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A C-terminal proline is required for bioluminescence of the Ca^{2+}-binding photoprotein, aequorin

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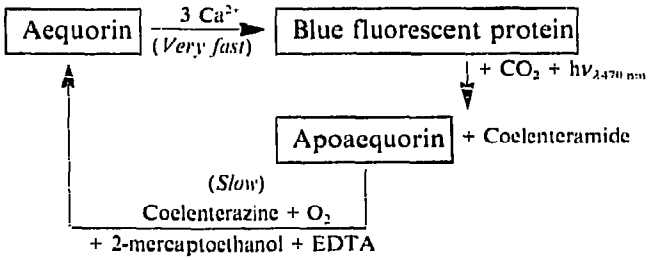
The requirement for a proline residue at the C-terminus of the Ca^{2+}-binding photoprotein, aequorin, was investigated by measuring luminescence activities of a series of C-terminal deletion mutants, substitution mutants and an addition mutant. CD spectral measurements of apoaequorin with the C-terminal proline deleted showed a small change in secondary structure. In all cases studied, the C-terminal proline was required for bioluminescence activity.

Aequorin; Ca^{2+}-binding protein; Bioluminescence; Carboxyl-terminus; Proline; Deletion mutant

1. INTRODUCTION

The jellyfish, *Aequorea victoria*, contains in the margin of its umbrella a small (M_r 21 400) monomeric Ca^{2+}-binding protein, aequorin, which emits light upon reacting with Ca^{2+} [1–5]. The protein is made up of apoaequorin (189 amino acid residues), coelenterazine (organic substrate, M_r 423) and molecular oxygen. Aequorin has three EF-hand structures characteristic of Ca^{2+}-binding sites. The binding of Ca^{2+} to aequorin induces a conformational change, resulting in the formation of an oxygenase (luciferase) which catalyzes the oxidation of coelenterazine via an intramolecular reaction [6–8]. Aequorin may be regenerated from apoaequorin by incubation with coelenterazine, dissolved oxygen, EDTA, and 2-mercaptoethanol [9].



Recently, amino acid modification studies by site-directed mutagenesis have been carried out involving the highly conserved glycine in each of the three Ca^{2+}-binding sites [10], the three cysteine residues [10,11] and a histidine residue in a hydrophobic region [10], yielding mutant aequorins with the same or reduced luminescence activities. Further, mutant aequorins with amino acid residues added to the N-terminal end, namely, M^{-7}TSK-NYSV^{1}KLTS...AVP^{189} and M^{-7}TMITPSSK^{2}LTS....AVP^{189}, and N-terminal fusion proteins of aequorin, have shown good luminescence activity [12–14], suggesting that the N-terminal residues are not crucial for the bioluminescence or regeneration of aequorin. However, a mutant aequorin with a modified C-terminal sequence, T^{175}MDRSSCLRKALWSCPLRSSTVVMHPRKMM^{204}, gave no activity [10]. This last observation prompted us to examine the possible involvement of the C-terminal amino acid residues in aequorin bioluminescence. Thus, C-terminal deletion mutants, substitution mutants and an addition mutant were constructed and analyzed. Surprisingly, the proline residue at the C-terminus was found to be absolutely essential for full bioluminescence activity.

2. MATERIALS AND METHODS

2.1. *Enzymes and chemicals*

Restriction enzymes and DNA ligase were purchased from Takara Shuzo (Kyoto, Japan). Radiolabeled compounds were obtained from Amersham. Oligonucleotides were synthesized by the phosphoramidite method [15] using an Applied Biosystems Model 380A DNA synthesizer. Coelenterazine, 2-(*p*-hydroxybenzyl)-6-(*p*-hydroxyphenyl)-3,7-dihydroxyimidazo[1,2-a]pyrazine-3-one, was chemically synthesized [16]. All other reagents were of analytical grade.

2.2. *Bacterial strain and plasmid*

The host strain was *E. coli* HB101 [F$^-$, hsdS20 (r_B^-, m_B^-), recA13 ara-14, proA2, lacY1, galK2, rpsL20(Sm^r), xyl-5, mtl-1, supE44, λ^-] [17] and the plasmid was piP-HE [18].

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2.3. *Growth of bacteria and preparation of apoaequorin samples for assay*

3 ml of LB medium, supplemented with 50 μg ampicillin/ml, was inoculated with transformed *E. coli* HB101 and incubated with shaking at 37°C for 16 h. The culture broth was centrifuged at 12 000 × *g* for 5 min at 4°C and 200 μl of the supernatant containing secreted apoaequorin was freeze-dried using a Speed-Vac concentrator (Savant Instruments, Farmingdale, NY). The residue was dissolved in 200 μl of 30 mM Tris-HCl, pH 7.6, 10 mM EDTA.

