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Cloning, expression and sequence analysis of cDNA for the Ca^{2+}-binding photoprotein, mitrocomin

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The primary structure of mitrocomin consists of 190 amino acid residues, with three Ca^{2+}-binding sites and a tyrosine residue at the C-terminus. Mitrocomin shows an amino acid sequence homology of 67.9% and 60.7% when compared with aequorin and clytin, respectively. The amino acid residues Cys^{152}, His^{58}, His^{169}, Trp^{12}, Trp^{86}, Trp^{108}, Trp^{129} and Trp^{173} are conserved in all three photoproteins, suggesting that they play a role in light emission.

Bioluminescence; *Halistaura*; Calcium; Aequorin; Clytin; C-terminal tyrosine

1. INTRODUCTION

The Class Hydrozoa, Phylum Cnidaria, contains at least four luminescent jellyfishes that produce light by utilizing a photoprotein [1,2]. These include *Obelia* [1–3], *Clytia* (formerly *Phialidium*) [4–6], *Aequorea* [7,8] and *Mitrocoma* (= *Halistaura*) [4,9]. The best studied example is *Aequorea victoria* [7,8,10,11] from Friday Harbor, Washington. Aequorea possesses in the margin of its umbrella, a small protein, aequorin, which emits light in the presence of Ca^{2+}. Aequorin is made up of apoaequorin (a monomer with M_r = 21,400), molecular oxygen and coelenterazine. Apoaequorin has 189 amino acid residues with three Ca^{2+}-binding sites [12,13]. The binding of Ca^{2+} to these sites causes a conformational change, converting the protein into an oxygenase (luciferase). Light emission (λ_{max} = 470 nm) occurs as a result of an intramolecular reaction in which coelenterazine (substrate) is oxidized to coelenteramide and CO_2 by the bound oxygen, catalyzed by luciferase. Apoaequorin may be regenerated into aequorin by incubation with coelenterazine:

$$\text{Aequorin} \xrightarrow{3\ Ca^{2+}} h\nu + \text{coelenteramide} + CO_2 + \text{apoaequorin}$$

$$\text{Apoaequorin} + \text{coelenterazine} \xrightarrow[+\ DTT]{O_2 + EDTA} \text{Aequorin}$$

Recently, a second Ca^{2+}-binding photoprotein, clytin, was described showing a high degree of amino acid sequence homology with aequorin [6]. Like aequorin, clytin consists of 189 amino acid residues, three Ca^{2+}-binding sites and a proline at the C-terminus. Since aequorin is fully active only when proline is at the C-terminus [14,15], it has been thought that a C-terminal proline is required by the other Ca^{2+}-binding photoproteins. We now report that a third Ca^{2+}-binding photoprotein, mitrocomin, which is composed of 190 amino acid residues and has three Ca^{2+}-binding sites, has a tyrosine at the C-terminus. Also, like apoaequorin and apoclytin, apomitrocomin can be regenerated into fully active mitrocomin with coelenterazine. Thus, it was of interest to compare the three photoproteins with respect to conserved amino acid residues possibly involved in light emission. The convention of naming mitrocomin after its source *Mitrocoma cellularia*, formerly *Halistaura mitrocoma*, is followed herein.

2. MATERIALS AND METHODS

2.1. *Materials*

Guanidinium thiocyanate, isopropyl β-D-thiogalactopyranoside (IPTG) and deionized formamide were obtained from Wako Pure Chemicals (Osaka, Japan). Oligotex-dT30 was from Takara Shuzo (Kyoto, Japan), and λZap II vector, Gigapack II Gold packaging kit, *E. coli* strains XL1-Blue and SOLR, and T_3 and T_7 primers were from Stratagene (La Jolla, CA). Dextran sulphate and Sepharose CL-4B were purchased from Pharmacia, salmon sperm DNA from Sigma Chemicals (St. Louis, MO), and [α-^{32}P]dCTP (3000 Ci/mmol) from NEN/DuPont. Coelenterazine was synthesized chemically [16].

