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Expression, purification and characterization of calcium-triggered luciferin-binding protein of *Renilla reniformis*

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Abstract

The Ca^{2+}-triggered luciferin-binding protein of *Renilla reniformis* (RLBP) is a non-covalent complex of apoprotein (apoRLBP) and coelenterazine (luciferin). The gene encoding apoRLBP with 552 nucleotides has been synthesized by assembly PCR methods with synthetic oligonucleotides, and the histidine-tagged apoRLBP expressed as a soluble form in the periplasmic space of *Escherichia coli* cells. The apoRLBP was purified by nickel chelate chromatography and the procedure yielded 18.2 mg of recombinant apoRLBP from 80 ml of cultured cells with purity greater than 95%. The purified apoRLBP was converted to RLBP by incubation with coelenterazine in the presence of dithiothreitol and the purity of recombinant RLBP was estimated to be over 95% by comparison with the absorption spectral data of native RLBP. When RLBP mixed with Ca^{2+}, coelenterazine was dissociated from RLBP and was utilized for the luminescence reaction of *Renilla* luciferase. Also semi-synthetic RLBPs with *h*-, *e*-, and *Bis*-coelenterazines were prepared and characterized.

Keywords: EF-hand; Coelenterazine; Secretion; Aequorin; *Anthozoa*; Bioluminescene; Gene synthesis

The bioluminescence of the sea pansy, *Renilla sp.* (class Anthozoa), is under the control of a nerve network [1–3] and is stimulated by changes of intracellular Ca^{2+} concentration [4–6]. The *in vivo* luminescence of *Renilla reniformis* involves two proteins, Ca^{2+}-triggered *Renilla* luciferin binding protein (RLBP)[1] [6,7] and *Renilla* luciferase [8,9]. RLBP is a dissociable complex of apoprotein (apoRLBP) and coelenterazine (luciferin) [7]. When RLBP reacts with Ca^{2+}, coelenterazine is released from RLBP and is used for the luminescence reaction of *Renilla* luciferase. *Renilla* luciferase catalyzes the oxidation of coelenterazine in the presence of molecular oxygen to produce coelenteramide, CO_2 and light (λmax = 480 nm), as in the following scheme.

$$\mathrm{RLBP} \xrightarrow{+Ca^{2+}} \mathrm{apoRLBP}(Ca^{2+}) + \mathrm{Coelenterazine}$$

$$\mathrm{Coelenterazine} \xrightarrow[+O_2]{\mathrm{Luciferase}} \mathrm{Coelenteramide} + CO_2 + \mathrm{light}(\lambda_{max} = 480\ \mathrm{nm})$$

Thus, the mechanism of Ca^{2+}-induced luminescence in *Renilla* is different from that of the Ca^{2+}-triggered photoproteins such as aequorin [10–12] in the class *Cindaria* of coelentrates.

$$\mathrm{Aequorin} \xrightarrow{+Ca^{2+}} \mathrm{apoAequorin}(Ca^{2+}) + \mathrm{Coelenteramide} + CO_2 + \mathrm{light}(\lambda_{max} = 460\ \mathrm{nm})$$

Native RLBP of *Renilla reniformis* has been isolated and characterized [6,7]. Further, the primary structure of RLBP was determined by direct amino-acid sequence analysis and the protein found to be composed of 184 amino acid residues in a single polypeptide chain [13]. RLBP has three EF-hand motifs characteristic of Ca^{2+}-binding site and is classified as a member of the EF-hand superfamily of

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[1] *Abbreviations used:* RLBP, Ca^{2+}-triggered luciferin-binding protein of *Renilla reniformis*; ApoAQ, apoprotein of Ca^{2+}-binding photoprotein aequorin; OmpA, outer membrane protein A; rlu, relative light unit.

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proteins. It is of interest that RLBP and aequorin show sequence similarity and bind coelenterazine and coelenterazine peroxide, respectively [11,13]. To understand the mechanism of Ca^{2+}-dependent coelenterazine releasing from RLBP and the structure–function relationship with aequorin, it is necessary to prepare fairly large amounts of protein, especially for X-ray crystallographic structural analysis. In this paper, the gene encoding RLBP was synthesized by assembly PCR methods using synthetic oligonucleotides, and histidine-tagged apoRLBP was expressed into the periplasmic space as a soluble form in *Escherichia coli* cells and purified. Recombinant RLBP was prepared from apoRLBP by incubation with coelenterazine and its analogues and characterized.

Materials and methods

Materials

Coelenterazine, *h*-coelenterazine and *Bis*-coelenterazine were chemically synthesized. *e*-Coelenterazine was kindly given by Dr. K. Teranishi (Mie Univ., Japan). Recombinant *Renilla* luciferase of *Renilla reniformis* was prepared as previously described [14]. The following materials were obtained from commercial sources: chelating Sepharose Fast Flow, Sephadex G25 (superfine grade) (Amersham Biosciences, Piscataway, NJ); imidazole, dithiothreitol (DTT), ethylenediamine tetraacetic acid (EDTA), $NiSO_4{\cdot}6H_2O$ (Wako Pure Chemicals, Osaka, Japan).