2.4. *Regeneration and assay of aequorin*

Aequorin was regenerated by mixing 1–5 μl of the sample from above (section 2.3) with 250 μl of 30 mM Tris-HCl, pH 7.6, 10 mM EDTA, 1 μl of 2-mercaptoethanol and 1 μl of coelenterazine (1 μg/μl, in absolute methanol) [16]. After standing in an ice-bath for 6 h, a small aliquot was transferred to a reaction cell and injected with 1.5 ml of 30 mM $CaCl_2$, 30 mM Tris-HCl, pH 7.6. The initial maximal light intensity was determined with a Labo Science (Tokyo) Model TD8000 photometer, previously calibrated with a carbon-14 light standard [19] and the results recorded with a Pantos Model U-212 chart recorder (Nippon Denki Kagaku, Tokyo). The recorded light intensity was converted to quanta/s to serve as a measure of activity.

2.5. *Purification of mutant pcdP and piP-HE apoaequorins*

The secreted pcdP apoaequorin was purified from the culture medium by ion-exchange [18] and size-exclusion chromatographies. HB101/pcdP strain was grown overnight at 30°C in 5 ml of LB medium supplemented with 50 μg of ampicillin/ml. The overnight culture was transferred to 40 ml of LB medium, supplemented with 50 μg of ampicillin/ml and incubated for 3 h. Subsequently, the culture was transferred to 400 ml of M9 medium [20] supplemented with 50 μg of

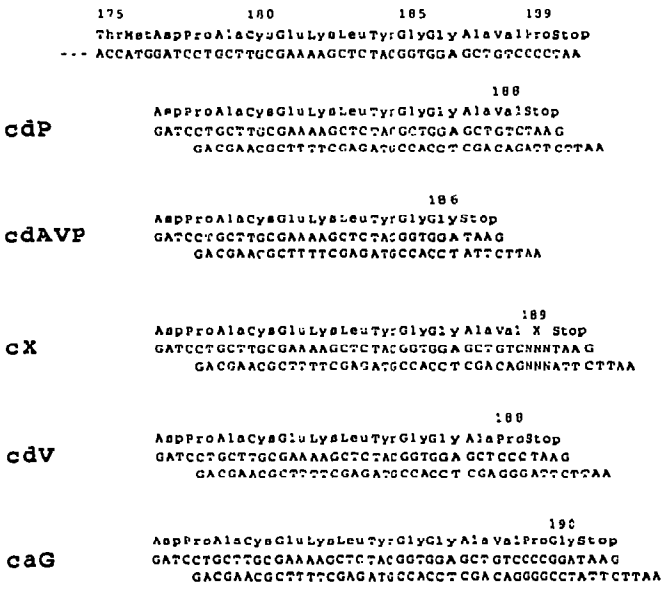

Fig. 1. Carboxyl-terminal end of piP-HE and synthetic DNA linkers used to obtain various mutant apoaequorins by site directed mutagenesis.

