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Bioluminescence of the Ca^{2+}-binding photoprotein, aequorin, after histidine modification

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Modification studies of the 5 histidine residues in aequorin employing site-directed mutagenesis and diethyl pyrocarbonate suggested that His^{169} may be the site of binding of molecular oxygen in aequorin. The modification of this residue led to complete loss of activity, whereas modification of the remaining 4 histidine residues yielded mutant aequorins with varying bioluminescence activities

Calcium; Apoaequorin; Coelenterazine; Diethyl pyrocarbonate; Oxygen binding

1. INTRODUCTION

The small monomeric Ca^{2+}-binding photoprotein aequorin (M_r 21,400), from the jellyfish *Aequorea victoria*, emits light by an intramolecular reaction when mixed with Ca^{2+} [1–3]. Aequorin consists of coelenterazine (substrate, m.wt. 423) and molecular oxygen bound tightly to apoaequorin (apoprotein). NMR studies have shown that one of the oxygens of molecular oxygen is attached to the C-2 carbon of coelenterazine [4] and the presumption is that the other oxygen is joined to an amino acid residue via a peroxide bridge [5]. Apoaequorin itself is made up of 189 amino acid residues, with 3 EF-hand structures characteristic of Ca^{2+}-binding sites [6,7]. When Ca^{2+} binds to aequorin, a conformational change takes place, converting the protein into a luciferase (oxygenase). The luciferase then catalyzes the oxidation of coelenterazine by the bound oxygen, yielding as products light (λ_{max} = 470 nm), CO_2 and coelenteramide [8]. The onset of light emission is very rapid [9] and the quantum yield is 0.23 at 25°C [10]. Apoaequorin may be regenerated into aequorin by incubation with coelenterazine, 2-mercaptoethanol, EDTA and dissolved oxygen (Scheme 1) [11].

Histidine residues are not commonly found in Ca^{2+}-binding proteins. For a Ca^{2+}-binding protein, aequorin has an unusually high content of histidine residues, namely 5 [6,7] compared to 1 each for bovine calmodulin [12] and troponin C [13]. Aequorin also has a relatively high number of cysteine and tryptophan residues and previous studies have shown that the 3 cysteine residues and at least 1 of the 6 tryptophan residues have a functional role in aequorin bioluminescence [14,15]. Thus, a study of the histidine residues may provide further insight into how light is generated in this protein. Modification of the 5 histidine residues by site-directed mutagenesis gave 4 mutant aequorins with varied bioluminescence activities and 1 with complete loss of activity, suggesting that the latter (residue 169) may be the binding site for molecular oxygen in aequorin.

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2. MATERIALS AND METHODS

2.1. *Enzymes and chemicals*

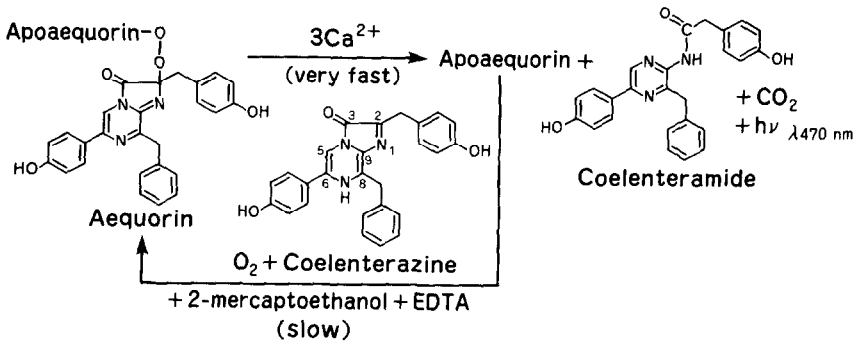
Restriction enzymes and *Escherichia coli* T4 DNA ligase were obtained from Takara Shuzo (Kyoto, Japan), and *Taq* polymerase and diethyl pyrocarbonate (DEPC) were from Wako Pure Chemicals (Osaka, Japan). Radiolabeled compounds were purchased from Amersham. Oligonucleotides were synthesized by the phosphoramidite method [16] using an Applied Biosystems (Foster City, CA) Model 380A DNA synthesizer. Coelenterazine was chemically synthesized as described [17]. All other chemicals were of the highest grade commercially available.

