[21] Addition of PCB to GA results in a reversibly photochromic chromoprotein, termed RGS (red–green switchable protein): state P_r ($\lambda_{max} = 650$ nm) is strongly fluorescent ($\Phi_F = 0.06$); it is reversibly converted by irradiation with red light into state P_g ($\lambda_{max} = 539$ nm), which has reduced and strongly blue-shifted fluorescence (Table 1, Figure 1 a). Photoswitching can be repeated many times; it is stable over a wide pH range, and is retained after RGS is embedded into polyvinyl alcohol (PVA) film (see Figures S1 and S2 in the Supporting Information).

Chromophorylated RGS can be produced in *E. coli*[11,13] that has been multiply transformed to produce the GAF3 apoprotein and two biosynthetic enzymes generating PCB from heme, that is, heme oxygenase (HO1) and the biliverdin reductase (PcyA). The cells show an intense red fluorescence that can be abolished by irradiation with red light and is regained with green light (see Figure S2 in the Supporting Information). When *pcyA* was replaced by *hy2,* the phytochromobilin chromophore (PΦB) was produced. The photochromic protein generated can be photoswitched reversibly between P_r ($\lambda_{max} = 663$ nm) and P_g ($\lambda_{max} = 573$ nm); in this case, both are moderately fluorescent (Table 1).

HO1 and PcyA are thought to be involved in substrate channeling[14] of the biliverdin produced by HO1; therefore, we fused the two genes and introduced the *ho1:pcyA* construct together with a plasmid containing the apoprotein gene, *gaf3*, into *E. coli*. These cells produced spectroscopically indistinguishable chromophorylated RGS in comparable yield (70–90%, Table 1) as previously with the separate plasmids. Finally, the gene *gaf3* coding for the apoprotein was fused to *ho1:pcyA* at the 5′-end. *E. coli* cells expressing the

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Table 1: Quantitative absorption and fluorescence data of persistently fluorescent and red–green photoswitchable (RGS) biliproteins.[a]

Biliprotein	Yield [mg L^{-1} culture]	Absorption λ_{max} [nm]		ε [M^{-1}cm^{-1}]×10^{-4}[b]		Fluorescence λ_{max} [nm]		Φ_F	
		15Z	15E	15Z	15E	15Z	15E	15Z	15E
Photoswitchable (RGS)									
PCB-GAF3:HO1:PcyA[c]	2.2	648	536	9.7	4.7	670	617	0.07	0.04
PCB-GAF3 (RGS)[c]	3.2	650	539	9.3	5.0	672	616	0.06	0.03
PΦB-GAF3[c]	n.d.	663	573	9.1	4.5	685	631	0.04	0.03
PΦB-GAF3:HO1:HY2[d]	n.d.	661	560	–	–	680	625	0.01	0.006
Persistently fluorescent									
PCB-ApcA:HO1:PcyA[c]	0.10	617, 635[e]	–	5.9	–	638	–	0.14	–
PCB-ApcE(1-258):HO1:PcyA[c]	0.15	658	–	9.9	–	672	–	0.15	–
PΦB-ApcE(1-258):HO1:HY2[d]	n.d.	670	–	–	–	680	–	0.005	–

[a] Spectra were recorded in potassium phosphate buffer (20 mM, pH 7.0). Data were averaged from two independent experiments. [b] The molar extinction coefficient of PΦB chromoproteins was calculated with the approximation that the molar extinction coefficient at 675 nm in acidic urea (8 M, pH 1.5) is the same as that of PCB chromoproteins at 660 nm in acidic urea (8 M, pH 1.5), that is, 35 500 M^{-1}cm^{-1}. [c] Spectra obtained with His$_6$-tagged chromoproteins after purification by Ni^{2+}-affinity chromatography. [d] Spectra obtained from supernatants of untagged chromoproteins. [e] Spectra differ from that of natural α-APC because of the lack of lyase.