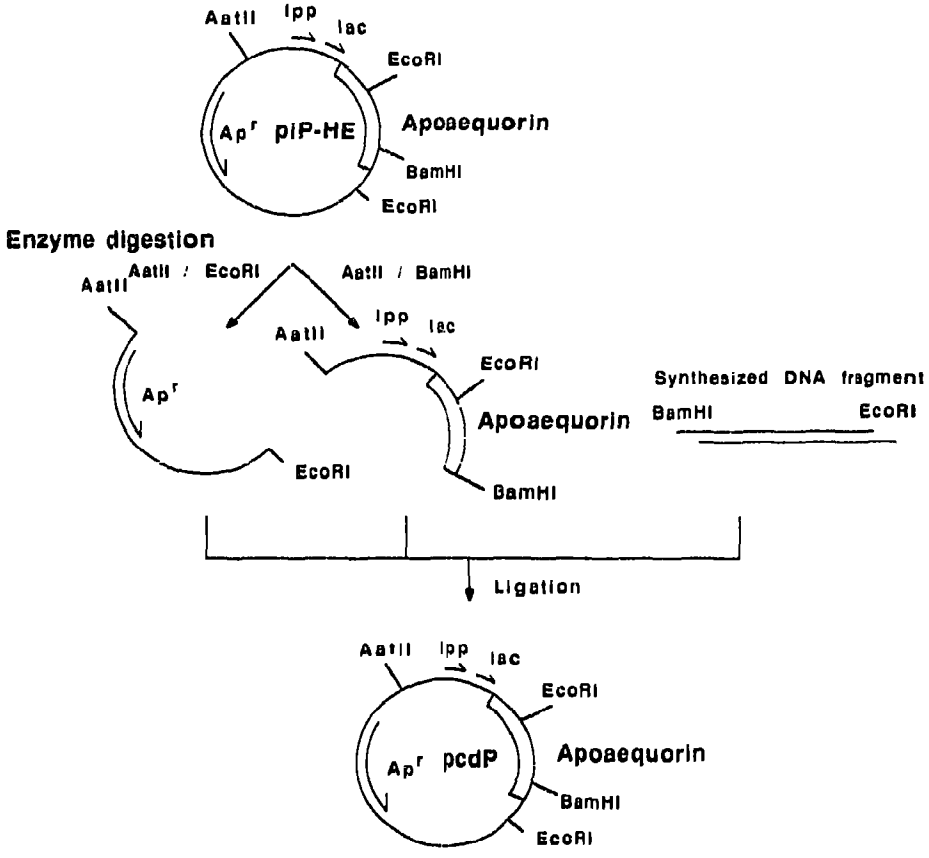

Fig. 2. Scheme used in constructing pcdP and various other expression plasmids.

ampicillin/ml and incubated at 37°C for 16 h. The final culture was centrifuged at 6000 × *g* for 10 min and the supernatant was acidified with 1 M acetic acid to pH 4.2. After standing at 4°C for 12 h, the precipitate was recovered by centrifugation at 9000 × *g* for 10 min. The precipitate was dissolved in 30 mM Tris-HCl, pH 7.6, 10 mM EDTA and applied directly to a Mono Q column (Pharmacia) equilibrated with 30 mM Tris-HCl, pH 7.6, 10 mM EDTA. The column was washed thoroughly with the same buffer to remove unadsorbed material and apoaequorin was eluted with a linear gradient of 0–0.4 M NaCl in 30 mM Tris-HCl, pH 7.6, 10 mM EDTA at a flow rate of 1 ml/min. The fractions corresponding to the apoaequorin peak (eluted at ~0.2 M NaCl) were pooled and subjected to size-exclusion HPLC using a Shodex (Tokyo) KW-803 column and an elution buffer consisting of 50 mM NaCl in 30 mM Tris-HCl, pH 7.6, at a flow rate of 0.6 ml/min. The fractions corresponding to the apoaequorin peak were pooled and subjected to SDS-PAGE. Apoaequorin concentration was determined by measuring absorbance at 280 nm and using the formula $A_{1\%,1\,cm}$=18.0 [21]. The yield of protein was <1 mg from 400 ml of culture medium. piP-HE apoaequorin was purified as previously described [18].

2.6. *CD spectra of pcdP and piP-HE apoaequorins*

CD spectra were measured in the absence and presence of Ca^{2+} using a JASCO (Tokyo) Model J-600 spectropolarimeter. The path length of the cell was 0.1 cm and the apoprotein concentration was 0.1 mg/ml in 30 mM Tris-HCl, pH 7.6, 10 mM EDTA. All spectra were taken at 4°C and represent the average of at least three scans.

3. RESULTS AND DISCUSSION

Two C-terminal DNA linkers were chemically synthesized (Fig. 1); one, cdp, to delete the C-terminal amino acid Pro^{189} and the other, cdAVP, to delete the three C-terminal amino acids $Ala^{187}Val^{188}Pro^{189}$. piP-HE is a plasmid that causes *E. coli* to overproduce and secrete apoaequorin into the culture medium [18]. piP-HE was digested with *Aat*II–*Bam*HI and *Aat*II–*Eco*RI, and the two DNA fragments were isolated and each ligated to the deletion linkers to obtain plasmids pcdP and pcdAVP (Fig. 2). Transformed *E. coli* HB101/pcdP and HB101/pcdAVP, and piP-HE were grown in LB medium. All three culture media, when analyzed by SDS-PAGE, revealed a characteristic 25 kDa apoaequorin band under reducing conditions [18] and showed luminescence activity (Table I). From the intensity of the bands, it was estimated that pcdP, pcdAVP and piP-HE apoaequorins were present in the ratio 0.33:1:1. Cells producing pcdP had large accumulations of apoaequorin in the periplasmic space, but low apoaequorin concentration in the culture medium. Sequencing of purified pcdP apoaequorin showed that the expressed protein was correctly cleaved during export, having a N- terminal sequence of Ala-Asn-Ser-Lys-Leu-Thr- (data not shown), as previously reported [18].

Table I

Luminescence activities of piP-HE and C-terminal deletion mutant apoaequorins

Apoaequorin	Initial activity (Quanta × 10^4/s)	Relative activity (%)
piP-HE	5451.0	100
pcdP	16.5	0.3
pcdAVP	1.1	0.02

Details of assay are described in Materials and Methods.