2.2. *Bacterial strain and plasmid*

The bacterial strain used was *Escherichia coli* D1210 and the plasmid was the previously described pIP-HE, consisting of the cDNA of apoaequorin fused to the *E. coli* outer membrane protein A (*ompA*) coding sequence under the control of the *lpp* promoter and *lac* operator [18].

2.3. *Growth of bacteria and purification of recombinant apoaequorin*

Transformed *E. coli* D1210 was grown at 30°C in 3 ml of LB medium, containing 50 μg/ml of ampicillin [18]. After overnight culture, the medium was transferred to 25 ml of fresh LB medium containing 50 μg/ml of ampicillin and incubated with shaking for 3 h at 30°C. The culture was then transferred to 1 l of fresh M9CA medium containing 50 μg/ml of ampicillin and incubated with shaking for 15 h at 37°C. The secreted recombinant apoaequorin was precipitated by adding 1 M acetic acid to the culture medium to a pH of 4.2, followed by gentle stirring for 12 h at 4°C. After centrifuging the medium at 9,000 × *g* for 20 min at 4°C, the sedimented apoaequorin was redis-



Scheme 1

solved in a small volume of 30 mM Tris-HCl, pH 7.6. After passing the apoaequorin solution through a PD-10 column (Pharmacia) equilibrated with the same Tris buffer, the apoaequorin solution was introduced into an anion exchange Mono Q column (Pharmacia) and the apoaequorin was eluted with a linear gradient of 0–0.3 M NaCl prepared in the same Tris buffer. The apoaequorin eluted as a single peak between 0.15 and 0.20 M NaCl and the fractions containing the apoaequorin were combined, dialyzed against distilled water, and freeze-dried. All of the purified apoaequorins gave a single band by SDS-PAGE (12.5%) and had purities of > 95%. The yields varied with the mutant protein and ranged from 1 to 30 mg per liter of culture medium. All molecular biology procedures were performed according to Sambrook et al. [19].

To obtain apoAQC145,152,180S, in which all three cysteine residues were replaced by serine, the aequorin gene in piP-HE [18] was replaced with the aequorin C145,152,180S (C1+2+3S) gene previously constructed and employed in piQ9-2HE [14]. Since the coding sequence for the *ompA* secretion signal is present in piP-HE, the expressed apoAQC145,152,180S was secreted into the culture medium, with the secretion signal cleaved, as reported previously [18]. The apoAQC145,152,180S was purified from the culture medium by acid precipitation and DEAE-cellulose chromatography [18].

2.4. *Modification of histidine residues by site directed mutagenesis*

Histidine modification was carried out by using the polymerase chain reaction (PCR) in which specific base substitutions were introduced as a mismatch between a PCR primer and the target sequence [20,21]. Besides wild type (piP-HE) apoaequorin (apoAQ), ten mutant apoaequorins were prepared: apoAQH16A, apoAQH16F, apoAQH18A, apoAQH27A, apoAQH27P, apoAQH58Y, apoAQH58F, apoAQH169A, apoAQH169F, and apoAQH169W. Each cDNA was sequenced by a modified dideoxynucleic acid sequencing method to confirm the presence of the mutation [22].

2.5. *Modification of histidine residues with DEPC*

DEPC, which reacts with the hydrogen of the N-3 nitrogen of histidine, was used to modify the histidine residues in wild type aequorin (piP-HE), wild type apoaequorin, aequorin AQC145,152,180S, and mutant apoaequorin apoAQC145,152,180S. Each protein was dissolved in a small volume of 66 mM potassium phosphate buffer, pH 7.6, passed through a NAP-5 column (Pharmacia) equilibrated with the same buffer, and the volume of the eluted solution was adjusted to give a protein concentration of 1 ng/μl. A 200 μl aliquot of the solution was mixed with 1–5 μl of stock DEPC solution freshly prepared in 66 mM potassium phosphate buffer, pH 7.6, to give the DEPC concentrations shown in Fig. 2. The mixture was incubated for 30 min at room temperature and 20 μl of 0.1 M histidine was added to terminate the modification reaction.