Gene design and synthesis

The gene coding apoRLBP (184 amino acids, swissprot: accession no. P05938) was designed using DNASIS software ver. 3.7 (Hitachi Software Engineering, Yokohama, Japan). We did not apply the optimal codon usage in *E. coli* and introduced 11 restriction enzyme sites in the apoRLBP sequence of 552 nucleotides. Oligonucleotides (40 mer × 28, 35 mer × 1), overlapped by 20 nucleotides, were synthesized by the phosphoramidate method using a Millipore DNA synthesizer (model Expedite) at a scale of 50 nmol and purified by gel purification, and then dried *in vacuo*. Gene assembly was carried out using PCR methodology [15] as follows; the dried-down oligos were resuspended in distilled water at a concentration of approx. 3.3 μg/μl (250 μM) and 1 μl each of the solutions of the internal oligos were combined, and the mixture (0.4 μl) was added to 40 μl of PCR mixture containing 0.25 mM of each dNTP, 5 units of ExTaq polymerase (Takara Shuzo, Kyoto, and Japan), and 4 μl of 10 × ExTaq buffer (the content of buffer is not shown in a manufacture sheet). The PCR program consisted of 55 cycles at 94 °C for 30 s, 50 °C for 30 s and 72 °C for 60 s (Perkin-Elmer). The assembly reaction mixture (2.5 μl) was used for amplification by both outside primer sets (3.3 μg); 15NL 5′ GGCAAGCTT-CCA-GAA-GTT-ACT-GCC-AGC-GAA-CGT-GCT-TAC-C 3′; *Hin*dIII site underlined and 33 RL 5‘GCCGGATCC-TTA-TAA-TAA-ATC-ACC-ATA-AAA-TGCATT-AGC-C 3′; *Bam*HI site underlined (30 cycles at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 60 s). The amplified fragment (approx. 550 bps) on a 1.2% agarose gel was eluted with 6 M NaI and purified using a PCR purification kit (QIAGEN). The isolated fragment was digested with *Hin*dIII and *Bam*HI, and then ligated to *Hin*dIII/*Bam*HI site of pUC9-2 [16] to give p92-RLBP. The DNA sequence was determined using an Applied Biosystems DNA sequencer (model 377 and 310).

Construction of apoRLBP expression vector and its purification

To express apoRLBP in the periplasmic space of *E. coli*, we made use of the bacterial secretory system by including the signal peptide sequence for the outer membrane protein A (OmpA) and six histidine residues under the control of lipoprotein promoter (lpp) and *lac* operator, derived from piP-HE [17]. Two constitutive expression plasmids, named piP-RLBP and piP-His-RLBP, were constructed by replacement of the *Hin*dIII/*Bam*HI fragment of apoaequorin cDNA in piP-HE [17] or piP-His-HE [Inouye, unpublished] with *Hin*dIII/*Bam*HI fragment of apoRLBP in p92-RLBP. The host strain used was *E. coli* strain BL21 (Amersham Bioscience, NJ, USA).

The seed culture of the bacterial strain possessing the expression plasmid was grown in 5 ml of Luria–Bertani medium with ampicillin (50 μg/ml) at 30 °C for 16 h and was added to 80 ml of LB medium in 500 ml of a Sakaguchi flask. After incubation for 16 h at 37 °C, the Klett unit of cell growth monitored with a Klett-Summerson photoelectric colorimeter (model 800-3) was 430. The cells (wet weight; 1.01 g) harvested by centrifugation at 5000*g* for 5min were suspended in 20 ml of 50 mM Tris–HCl (pH 7.6) and then disrupted by sonication with a Branson model 250 sonifire in an ice bath. The resultant supernatant (20 ml, 61.0 mg protein) obtained by centrifugation at 12,000*g* for 10 min was applied on a Ni-chelate column (1.5 × 4 cm), equilibrated with 50 mM Tris–HCl (pH 7.6). The column was washed with 50 ml of 50 mM Tris–HCl (pH 7.6) and the proteins absorbed were eluted stepwise by 20 ml aliquots of 0.05, 0.1, 0.3, 0.5 and 1 M imidazole in 50 mM Tris–HCl (pH 7.6). The fractions of histidine-tagged apoRLBP were eluted at the concentration of 0.1–0.3 M imidazole, determined by SDS–PAGE analysis. The apoRLBP fractions were combined, dialyzed against 4 L of 50 mM ammonium bicarbonate (pH 8.3) and stored at −80 °C. The yield of purified apoRLBP was 18.2 mg from 80 ml cultured cells and the amount of the expressed apoRLBP was 29.8% in the soluble fraction extracted from bacterial cells.

Preparation of RLBP from apoRLBP

The purified apoRLBP (1.0 mg = 46 nmol) was dissolved in 5 ml of 30 mM Tris–HCl (pH 7.6) containing