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蓝藻红色荧光蛋白 All1280 GAF2 在 *E. coli* BL21 (DE3)中的表达及其突变体构建

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摘 要: 采用 PCR 技术从鱼腥藻(*Anabaena* sp.) PCC 7120 中扩增获得红色荧光蛋白基因 *all1280 gaf2*, 并利用 *Bam*H Ⅰ和 *Sal* Ⅰ酶切位点, 将该基因插入到 pET-30a(+)中, 构建表达载体 pET-*all1280 gaf2*。将该表达载体与藻胆色素生物合成质粒 pACYC-*ho1-pcyA* 同时转化到大肠杆菌 *E. coli* BL21 (DE3), 表达后获得大肠杆菌色素细胞。结果显示, 该色素细胞在荧光显微镜下具有红色荧光, 且在 *15E/15Z* 态之间具有可逆光效应。进一步以 pET-*all1280 gaf2* 为模板, 通过定点突变技术在 *all1280 gaf2* 基因中引入 C53A 突变, 获得了突变体 All1280 GAF2 (C53A)。将 All1280 GAF2 (C53A)与藻胆色素在 *E. coli* BL21 (DE3)中共表达, 获得了比野生型红色荧光更强的大肠杆菌色素细胞。研究结果表明, 与野生型相比, All1280 GAF2 (C53A)具有较高的摩尔消光系数和荧光量子产率, 红色荧光更强。

关键词: 蓝藻光敏色素; 荧光蛋白; 探针; 可逆效应
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Expression of red fluorescent protein All1280 GAF2 in *E. coli* and constructing mutation of cyanobacteriochrome

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Abstract: Cyanobacteriochromes contain GAF (cGMP phosphodiesterase, adenylyl cyclase, and FhlA protein) domains in the N-terminal region that bind phycocyanobilin autocatalytically. In the current study, we amplified the cyanobacteriochrome gene fragment of *all1280 gaf2* from *Anabaena* sp. PCC 7120 using PCR, and then inserted it into pET-30a(+). For over-expression, both pET-*all1280 gaf2* and pACYC-*ho1-pcyA*, which catalyze phycocyanobilin (PCB) biosynthesis, were transformed into *E. coli* BL21 (DE3). Cells harboring pET-*all1280 gaf2* and pACYC-*ho1-pcyA* expressed chromophorylated All1280 GAF2 successfully. Results

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showed that All1280 GAF2 underwent reversible photoconversion between the *15E* form (λ_{max} = 560 nm) and *15Z* form (λ_{max} = 413 nm). Using fluorescent microscopy, we detected a red fluorescence/no fluorescence reversible photoconversion of All1280 GAF2 in *E. coli* BL21 (DE3). Cys53 was essential for photoconversion of All1280 GAF2 because its mutagenesis resulted in a PCB adduct, which exhibited no photoconversion but stable red fluorescence. Compared with the wild-type, All1280 GAF2(C53A) had stronger red fluorescence with higher extinction coefficients and fluorescence yields. It is expected that these two constructs could serve well in the labeling of living cells.

Key words: Cyanobacteriochrome; Fluorescent chromoprotein; Probe; Photoreversibility

Cyanobacteriochromes are important photochromic photoreceptors of the light harvesting system in cyanobacteria. At the N-terminus, cyanobacteriochromes contain one or several GAF (cGMP phosphodiesterase, adenylyl cyclase, and FhlA protein) domains, which are covalently linked with phycocyanobilin (PCB) by a thioethel bond[1]. Cyanobacteriochromes can absorb light of specific wavelength and generate strong fluorescence, thus emerging as a promising fluorescent protein probe in organisms[2].