2.6. *Regeneration of aequorin and assay for activity*

The regeneration of apoaequorin in Table I was carried out by dissolving apoaequorin in 66 mM potassium phosphate buffer, pH 7.6/1 mM EDTA, at a concentration of 1 μg/ml and mixing 200 μl of the solution with 2 μg of coelenterazine and 2 μl of 2-mercaptoethanol [11]. Regeneration of the 220 μl of DEPC-treated wild type apoaequorin (Fig. 2) was performed by mixing the solution with 2 μg of coelenterazine and 2 μl of 2-mercaptoethanol; regeneration of DEPC-treated apoAQC145,152,180S was carried out with the 2-mercaptoethanol omitted [14]. CD measurements were performed at an aequorin concentration of 0.2 mg/ml, using proportionately higher concentrations of apoaequorin, coelenterazine, EDTA, and 2-mercaptoethanol in the regeneration mixture. All regenerations were carried out for 3 h in an ice bath. Luminescence activity was determined by transferring 50 μl of the regeneration mixture to a glass reaction cell and injecting 1.5 ml of 30 mM $CaCl_2$/30 mM Tris-HCl, pH 7.60. The initial maximal light intensity was read with a Labo Science (Tokyo) Model TD-8000 photomultiplier photometer, calibrated with a carbon-14 light standard [23].

2.7. *CD spectra of aequorin*

CD spectra of the aequorin solutions were measured with a Jasco (Tokyo) Model J-600 spectropolarimeter using a cell with a path length of 0.1 cm. The concentration of aequorin was 0.2 mg/ml in 66 mM potassium phosphate buffer, pH 7.6, estimated before regeneration of the apoaequorin by using an extinction coefficient of $E_{1\%,1.0\,cm}^{280\,nm} = 18.0$ at 280 nm [24]. The CD spectra was read against a control consisting of reagents used in the regeneration of

Table I

Bioluminescence activities of wild type and mutant aequorins

Aequorin		Luminescence activity[a] (%)	λ_{max} (nm)
Wild type (piP-HE)		100	465
AQH16A	$His^{16} \rightarrow Ala$	25.7	465
AQH16F	$His^{16} \rightarrow Phe$	0.1	–
AQH18A	$His^{18} \rightarrow Ala$	20.0	455
AQH27A	$His^{27} \rightarrow Ala$	69.8	460
AQH27P	$His^{27} \rightarrow Pro$	67.2	462
AQH58Y	$His^{58} \rightarrow Tyr$	14.9	470
AQH58F	$His^{58} \rightarrow Phe$	1.4	–
AQH169A	$His^{169} \rightarrow Ala$	1.1	470
AQH169F	$His^{169} \rightarrow Phe$	0.1	–
AQH169W	$His^{169} \rightarrow Trp$	0.0	–

[a] Luminescence activity was calculated as the ratio of the initial maximal light intensity relative to aequorin.

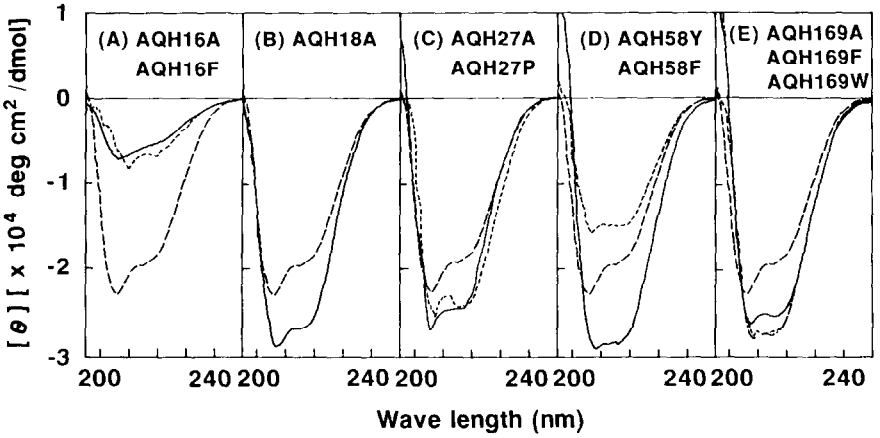


Fig. 1. CD spectra of aequorin (long dashed line) and modified aequorins. (A) AQH16F (solid line), AQH16A (short dashed line); (B) AQH18A; (C) AQH27P (solid line), AQH27A (short dashed line); (D) AQH58Y (solid line), AQH58F (short dashed line); and (E) AQH169A (solid line), AQH169F (short dashed line), AQH169W (dashed dotted dashed line). Modified apoaequorins were regenerated into their respective aequorins by incubation with coelenterazine for 3 h in an ice bath and CD spectra were taken as described in section 2.7.

aequorin. All measurements were performed at 4°C and each spectrum represented an average of at least five scans.