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2.2. *Construction of cDNA library*

Specimens of *Mitrocoma cellularia* (30–50 mm diam.) were collected by dip netting at Friday Harbor, Washington, in the fall of 1991. Circumferential rings, containing the photocytes, were cut with a pair of scissors and quickly frozen in liquid nitrogen. The rings were stored at −80°C until used. Total RNA was prepared by the guanidine isothiocyanate method [17]. One gram of rings was homogenized in 10 ml of extraction solution (4 M guanidium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl and 0.1 M 2-mercaptoethanol) for 10 s using a Janke and Junkel Ultra-Turrax T25 (Staufen, Germany) homogenizer. The mixture was cooled in an ice bath and the homogenization repeated, after which the following solutions were added in sequence accompanied by thorough mixing: 1 ml of 2 M sodium acetate, pH 4.0, 10 ml of water-saturated phenol and 2 ml of chloroform/isoamyl alcohol (49 : 1). The final mixture was mixed vigorously for 10 s, cooled in an ice bath for 15 min and centrifuged at 10,000 × *g* for 20 min at 4°C. The aqueous layer (blue) was recovered and the RNA precipitated by adding an equal volume of isopropyl alcohol at −20°C. The solution was centrifuged, the pellet resuspended in 3 ml of extraction solution and the isopropanol precipitation repeated. The sedimented RNA pellet was washed with 70% ethyl alcohol, dried and redissolved in RNase-free sterile distilled water. The yield of total RNA was 800 μg based on absorbance at 260 nm. Poly(A)$^+$ RNA was isolated using oligodT-labeled latex beads (Oligotex-dT30) [18]. A 2.4 μg aliquot of poly(A)$^+$ RNA was employed to synthesize cDNA by the method of Kakizuka et al. [18] using a Pharmacia cDNA synthesis kit. First and second strand synthesis was monitored by measuring the incorporation of ^{32}P from [α-^{32}P]dCTP. After completion of the second strand synthesis, the blunt-ended cDNAs were ligated to *Eco*RI/*Not*I adaptors. Subsequently, the 5′ ends were phosphorylated and the excess removed using Sepharose CL-4B. Ligation of 11.5 ng of cDNA with 1 μg of *Eco*RI digested/calf intestinal alkaline phosphatase-treated λZap II vector [19] was carried out at 4°C in a total volume of 5 μl. After two days, 2.5 μl was packaged using Gigapack II Gold packaging kit, yielding 6.7 × 10^5 pfu. The host strain was *E. coli* XL1-blue. PCR analysis [20] using T_3 and T_7 primers and a Perkin-Elmer DNA Thermal Cycler operating for 35 cycles (denaturation at 94°C × 1 min; annealing at 40°C × 2 min; elongation at 72°C × 3 min) showed that the insert size ranged from 200 to 4K bp.

2.3. *Isolation and sequence determination of mitrocomin cDNA clone*

The library was amplified once and 5 × 10^5 plaques growing on ten 15-cm LB plates were screened by the plaque hybridization method [21]. Plaques were transferred to nylon membranes (Genescreen Plus, DuPont) according to the manufacturer's directions. The probe was a 550 bp segment of the piP-HE plasmid containing the apoaequorin cDNA [22]. The segment was amplified by PCR using flanking 17-mer primers and purified by means of gel electrophoresis and Sephaglas BandPrep (Pharmacia). The probe was labeled with [α-^{32}P]dCTP using an Amersham Multiprime DNA labeling kit. Hybridization and washing were carried out as previously described [6]. Positive plaques were removed, rescreened, isolated and inserts excised as the pBluescript phagemid using ex-assist helper phage [19]. The host strain was *E. coli* SOLR. The nucleotide sequence of the purified plasmid DNA was determined by a modified dideoxy termination method [23], using a Taq DyeDeoxy Terminator Cycle sequencing kit (Applied Biosystems, Foster City, CA), and with an Applied Biosystems 370A DNA sequencer. An Applied Biosystems 392 DNA synthesizer was employed to synthesize the sequencing primers: MI185, 5′-TGGAGCAACAGAAGAAC-3′; MI507, 5′-TCAAGTTTACCGTCACC-3′; MI28, 5′-GCAGCAGATACGCAGTC-3′; and MI145, 5′-AAATGGTCCATAAGGCT-3′.