Fluorescent proteins constructed from cyanobacteriochromes can emit fluorescence upon illumination with specific light. This fluorescence effect can be used to develop a novel detection system for tracking temporal and spatial changes in living cells[3]. Earlier studies have mainly focused on the utility of green fluorescent protein (GFP) and its derivatives[4,5]. However, the emission wavelengths of GFP derivatives are limited within the range of 440–550 nm[6]. Compared with GFP derivatives, red fluorescent proteins from cyanobacteriochromes show longer emission wavelengths, lower image backgrounds, and lower absorption in living cells[7]. In addition, GAF domains of cyanobacteriochromes can be self-catalyzed to bind to PCB without the catalysis of lyase[8]. These natural properties render cyanobacteriochromes as excellent biomarkers and photochemical material.

Based on bioinformatics, All1280 from *Anabaena* sp. PCC 7120 is considered a putative cyanobacteriochrome apoprotein composed two GAF domains in the N-terminal region. Previous analysis has also shown All1280 GAF2 to have a 42% homology with red fluorescent protein (RGS) (coded by *slr1393* gene)[9]. To study the fluorescence properties of All1280 GAF2, we co-expressed All1280 GAF2 with an enzyme that catalyzed PCB biosynthesis in *E. coli* BL21 (DE3). The red fluorescence of *E. coli* cells expressing chromoprotein All1280 GAF2 was then detected. We also performed site-direct mutagenesis of Cys53 in All1280 GAF2. Results showed that mutagenesis of Cys53, which was conserved in a subclass of cyanobacteriochromes, abolished *15Z/15E* photoconversion of All1280 GAF2, thereby yielding stable red fluorescent protein.

1 Materials and methods

1.1 Reagents, cells, and plasmid vectors

Expression vector pET-30a (+) was purchased from Novagen Corporation. *Anabaena* sp. PCC 7120 was purchased from the Freshwater Algae Culture Collection of the Chinese Academy of Sciences. The site-directed mutagenesis kit was obtained from Takara. pACYC-*ho1-pcy*A, a PCB biosynthesis plasmid whose products generated chromophores from endogenous heme HO1 and biliverdin reductase PcyA in *E. coli* BL21 (DE3), and *E. coli* BL21(DE3) were obtained from the Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University.

1.2 Construction of expression plasmids

Primers were designed based on the nucleotide sequence of *all1280 gaf2* (Table 1). Using the genomic DNA of *Anabaena* sp. PCC 7120 as a template, *all1280 gaf2* was amplified with primers P1－P2. The *all1280 gaf2* was then inserted into pET- 30a(+) using *Bam*H Ⅰ and *Sal* Ⅰ enzyme sites, with pET-*all1280 gaf2* thus obtained. For site-directed mutagenesis, P3 was designed as the conserved *C53* replaced with A53. Using the site-directed mutagenesis kit, *all1280 gaf2* (C53A) was amplified with pET-*all1280 gaf2* as the template, P3 and P4 as the primers. The *all1280 gaf2*(C53A) was then inserted into pET-30a(+) using *Bam*H Ⅰ and *Sal* Ⅰ, with pET-*all1280 gaf2*(C53A) thus constructed. Nucleotide sequencing identified that *all1280 gaf2* and *all1280 gaf2*(*C53A*) fragments were successfully cloned into the pET-30a (+) vector.

Table 1　Primers for construction of plasmids

Primer	Sequence	DNA
P1	5′-CCCGGATCCCAATCGCTGAATCTA-CAAAAT-3′	*all1280 gaf2*
P2	5′-GGCGTCGACTTACAATTCATTCT-GGGCTTG-3′	
P3	5′-AATGCATTTCAGTCAGGAGCAGGA-3′	*all1280 gaf2* (*C53A*)
P4	5′-ATCTTGAATATCTGCGCCTAGAGC-3′	

