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Aequorea green fluorescent protein

Expression of the gene and fluorescence characteristics of the recombinant protein

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Abstract

Expression of the cDNA for *Aequorea* green fluorescent protein in *E. coli* yielded a fused protein with fluorescence excitation and emission spectra virtually identical to those of the native green fluorescent protein. Further, a solution of the protein, when mixed with aequorin and calcium ion, emitted a greenish luminescence characteristic of the in vivo luminescence of the animal, indicating a radiationless energy transfer to the protein.

Key words: Bioluminescence; Aequorin; Calcium; Chromophore; Energy transfer

1. Introduction

The jellyfish, *Aequorea victoria*, emits a bluish-green light from the margin of its umbrella [1]. The light is due to the presence of two closely associated proteins: aequorin (21.4 kDa), consisting of a Ca^{2+}-binding apoprotein (apoaequorin), coelenterazine (organic substrate) and molecular oxygen [2–4] and a green fluorescent protein (GFP, 27 kDa, fluorescence λ_{max} = 508 nm), containing a modified hexapeptide as a chromophore [5–10]. In vitro, the binding of Ca^{2+} to aequorin triggers an intramolecular reaction in which coelenterazine is oxidized to coelenteramide, yielding as products a blue fluorescent protein (fluorescence λ_{max} = 470 nm), CO_2 and light (λ_{max} = 470 nm) [2]. The blue fluorescent protein is made up of coelenteramide bound to apoaequorin and the excited state coelenteramide is the emitter in the reaction. If GFP is added to the reaction mixture, however, a bluish-green light (λ_{max} = 508 nm), identical to that of the in vivo luminescence of the animal, is observed due to a radiationless energy transfer from the blue fluorescent protein to GFP [5,6,11]. The green light emission may be schematically outlined, as follows:

$$\text{Aequorin} \xrightarrow{Ca^{2+}} \text{Blue fluorescent protein} + h\nu_{\lambda max\ 470\ nm} + CO_2$$
$$\Big\downarrow + \text{GFP}$$
$$h\nu_{\lambda max\ 508\ nm}$$

The genes for apoaequorin and GFP have been cloned and the primary structure of the proteins deduced from the nucleotide sequence [3,12]. Both proteins are made up of a single polypeptide chain, with apoaequorin having 3 EF-hand structures (Ca^{2+}-binding sites) [3,4,12]. The expression of the apoaequorin gene has been studied in some detail [3,13–18], but no comparable studies have yet been reported for the GFP gene. We now report on the expression of the GFP gene in *E. coli* and show that the expressed protein exhibits fluorescence characteristics similar, if not identical, to those of *Aequorea* GFP, including its capability to serve as an energy acceptor in the aequorin bioluminescence reaction. The finding that the expressed protein is fluorescent suggests that the primary structure of the protein undergoes modification to form a chromophore during expression. Because of the marked intrinsic fluorescence of the protein, it may also serve as a reporter or marker in gene expression studies.

2. Materials and methods

2.1. Materials

The following materials were obtained from commercial sources: Chelating Sepharose-Fast Flow, Pharmacia-LKB, Piscataway, NJ; imidazole, Sigma, St. Louis, MO; $NiCl_2$, Merck; plasmid pTrcHis-C and *E. coli* strain Top10, Invitrogen, San Diego, CA; IPTG, Boehringer

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Mannheim, Indianapolis, IN; restriction and modification enzymes, Stratagene, San Diego, CA; and SDS-PAGE gel and buffer, Novex, San Diego, CA. Recombinant aequorin was prepared as previously described [14,17].