2.4. *Partial purification of expressed mitrocomin*

Twenty milliliters of LB medium containing 50 μg/ml of ampicillin was inoculated with *E. coli* SOLR, transformed with expression plasmid pMI17. After incubating overnight at 37°C with constant shaking, the medium was used to seed 2 l of LB medium containing 0.5 mM IPTG. The culture was grown overnight and the cells were harvested by centrifugation at 8,000 × *g* for 15 min. The pellet was washed with TE buffer (30 mM Tris-HCl/10 mM EDTA, pH 7.6), resuspended in 30 ml of the same buffer and disrupted in an ice bath with a Heat Systems Ultrasonics (Farmingdale, N.Y.) W-385 sonicator (15 × 10 s). After centrifugation at 10,000 × *g* for 20 min, the supernatant was concentrated to 3 ml with an ultrafilter (Centriprep 10, Amicon, Beverly, MA). One ml of the concentrate was applied to a Mono Q FPLC column (Pharmacia), previously equilibrated with TE buffer. The column was washed with 30 ml of TE buffer, then with 40 ml of TE buffer containing 0.1 M NaCl and the apomitrocomin was eluted with a linear gradient of 0.1 to 0.6 M NaCl in TE buffer. Active fractions, which eluted between 0.25–0.43 M NaCl, were pooled, dialyzed overnight against two changes of 1 l each of TE buffer and concentrated to 1.0 ml by ultrafiltration.

2.5. *Purification of native mitrocomin*

Circumferential rings from 75 specimens of *Mitrocoma cellularia* were washed by soaking for a few minutes in 50 mM EDTA, pH 8.0, saturated with $(NH_4)_2SO_4$. Extraction of mitrocomin was carried out by macerating the rings in 100 ml of 10 mM EDTA and filtering. Thereafter, the extraction of the macerated rings was repeated until no further activity was obtained. Assays for activity and purification of mitrocomin in the extracts were carried out essentially as previously described for aequorin [24]. The purification involved: (1) gel filtration with a Sephadex G-50 (fine) column (10 cm × 100 cm), equilibrated and eluted with 10 mM EDTA, pH 6.5, and (2) ion-exchange column (4.5 cm diam.) chromatography with QAE Sephadex A-50 (7.5 g, dry weight) equilibrated with 10 mM EDTA, pH 6.5, and eluted with a

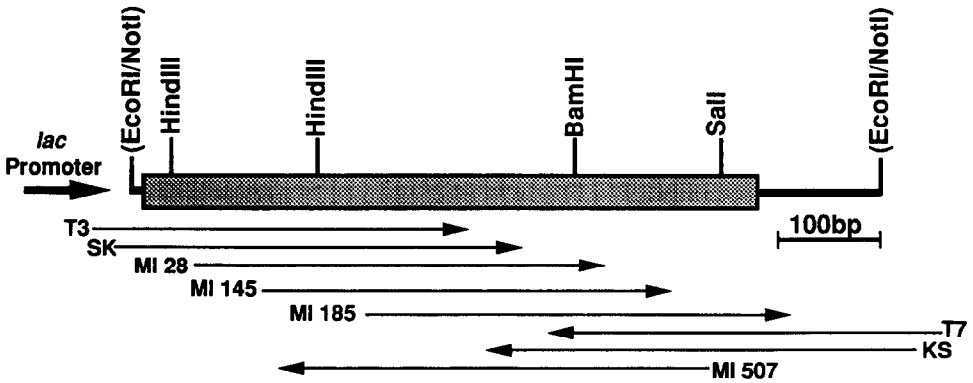

Fig. 1. Restriction map and sequencing strategy of the mitrocomin cDNA clone pMI17. Horizontal arrows show the sequenced region and the numbers refer to the synthetic primers used in sequencing. The coding region is shown by the shaded box and the bold arrow indicates the direction of the *lac* promoter.