1.3 Expression and purification of chromoprotein

The pET expression vector and PCB biosynthesis plasmid pACYC-*ho*1-*pcyA* were transformed into *E. coli* BL21 (DE3) and cultivated on LB agar medium containing chloramphenicol (20 μg/mL) and kanamycin (40 μg/mL). The positive clones were isolated and later cultivated in LB liquid medium. When OD_{600} reached 0.8, the expression of chromoprotein was induced by 1 mmol/L isopropyl β-D-thiogalactoside (IPTG). After expression for 14 h at 18℃ and 150 r/min in the dark, cells were collected by centrifugation and washed with distilled water. The cells were pthen re-suspended in potassium phosphate buffer (KPB, 20 mmol/L, pH 7.2) and broken with sonication for 3 min at 50 W. The suspension was centrifugated at 9000 *g* for 15 min at 4℃. The supernatant was then purified via Ni^{2+} affinity chromatography. We used KPB (20 mmol/L, pH 7.2) containing 0.05 mol/L imidazole for impurity removal and KPB containing 0.5 mol/L imidazole for elution of the target protein[10].

1.4 Protein assay

Protein concentrations were determined by the Bradford assay, calibrated with bovine serum albumin. SDS-PAGE was performed with the buffer system of Laemmli[10]. Proteins were stained with 1.5 mol/L zinc acetate solution for 5 min, and then stained with Coomassie brilliant blue.

1.5 Spectrum analysis of chromoprotein

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 400 or 570 nm interference filters. The absorption spectra were obtained using a UV-Vis spectrometer (Perkin-Elmer Lambda 25) within the 300－800 nm scanning range. The fluorescence spectra were measured using a fluorescence spectrometer (Hitachi F4500). The fluorescence quantum yield of the chromoprotein, ΦF, was calculated based on the absorption and fluorescence properties of the chromoprotein[11].

1.6 Chromoprotein denaturation

To determine the type of chromophore, chromoproteins were denatured with acidic urea (8 mol/L, pH 1.5). After denaturation, chromophores were free from apoproteins. The absorption spectra of the denatured supernatants were then measured, and the types of chromophores were determined according to the absorption spectra. In addition, the molar extinction coefficient, ε_{Vis}, of the chromoprotein was calculated using Lambert-Beer's law based on the absorption spectra of the natural chromoprotein and denatured solution[10].

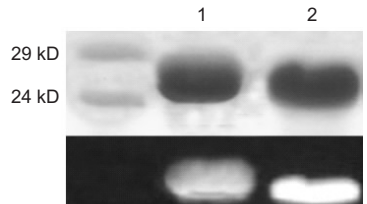
1.7 Fluorescence detection of cells expressing chromoprotein

Cells expressing chromoprotein were obtained via co-expressing apoprotein with PCB in *E. coli*. Cells were washed twice with distilled water and then suspended with 0.05% agarose. Samples were deposited on glass slides at room temperature for 10 min. Bright-field micrographs were taken under white light and fluorescence micrographs were taken under cold light via fluorescence microscopy[11].

2 Results

2.1 Identification of chromophorylated All1280 GAF2 and All1280 GAF2(C53A)

All1280 GAF2 and All1280 GAF2 (C53A) were co-expressed with PCB in *E. coli* BL21 (DE3), respectively. The purified chromoproteins, All1280 GAF2 and All1280 GAF2(C53A), were then subjected to SDS-PAGE, followed by Zn^{2+} staining or Coomassie brilliant blue staining. The corresponding bands of All1280 GAF2 and All1280 GAF2 (C53A) were detected in SDS-PAGE, consistent with its molecular weight of about 26 kD (Fig.1). All1280 GAF2 and All1280 GAF2(C53A) both showed Zn^{2+}-induced fluorescent bands, indicating these two proteins were successfully expressed in *E. coli* BL21(DE3) and could bind to PCB covalently.



1: All1280 GAF2; 2: All1280 GAF2(C53A).