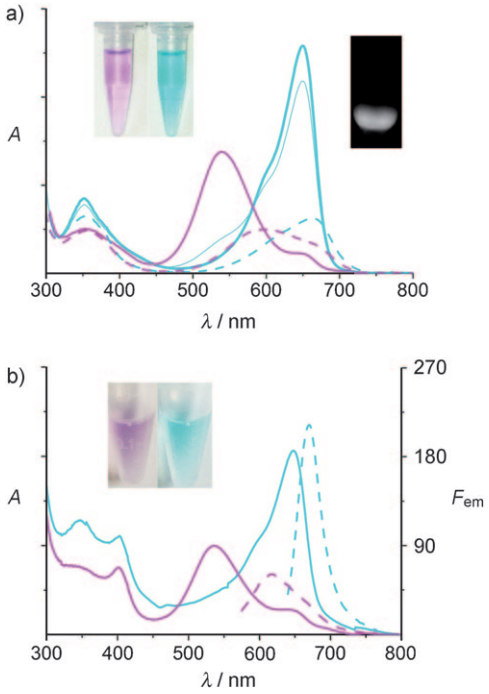

Figure 1. Photochromism of His$_6$-tagged RGS biliproteins. a) PCB-GAF3. Absorption spectra of the native biliprotein in the P$_r$ state (15Z chromophore, thick solid blue line) obtained after irradiation with 500 nm light, in the P$_g$ state (15E chromophore, thick solid pink line) obtained with 650 nm light, and of the mixture (mostly 15Z, thin solid blue line) obtained with white light, and of denatured P$_r$ (dashed blue line) and P$_g$ forms (dashed pink line). Left insert: tubes containing P$_r$ (green) and P$_g$ (pink); right insert: Zn^{2+}-induced fluorescence in SDS-PAGE. b) PCB-GAF3:HO1:PcyA: Absorption (solid blue line) and fluorescence emission spectra (dashed blue line) of the P$_r$ state (15Z chromophore) obtained after irradiation with 500 nm light (fluorescence excitation at 620 nm), and of the P$_g$ state (15E chromophore) obtained after irradiation with 650 nm light (absorption (solid pink line), and fluorescence (dashed pink line) excited at 550 nm). Insert shows tubes with P$_g$ (left) and P$_r$ (right). All samples were purified by Ni^{2+}-affinity chromatography; spectra of native biliproteins were recorded in potassium phosphate buffer (20 mM , pH 7.0) containing 0.5 M NaCl, those of denatured biliproteins in 8 M urea, pH 1.5.

trigenic construct *gaf3*:*ho1*:*pcyA* were red-fluorescing (Figure 1) and produced chromophorylated RGS:HO1:PcyA in good yield (Table 1). Its spectroscopic properties are like those of RGS (Figure 1 b); the fluorescence yield and photochemistry are comparable (Table 1). Also this fusion protein retained its photochromicity in PVA film (Figure 2). Replacing *pcyA* by *hy2* again generated a photochromic chromoprotein carrying a PΦB chromophore that has red-shifted, but otherwise identical, spectra (Table 1 and Figure S3 in the Supporting Information).

The gene-fusion approach can also be used to generate persistently fluorescent proteins from a single plasmid. In the first example, the GAF3 apoprotein is replaced by the chromophore domain (aa 1-258) of the phycobilisome linker ApcE, which attaches PCB autocatalytically.[15] *E. coli* cells transformed with *apcE*(1-258):*ho1*:*pcyA* showed a stable red fluorescence (Table 1, Figure S4 in the Supporting Information). Yet another persistently red-fluorescent protein was