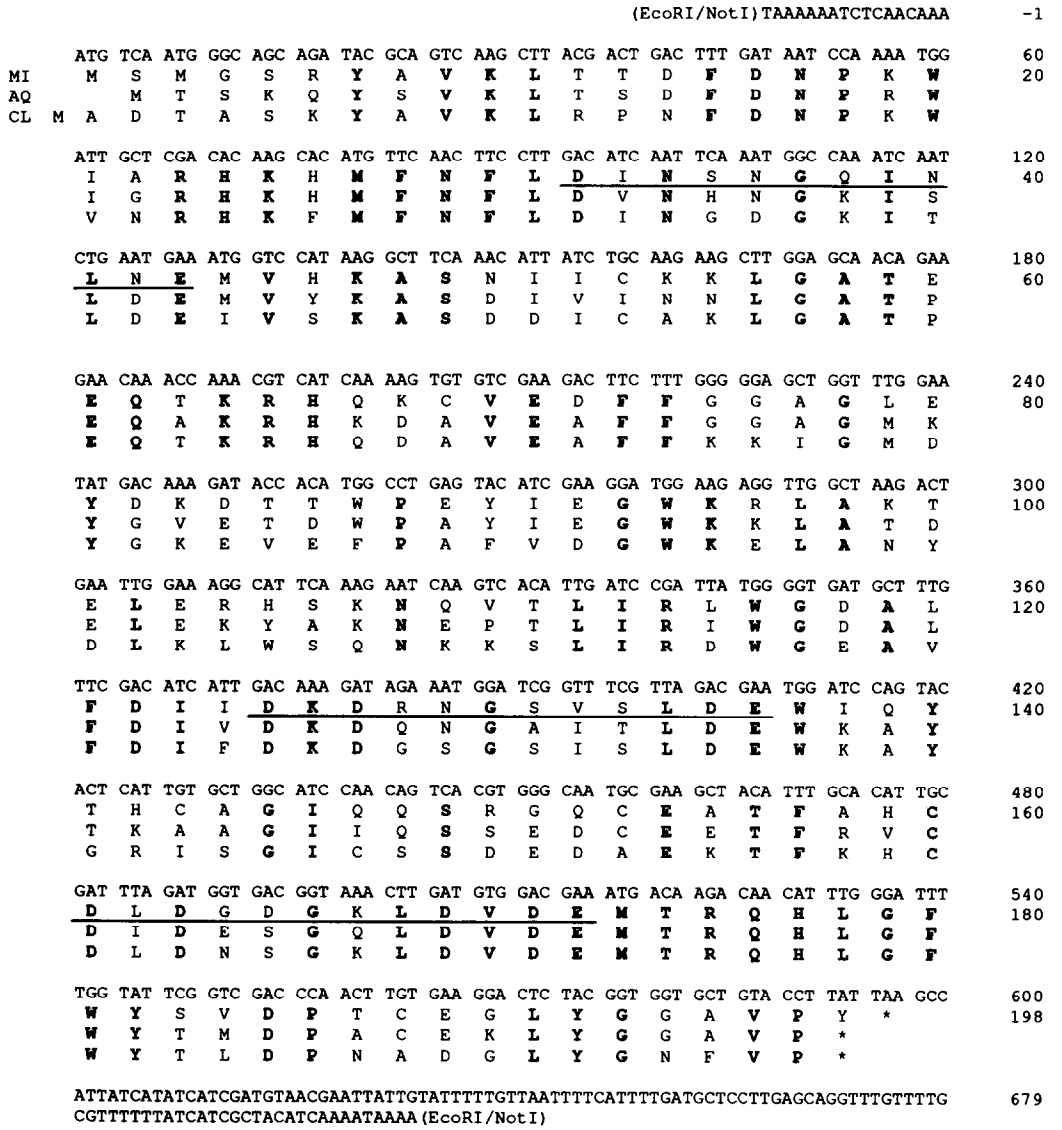

Fig. 2. Nucleotide sequence of the mitrocomin cDNA clone pMI17, the deduced amino acid sequence of mitrocomin, and the amino acid sequences of aequorin, AQ [12] and clytin, CL [6]. The putative Ca^{2+}-binding sites are underlined. Bold face type denotes identical amino acid residues in the same position in all three photoproteins.