Fig. 1 SDS-PAGE profiles with Zn^{2+} staining and Coomassie brilliant blue staining

2.2 Chromophorylated All1280 GAF2 is photoreversible and fluorescent

Chromophorylated All1280 GAF2 demonstrated reversibility between *15E/15Z* forms with irradiation of certain wavelength light. After irradiation with a 400-nm light, chromophorylated All1280 GAF2 was observed in the *15E* photo-excited form. Furthermore, it exhibited remarkable absorption at 560 nm (Fig.2: a), maximum fluorescence emission at 635 nm (Fig.2: b), and fluorescence quantum yield of 0.07 (Table 2), indicating relatively high fluorescence activity. After irradiation with a 570-nm light, chromophorylated All1280 GAF2 transformed from the *15E* photo-excited form to *15Z* dark form. The most intensive absorption peak of *15Z*-All1280 GAF2 was observed at 413 nm (Fig.2: a). At the same time, its fluorescence emission peak at 635 nm disappeared (Fig.2: b) and fluorescence quantum yield was zero (Table 2). Via recurrent irradiation with 400-nm/570-nm light, the absorption spectra of chromophorylated All1280 GAF2 demonstrated reversibility between 413 nm and 560 nm. The fluorescence emission peak at 635 nm was also reversible between the red fluorescence form and non-fluorescence form.

The binding of apoprotein with chromophore is autocatalytic. Some cyanobacteriochromes can isomerize PCB into phycoviolobilin (PVB) during the binding process[11]. In the current study, All1280 GAF2 was found to partially isomerize PCB into PVB (Table 2).

2.3 Fluorescence properties of *E. coli* cells expressing chromophorylated All1280 GAF2

The *E. coli* cells expressing chromophorylated All1280 GAF2 generated red fluorescence under 400-nm light irradiation, and the red fluorescent proteins were distributed over the entire cell (Fig.3: a, b). However, the red fluorescence of chromophorylated All1280 GAF2 cells disappeared completely under 570-nm light irradiation (Fig.3: c). These results showed that the red fluorescence reversibility of *E. coli* expressing All1280 GAF2 could be clearly observed under a fluorescent microscope. Thus, the successful construction of red fluorescent All1280 GAF2 could serve well in the labeling of living cells.

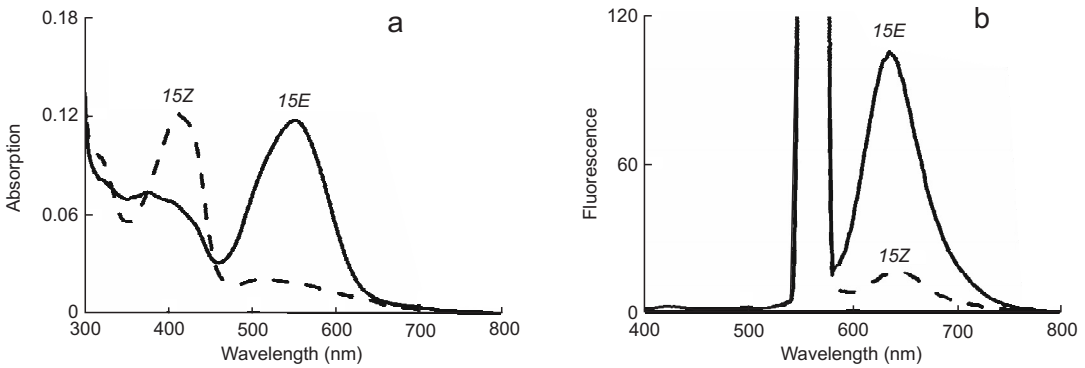
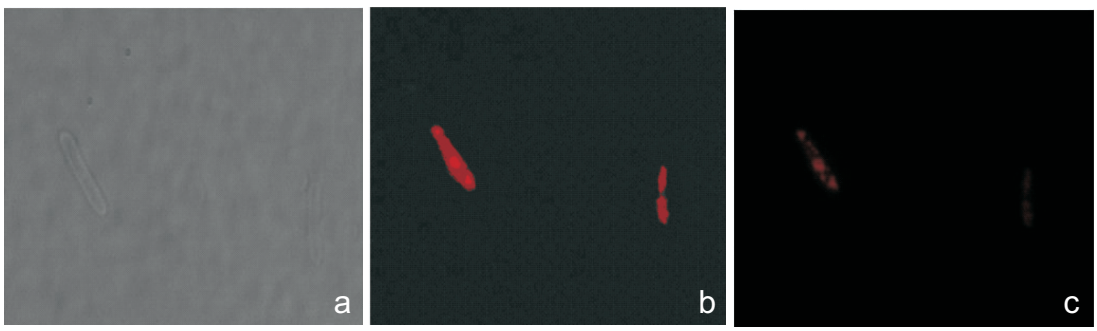