2.2. Isolation of cDNA clone of Aequorea GFP

The cDNA clone of GFP was isolated directly from a cDNA library of *Aequorea victoria* using the polymerase chain reaction [19]. Based on the known sequence of the 5′- and 3′-regions of the cDNA of GFP [12], two primers were synthesized using a Cyclone Plus DNA synthesizer (MilliGen/Biosearch, Novato, CA), as follows: GFP-A (5′-region, 5′-GGCGGGATCCCAAAG-ATG-AGT-AAA-GGA-GAA-GAA-CTT-TTC-AC-TGG) and GFP-B (3′-region, 5′-CCGGGTCGACG-GACAT-TTA-TTT-GTA-TAG-TTC-ATC-CAT-GCC-ATG-TG). A λZapII cDNA library of *Aequorea victoria* was constructed by the same method as described previously [20,21]. Using a GeneAmp PCR Reagent Kit (Perkin-Elmer-Cetus), library phages (1×10^7 pfu) and 4 μg of each primer, gene amplification was carried out for 30 cycles in a Perkin-Elmer (Norwalk, CT) DNA Thermal Cycler (denaturation at 94°C for 1.4 min, annealing at 40°C for 2 min, elongation at 72°C for 3 min). The amplified DNA fragment (approx. 740 bp) was isolated, digested with *Bam*HI/*Sal*I and ligated into the *Bam*HI/*Sal*I site of pBluescript SK(+) [22] to yield the plasmid, pAGP. The cloned cDNA of GFP was confirmed by DNA sequencing [23] using T3 and T7 primers (Stratagene) and AGF-3(+) primer (5′-GGTGTTCAATGCT-TTTCAAGA).

2.3 Expression and purification of expressed GFP

The expression vector pTrcHis-C, which is controlled by *trc* promoter and *lac* operator in *E. coli*, encodes for a 34 amino acid polypeptide possessing six contiguous histidine affinity groups at its N-terminus which can be used in immobilized metal affinity chromatography. The *Bam*HI/*Sal*I fragment of the GFP cDNA was inserted into the *Bam*HI/*Xho*I site of pTrcHis-C to give the GFP expression vector, pHis-AGP. For overexpression of the protein, the *E. coli* strain Top10, transformed with pHis-AGP plasmid, was grown at 37°C in 50 ml Luria-Bertani broth in a 500 ml Erlenmyer flask with vigorous shaking using a rotary incubator (300 rpm/min). When the absorbance (600 nm) of the cells reached 0.3, IPTG was added to a final concentration of 0.2 mM and the incubation continued for another 4 h. The increase in GFP concentration was monitored with a long wavelength UV lamp (Ultraviolet Products, San Gabriel, CA). The cells were harvested by centrifugation and suspended in 5 ml of 100 mM Na-phosphate buffer, pH 8.0. The cells were disrupted by sonication (6×30 s, in an ice bath) using a Branson (Danbury, CT) model J-17A sonifier. After centrifugation at $12{,}000 \times g$ for 20 min at 4°C in a Beckman Model J-21B refrigerated centrifuge, the supernatant (4.5 ml) was stored at −80°C until used.

Purification of the expressed protein was carried out at room temperature employing immobilized metal ion affinity chromatography with immobilized Ni(II) serving as the adsorbent [24]. The nickel was immobilized by washing Chelating Sepharose-Fast Flow with distilled water and then with 100 mM $NiCl_2$. The nickel-loaded gel (1.0 ml) was placed in a small column (0.8 cm diam.) and equilibrated with 100 mM Na-phosphate buffer, pH 8.0/0.5 M NaCl. The supernatant (4.5 ml), with NaCl added to a concentration of 0.5 M, was applied to the gel and the gel was washed with the same buffer until the absorbance (280 nm) reached below 0.005. The adsorbed protein was eluted with 100 mM imidazole, dissolved in the same buffer.

2.4. Protein analysis

SDS-PAGE was carried out under non-reducing conditions using a 4–12% gradient gel according to Laemmli [25]. The protein sample was dissolved in denaturing, non-reducing sample buffer (Novex) with heat treatment for 5 min at 95°C [26] or without heat treatment before being applied to the gel. Electrophoresis was run at 25 mA for 2 h and the gels were stained with 0.1% Coomassie brilliant blue R250 (Bio-Rad, Richmond, CA) dissolved in 50% trichloroacetic acid/water (w/v) and destained in ethanol/acetic acid/water (1:1:8) by gently shaking at room temperature.