linear gradient of 0 to 1.0 M NaCl in 10 mM EDTA, pH 6.5. An Amicon YM10 ultrafilter was used to concentrate the active fractions. The final purification was carried out by repeating the previous steps, except that a 4.5 cm × 100 cm column of Sephadex G-50 (fine) was used in step 1. The purified preparation contained 2.5 μg of mitrocomin/100 μl. HPLC and SDS-PAGE/Western blot analyses of the preparation showed that it contained approximately six isospecies.

2.6. *Measurement of bioluminescence emission spectra after regeneration with coelenterazine*

Recombinant mitrocomin and aequorin were regenerated by incubating each apoprotein overnight with coelenterazine at a temperature of 4°C. For mitrocomin: 200 μl of apomitrocomin ultrafiltrate + dissolved oxygen + 10 μg coelenterazine + 2.5 mM DTT [25]; for aequorin: 200 μl of a 0.89 mg/ml solution of purified apoaequorin [26] in TE buffer + dissolved oxygen + 10 μg coelenterazine + 2.5 mM DTT. Bioluminescence emission spectra of recombinant mitrocomin, recombinant aequorin and native mitrocomin were measured using a Model I-MCPD multichannel photodetection system (Otsuka Electronics, Osaka, Japan).

3. RESULTS AND DISCUSSION

The cDNA library was screened using an aequorin fragment (550 bp) prepared by amplifying the apoaequorin cDNA in piP-HE by PCR. Seven positive clones were obtained from 5×10^5 clones. Restriction enzyme analysis gave the map shown in Fig. 1. The positive clones were examined for expression of apomitrocomin using *E. coli* SOLR as the host strain. Expression of apomitrocomin would be expected to occur in clones possessing the *lac* promoter under the control of the *lac* operator in the pBluescript vector. Extracts of *E. coli* were prepared by disrupting the cells with a sonicator and centrifuging the lysate. After incubating the supernatants with coelenterazine, dissolved O_2, EDTA and DTT, each mixture was injected with 30 mM $CaCl_2$/10 mM Tris-HCl, pH 7.6 and the light intensity measured.