Fig. 2　Reversible absorption（a）and fluorescence spectra（b）of All1280 GAF2

Table 2　Quantitative absorption and fluorescence data of chromophorylated proteins

Chromoprotein	Chromophore	Absorption				Fluorescence		Brightness	Yield (mg/L)
		λ_{max} (nm)		ε_{Vis} (mmol/cm)		λ_{max} (nm)	ΦF	ε_{Vis}ΦF	
		15*Z*	15*E*	15*Z*	15*E*	15*E*	15*E*		
All1280 GAF2	PVB/PCB	413	560	44	55	635	0.07	3.85	1.6
All1280 GAF2（C53A）	PCB	–	580 635w	–	– 58	– 656	– 0.12	– 6.96	– 2.4

Notes：Spectra were recorded in potassium phosphate buffer（KPB，20 mmol/L，pH 7.2）. Data were averaged from three independent experiments. "w" denotes weak peaks.



a：Bright-field micrograph of All1280 GAF2；b：Fluorescence micrograph of *E. coli* containing *15E*-All1280 GAF2；c：Fluorescence micrograph of *E. coli* containing *15Z*-All1280 GAF2.

Fig. 3　Fluorescent photoconversion of All1280 GAF2 in *E. coli*

2.4　Chromophorylated All1280 GAF2（C53A）is not photoreversible but is highly fluorescent

Some CBCRs have conserved DXCF motifs，which contain a conserved cysteine forming a second bond to the chromophore. Loss of the second cysteine linkage can result in increased formation of chromophores[12]. C53 is the second cysteine in the DXCF motif and is conserved in a subclass of cyanobacteriochromes，including All1280 GAF2，SyPixJ1，All2239，Alr2279，and Tlr0924（Fig.4）. The second cysteine in the conserved DXCF motif is usually replaced with aliphatic amino acid，alanine（Ala），or aspartic acid（Asp）[13]，which may be equivalently positioned in CBCRs.

When A53 was substituted for C53 in All1280 GAF2，All1280 GAF2（C53A）exhibited a maximum absorption peak at 580 nm and a weak peak at 635 nm（Table 2），resulting in a strong fluorescence emission peak at 656 nm（Fig.5）. Exhaustive irradiation of All1280 GAF2（C53A）with green or red light failed to produce detectable

DXCF highlighted in dark grey represents conserved Asp-Xaa-Cys-Phe motifs. CH highlighted in light grey represents conserved Cys-His motifs.

Fig. 4 Multiple sequence alignment of GAF domains of DXCF CBCRs

changes in absorption or fluorescence spectra, suggesting that replacement of C53 with A53 ablated *15Z/15E* photoreversibility of All1280 GAF2, resulting in the formation of a PCB adduct that exhibited stable photochemical properties, similar to Tlr0924(C499D)[14].