2.5. Measurement of fluorescence spectra

Fluorescence emission and excitation spectra were measured using a Perkin-Elmer MPF-4 Fluorescence Spectrophotometer.

3. Results and discussion

The amino acid sequence of *Aequorea* GFP, deduced from the nucleotide sequence, is shown in Fig. 1. When compared with the sequence reported previously for GFP [12], the two sequences were found to have 97.5% homology, with neutral amino acid changes occurring in 8 positions. The calculated molecular weight was 26,862.

The expression vector, pHis-AGP, produced a fused protein of approximately 31 kDa in *E. coli*. The protein consisted of the 34 amino acid polypeptide having 6 histidine residues in tandem at the N-terminus, plus 3 amino acid residues originating from the PCR primer at the fusion junction and the GFP. The expressed protein was efficiently adsorbed by the Ni-loaded gel and formed a bright greenish fluorescent band, which was readily eluted with imidazole. The protein was subjected to SDS-PAGE analysis [25] under varying conditions (Fig. 2). Electrophoreses were run on aliquots of cell-free extracts of transformed *E.coli*, without heat treatment (lanes 1

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                .         .         .         .         .
GFP   MSKGEELFTG VVPILVELDG DVNGQKFSVS GEGEGDATYG KLTLKFICTT  50
gfp2  .......... .......... ....H..... .......... ..........
gfp10 .......... .......... ....H..... .......... ..........

      GKLPVPWPTL VTTFSYGVQC FSRYPDHMKQ HDFFKSAMPE GYVQERTIFF 100
      .......... .......... .......... .......... .........Y
      .......... .......... .......... .......... ..........

      KDDGNYKTRA EVKFEGDTLV NRIELKGIDF KEDGNILGHK MEYNYNSHNV 150
      .......S.. .......... .......... .......... ..........
      .......... .......... .......... .......... L.........

      YIMADKPKNG IKVNFKIRHN IKDGSVQLAD HYQQNTPIGD GPVLLPDNHY 200
      ......Q... .......... .E........ .......... ..........
      ......Q... .......... .E........ .......... ..........

      LSTQSALSKD PNEKRDHMIL LEFVTAAGIT HGMDELYK
      .......... .......... .......... ........
      .......... ........V. .......... ........
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Fig. 1. Amino acid sequence of *Aequorea* GFP deduced from the nucleotide sequence. The amino acid sequence of GFP obtained by PCR cloning is shown at top. The clones *gfp*2 and *gfp*10 [12] are aligned with GFP to show sequence homology. Underline indicates the residues presumably involved in chromophore formation.

and 3) and after heat treatment (lanes 2 and 4), and examined by staining with Coomassie brilliant blue (lanes 1 and 2) and under UV light (lanes 3 and 4) (Fig. 2A). Lane 1 is seen to have a prominent band at 43 kDa, which is missing in lane 2. Since native GFP is known to self-associate [6,27], the 43 kDa band is most likely due to the presence of a partially denatured GFP dimer, which is converted to a monomer (31 kDa) by denaturation in lane 2. This would account for the bright greenish fluorescence of the 43 kDa band in lane 3 [6], and for its absence in lane 4 as a fluorescent band and in lane 2 as a stained band. On denaturation, GFP is known to become completely non-fluorescent [26,28]. The purified fused protein is shown as a single 31 kDa band, in Fig. 2B, lane 2. The purity of the protein was estimated to be approximately 80%.

The fluorescence excitation maxima of the affinity purified protein were recorded at 395 nm and 478 nm, whereas the fluorescence emission maximum was at 507 nm, with a shoulder at 545 nm (Fig. 3). The observed maxima and shapes of the spectra were virtually the same as to those that have been previously reported for native GFP [6,26]. The fluorescence of native GFP has been attributed to the presence of an hexapeptide chromophore (Fig. 1) in the protein [8,10]. Thus, it is assumed that a post-translational modification of a covalent nature has occurred in the primary structure of the protein during expression.