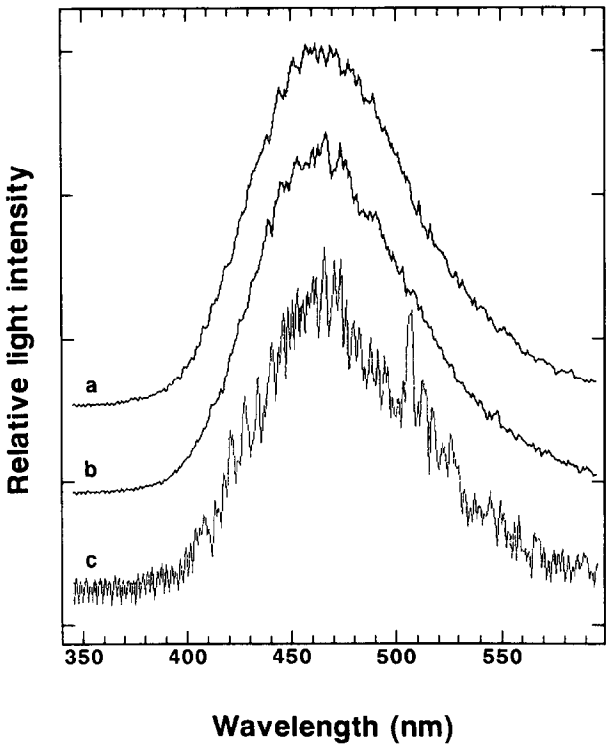

Fig. 3. Bioluminescence emission spectra of recombinant mitrocomin, recombinant aequorin and native mitrocomin. Amount of photoprotein used: (a) recombinant mitrocomin, 50 μl; (b) recombinant aequorin, 5 μl (4.45 μg); and (c) native mitrocomin, 50 μl (1.25 μg). The three photoproteins were prepared and regenerated as described in section 2. Each solution was placed in a 1.0 ml polystyrene cuvette in the sample compartment of the multichannel photodetector and injected with 0.75 ml of 30 mM $CaCl_2$/10 mM Tris-HCl, pH 7.6. Data collection was for 166 milliseconds. When the light reactions were measured in a photometer calibrated with a C-14 light standard [6], the three mixtures gave the following peak light intensities: (a) recombinant mitrocomin, 5.5×10^{12} q/s; (b) recombinant aequorin, 10×10^{12} q/s; and (c) native mitrocomin, 1×10^{12} q/s.

Of the seven positive clones examined, one clone, pMI17, was observed to be highly active and this clone was subjected to further study.

Fig. 2 shows the DNA sequence of mitrocomin and its amino acid sequence, deduced from the nucleotide sequence. The primary structure consisted of 190 amino acid residues, with three Ca^{2+}-binding sites. The calculated molecular weight was 21,833. The N-terminus of the mature protein was assumed to be valine since it was found previously from direct sequencing of native aequorin that the N-terminal residue is valine [12]. The function of the leader peptide, MSMGSRYA, is unknown, as is the case for aequorin and clytin. Alignment of the amino acid sequence of mitrocomin with those of aequorin and clytin showed 67.9% homology with aequorin and 60.7% homology with clytin. The C-terminal amino acid residue in mitrocomin was tyrosine, whereas in aequorin and clytin it has been shown to be proline [6,12]. However, since Tyr^{190} in mitrocomin is preceded by Pro^{189}, and Pro^{189} is the C-terminal residue in aequorin and clytin, Pro^{189} is conserved in all three photoproteins. In aequorin, a C-terminal proline is known to be essential for full bioluminescence activity [14]. Interestingly, cell extracts of transformed *E. coli* containing mutant aequorin with a tyrosine at the C-terminus, viz., Pro^{189} Tyr^{190}, had 10% of the activity of the wild type aequorin control, whereas a mutant mitrocomin with the C-terminal tyrosine deleted, viz., Val^{188} Pro^{189}, had substantial activity when compared to the control (data not shown).

Fig. 3 shows the bioluminescence emission spectra of recombinant mitrocomin, recombinant aequorin and native mitrocomin. The spectra are observed to be virtually identical with a peak at 470 nm. The results suggest that in mitrocomin and aequorin the proteins function similarly in light emission.

Cysteine, tryptophan and histidine residues are not commonly found in Ca^{2+}-binding proteins such as calmodulin, parvalbumin and troponin C [27]. In aequorin and clytin, however, the three amino acids occur in unusually high numbers [6,12]; this also is seen for mitrocomin, such that the residues are found in the ratios of 6, 6 and 8 in mitrocomin, 3, 6 and 5 in aequorin and 3, 6 and 4 in clytin, respectively. Among the three amino acid residues, the following are conserved in all three photoproteins: Cys^{152}, Trp^{12}, Trp^{86}, Trp^{108}, Trp^{129}, Trp^{176}, His^{16}, His^{58} and His^{169}. Previously, we showed that the cysteine residues may be involved in the regeneration of apoaequorin into aequorin [25]. Besides the above five conserved tryptophan residues, the sixth is present in aequorin as Trp^{79}, in mitrocomin as Trp^{79} and in clytin as Trp^{97}, with position 79 in clytin being occupied by phenylalanine (Fig. 2). Recent tryptophan modification studies have shown that two excited states are induced when Trp^{86} in aequorin is modified to phenylalanine [28]. Thus, it is conceivable that the tryptophan residues are involved in the generation of the product excited state. Earlier studies have suggested that His^{58} may be the binding site for molecular oxygen [27], but a more probable site now may be His^{169} [29]. Finally, two regions in the aequorin, mitrocomin and clytin molecules, comprising residues 7 to 24 and 158 to 174, appear to be highly conserved, suggesting that they also play a role in light emission.

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