Table II

Luminescence activities of mutant apoaequorins with modified C-terminal amino acid residues

Apoaequorin	C-terminal amino acid residues	Relative activity (%)
pip-HE	Pro	100
pcX1 - 4	Pro	80,52,45,33
pcX5 - 8	Gln	2.8,2.5,2.3,2.0
pcX9	Ile	2.3
pcX10	Lys	1.6
pcX11	Val	1.7
pcX12 - 13	Thr	1.4,1.0
pcX14 - 15	Ser	0.9,0.8
pcX16	Leu	0.7
pcX17	Asn	0.4
pcX18	Glu	0.3
pcX19 - 20	Stop	0.2
pcX21 - 54	unknown	2.8 - 0.2

Details of assay are described in Materials and Methods.

A mixed linker, cX, having the sequence NNN at the site for C-terminal proline, was chemically synthesized (Fig. 1). The linker allowed for all 20 different amino acids to be substituted for proline at the C-terminus. Fifty-four transformants were isolated and cultured. Apoaequorin expression was estimated from the 25 kDa band densities of SDS-PAGE gels. Luminescence activity was determined on identical aliquots of culture medium after regenerating aequorin with coelenterazine. Out of the 54 clones examined, 4 clones showed relatively high luminescence activities, with values of 33, 45, 52 and 80% compared to piP-HE (Table II). The remaining 50 clones had activities ranging from 0.2–2.8%. The mutagenized DNAs of 20 of the clones, including the 4 with high activity, were sequenced [22], and the C-terminal sequence deduced from the nucleotide sequence. A C-terminal proline was found in all the 4 clones possessing high activity, whereas the remaining 16 had 9 different C-terminal amino acid residues.

Two linkers, cdV and caG, were chemically synthesized (Fig. 1). pcdV, in which Pro^{189} became Pro^{188} by deletion of Val^{188}, was used to examine the effect of the position of proline. This mutant, which would also be a valine deletion mutant, and pcaG, in which glycine

Table III

Luminescence activities of pcdV and pcaG apoaequorin

Apoaequorin	C-terminal sequence	Relative activity (%)
piP-HE	-GGAVP^{189}	100.0
pcdV	-GGAP^{188}	< 0.1
pcaG	-GGAVPG^{190}	1.4

Details of assay are described in Materials and Methods.

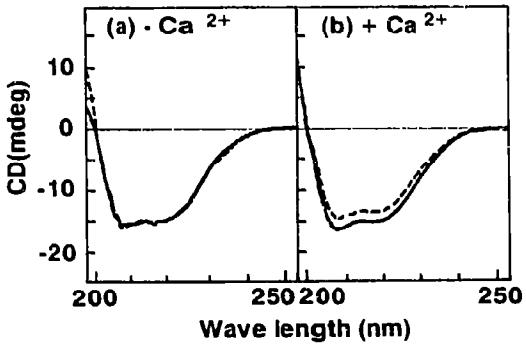

Fig. 3. CD spectra of pcdP (solid line) and piP-HE (dashed line) apoaequorins, in the absence (a) and presence (b) of calcium. Details of measurements are in the text.

was added to Pro^{189} to give Gly^{190}, showed low activities (Table III).

Aequorin has Ca^{2+}-binding sites that are homologous to those of bovine calmodulin, which has 4 Ca^{2+}-binding sites in a chain of 148 amino acid residues [23]. If the amino acid residues of the two proteins are aligned for sequence homology, aequorin is found to lack a Ca^{2+}-binding site near residues 65–76, which would correspond to the second Ca^{2+}-binding site of calmodulin. This region, therefore, is suspected of being involved in coelenterazine binding or active site formation. Further, the distance between the 2nd and 3rd Ca^{2+}-binding sites of aequorin and the 3rd and 4th Ca^{2+}-binding sites of calmodulin are identical, indicating that the two proteins have a common evolutionary origin [4]. It is seen that the C-terminal end extends for 25 amino acid residues beyond the last Ca^{2+}-binding site in aequorin, whereas the C-terminal end of calmodulin extends for only 8 amino acid residues in an α-helix. In contrast, the first Ca^{2+}-binding site at the N-terminal end of aequorin is preceded by 23 amino acid residues, whilst the number is almost the same for calmodulin, i.e. 19. Thus, the C-terminal proline, which is present as a nonpolar hydrophobic group at the end of the long tail, may have a functional role such as being involved in the formation of the catalytic site or in the assembly of aequorin from coelenterazine and molecular oxygen. Whatever the role, it is apparent that the deletion of the C-terminal proline causes a small alteration in the secondary structure (Fig. 3b) leading to almost complete loss of bioluminescence activity.

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