It is apparent that the fluorescence characteristics of the expressed protein are not affected by the fusion of the 37 amino acid polypeptide to the N-terminus of GFP.

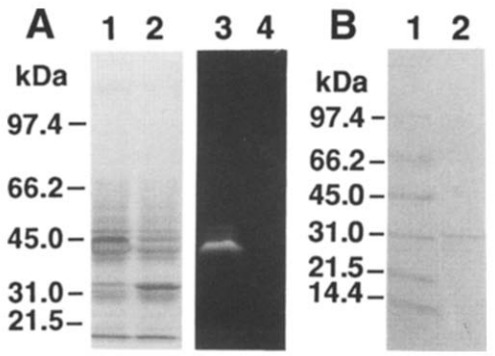

Fig. 2. SDS-PAGE and fluorescence analyses of expressed GFP. (A) Cell free extracts of transformed *E. coli*. The gels were run under non-reducing conditions with/without heat treatment (95°C, 5 min [26]). Lanes 1 and 3: 12,000 × *g* supernatant, 10 μl, without heat treatment; lanes 2 and 4: 12,000 × *g* supernatant, 10 μl, after heat treatment. Lanes 1 and 2 were stained with Coomassie brilliant blue R250 and lanes 2 and 4 were examined with short UV light (312 nm) using a Model FBTIV-88 (Fischer Scientific, Pittsburgh, PA) transilluminator. The numbers on the left margin represent molecular weight markers (Bio-Rad): phosphorylase b (97,400), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and lysozyme (14,400). (B) Partially purified GFP. Lane 1: molecular weight markers as described in (A); lane 2: eluent from Ni-ion affinity column (5 μl of a 500 μl fraction), treated with heat as described in (A).

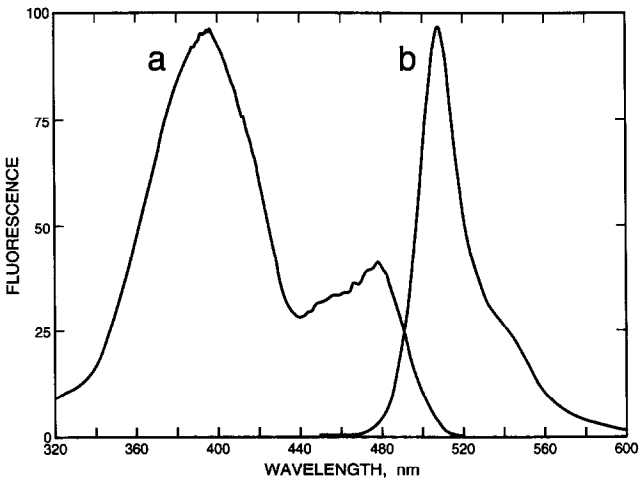

Fig. 3. Fluorescence excitation and emission spectra of GFP. Curve a, fluorescence excitation spectrum, measured with excitation monochromator set at a band pass of 3 nm and the emission wave length fixed at 530 nm; curve b, fluorescence emission spectrum, measured with the emission monochromator set at a band pass of 3 nm and the excitation wave length fixed at 400 nm. Recombinant GFP concentration was approximately 1 mg/ml and was used after dialysis against 100 mM ammonium bicarbonate, pH 7.8. Recording condition: scan speed was 60 nm/min; measurement was carried out at room temperature in a 0.5 ml microcell with a light path of 5 mm. Fluorescence intensity is in arbitrary units and the spectra are uncorrected.

Further, when 50 μl of 0.3 mM $CaCl_2$ was added in the dark to a mixture consisting of 11 μg of regenerated recombinant aequorin [14,17] and 100 μg of the purified expressed GFP dissolved in 150 μl of 30 mM Tris-HCl, pH 7.6/0.1 mM EDTA, a greenish luminescence was produced that was clearly visible to the eye. This observation indicates that, even with the 37 amino acid residues fused to the N-terminus, the expressed GFP is functionally active as an energy acceptor in the aequorin reaction. The intense fluorescence emitted by GFP, therefore, may also prove useful as a reporter in gene expression studies.

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