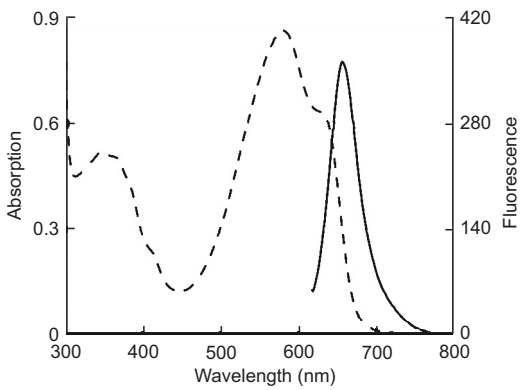
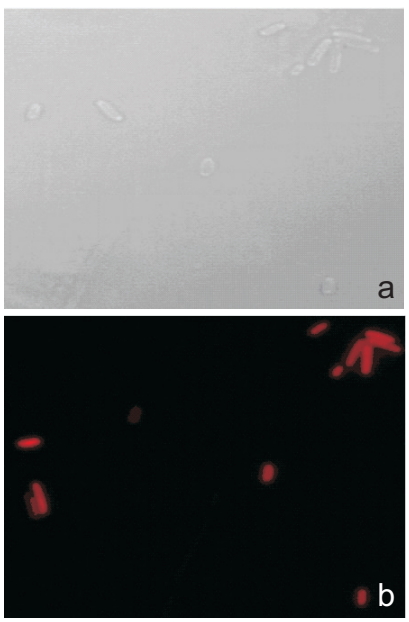


Fig. 5 Absorption (dashed) and fluorescence (solid) spectra of All1280 GAF2(C53A)

To obtain chromophorylated protein-expressed cells, All1280 GAF2(C53A) was also co-expressed with PCB in *E. coli*. The fluorescence microscopy images were captured. All1280 GAF2(C53A) pigment cells showed strong and stable red fluorescence (Fig.6). Compared to the wild-type All1280 GAF2, All1280 GAF2(C53A) possessed a higher molar extinction coefficient ($\varepsilon_{Vis} = 58 \times 10^3$), fluorescence quantum yield ($\Phi_F = 0.12$), pigment cell yield (2.4 mg/L), and brightness value (6.96) (Table 2). These results indicated that mutagenesis of C53 changed the structure of All1280 GAF2, which contributed to an increased ratio of All1280 GAF2(C53A) binding with PCB and elevation of the fluorescence quantum yield. As a result, the red fluorescence of *E. coli* expressing All1280 GAF2 (C53A) was stronger than that of All1280 GAF2.



a: Right-field micrograph of All1280 GAF2 (C53A) in *E. coli*. b: Fluorescence micrograph of All1280 GAF2 (C53A) *E. coli*.

Fig. 6 Micrograph of All1280 GAF2 mutants in *E. coli*

3 Discussion

GAFs of cyanobacteriochromes compare favorably with GFP derivatives with respect to both wavelengths of excitation and emission. In addi-

tion, GAFs can bind bilins autocatalytically, such that chromophorylation does not depend on lyases[13,14]. Applications have been limited, however, as chromophores must be provided separately or co-expressed with apoprotein in living cells[15].

Chromophorylated All1280 GAF2 exhibited photo-switching reversibility between red fluorescence and non-fluorescence states. This reversibility was based on *15Z/15E* isomerism of PCB under certain light irradiation. To increase red fluorescence of All1280 GAF2, site-directed mutagenesis was applied. Results indicated that Cys53 was essential for the reversible photoconversion of All1280 GAF2 because its mutagenesis resulted in a PCB adduct that showed no photoconversion. All1280 GAF2（C53A）incorporated PCB more efficiently, resulting in chromophorylated All1280 GAF2（C53A）with a higher molar extinction coefficient and fluorescence quantum yield.

This study provides new concepts and methods for the generation of red fluorescent proteins from cyanobacteriochromes. Because of its many advantages, red fluorescent protein from cyanobacteriochromes has the potential to serve as a promising molecular probe. With the development of photochemical biomaterials, further studies should enhance the brightness, stability, and yield of red fluorescent protein from cyanobacteriochromes, therefore broadening its prospective applications in biology.

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