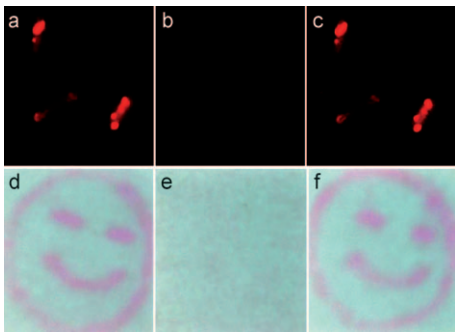

Figure 2. Photochromism of His$_6$-tagged PCB-GAF3:HO1:PcyA in *E. coli* cells and PVA film. Fluorescence micrographs were recorded: a) after irradiation with green light (band-pass filter 540–555 nm) using a 665–715 nm fluorescence filter; b) after irradiation with red light (640–660 nm) using a 685–735 nm fluorescence filter; and, c) after re-irradiation with green light (540–555 nm light) using a 665–715 nm fluorescence filter. d) A smiley face was drawn on the PVA film containing PCB-GAF3:HO1:PcyA with a 650 nm red laser pen; the image could be erased by irradiation with green light (e), and drawn again with the red laser pen (f).

obtained by replacing *apcE(1-258)* by *apcA,* which codes for the α subunit of allophycocyanin (Table 1).

When the ubiquitous endogenous heme is used as a precursor for bilin chromophores, the introduction of a single, multigenic construct is obviously sufficient for generating biliproteins which, depending on the apoprotein used, are persistently fluorescent or photoswitchable between two states. Although the chromophore is not generated from the protein as in GFP-like proteins,[1] the constructs should be usable as a reporter in many organisms. At least in *E. coli*, there was no obvious effect of diverting heme to bilins, but this needs to be ascertained in other cases.

In RGS, the red fluorescence is turned on and off with strong red and green light pulses, respectively. Very little overlap exists between the two states ($\Delta\lambda = 110$ nm), and broad-band filters can be used for irradiation and detection. In many phytochromes and cyanobacteriochromes, one of the states (generally P_{fr} containing the 15E chromophore) also reverts thermally, which is desirable for certain microscopic applications.[3] A nearly complete coverage of the visible spectrum (430–750 nm) is possible with chromophore domains from known cyanobacteriochromes and phytochromes,[7,9] and further extensions are expected to be found in nature, or generated by mutagenesis of the apoproteins, or proper choice of reductases. This also allows for changing the photophysical properties of the products, such as the fluorescence yields in the two states, the direction of the absorption or fluorescence shift, or the thermal stability of the 15E states (see Table 1). Currently, controlled mutagenesis of biliproteins for particular biophysical properties is still largely by trial and error, but some principles are emerging, and expected to develop rapidly in the near future. An obvious extension to phycobiliproteins that do not bind the chromophore autocatalytically is the further fusion to lyases for chromophore attachment.[12] Last, but not least, the use of fused biosynthetic genes like *ho1:pcyA* is valuable for a synthon approach to the assembly of phycobilisomes.

Experimental Section

The *gaf3* DNA fragment of *slr1393* was amplified from genomic DNA of *Synechocystis sp.* PCC6803 by PCR and inserted into pET30 or, for co-transformation with pET-*ho1*:pcyA*, into pCDF. The *Arabidopsis* gene *hy2* was obtained from *Tair* (http://www.arabidopsis.org/) and subcloned in pACYC-*ho1*[16] to yield pACYC-*ho1-hy2*. The expression vectors were transformed into *E. coli* Tuner (DE3) (Novagen) for respective overexpression. All sequences were verified.

Micrographs of cells were recorded with a fluorescence microscope; switching between photochemically reversible chromoprotein states was done with the microscope lamp using red (640–660 nm) or green band-pass filters (540–555 nm).

Chromoproteins were purified by Ni^{2+}-affinity chromatography on chelating Sepharose; they were analyzed spectroscopically using the extinction coefficient of protein-bound PCB in 8 M acidic urea[17] and the fluorescence yield of phycocyanin from *Nostoc*[18] as standards. Photoreactions were induced with a fiber optical cold-light source equipped with suitable interference filters.[19]

Chromoproteins were immobilized in polyvinyl alcohol film by mixing an aqueous PVA solution (7%) with the same volume of the sample (0.7 mM in 20 mM KPB containing 0.5 M NaCl, pH 7.0).[20]

Further details are given in the Supporting Information.

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