3. RESULTS AND DISCUSSION

Table I shows the bioluminescence activities of mutant aequorins with His^{16} and His^{18} replaced by Ala, His^{27} replaced by Ala and Pro and His^{58} replaced by Tyr. All showed fair to good (14.9–69.8%) luminescence activities. However, mutant aequorins with His^{169} replaced by Ala, Phe and Trp had very slight or no activity. Previously, aequorin with His^{58} replaced by Phe was observed to lack activity [25]. Mutant aequorins with the smallest changes in CD spectra (AQH18A, AQH27P and AQH27A), excluding AQH169A, AQH169F and AQH169W, also gave fair to good luminescence activities (Fig. 1 and Table I). The result with AQH16A is inexplicable, but His^{169} was investigated further as it seemed to be a good candidate for the site of oxygen binding.

Fig. 2 shows the effect of increasing concentrations of DEPC on the bioluminescence activities of aequorin and apoaequorin. Fig. 2A shows the luminescence activity of wild type aequorin, regenerated from DEPC-treated apoaequorin (lower curve), and DEPC-treated aequorin (upper curve). The same experiment was repeated (Fig. 2B) with apoAQC145,152,180S (lower curve) and AQC145,152,180S (upper curve). It is seen that in both experiments apoaequorin is highly sensitive to inactivation by DEPC, being completely inactivated at a concentration of 0.1 mM, whereas strong protection of aequorin from inactivation was observed when coelenterazine and molecular oxygen were incorporated into the protein. Since apoAQC145,152,180S and AQC145,152,180S did not contain any cysteine residues, it is reasonable to assume that the DEPC reaction involved almost exclusively the histidine residues. The above retardation of aequorin inactivation therefore may be due to specific interference of DEPC by bound coelenterazine and/or molecular oxygen. Further, since

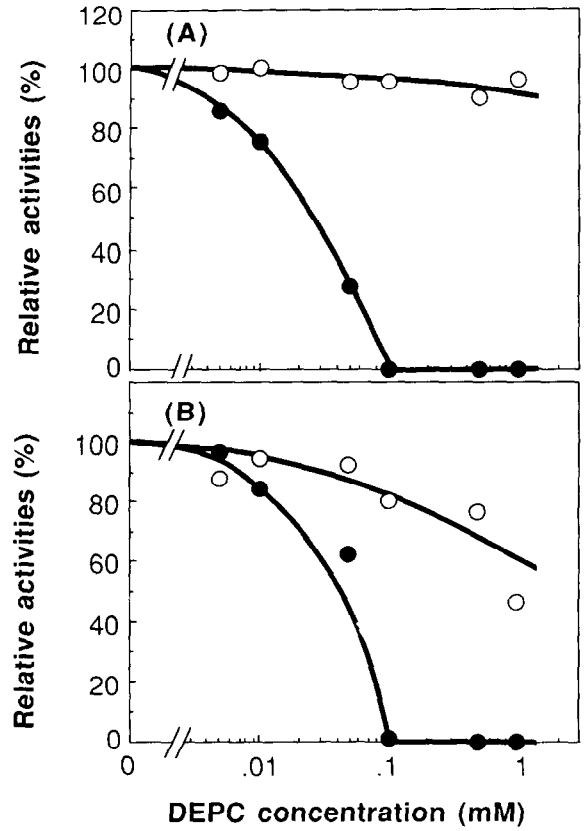


Fig 2. Effect of increasing concentrations of DEPC on the bioluminescence activity of aequorin and apoaequorin subsequently regenerated into aequorin. (A) Wild type aequorin (piP-HE) (○), wild type apoaequorin (●). (B) AQC145,152,180S (○), apoAQC145,152,180S (●). Details of experiment are described in section 2.5.

wild type apoaequorin and apoAQC145,152,180S, upon reacting with DEPC, did not show any significant change in CD spectra (data not shown), the loss of activity must be presumed to be due to histidine modification and not to a change in secondary structure.

The problem of the binding of molecular oxygen in aequorin has been discussed previously [4,25–27]. Because aequorin light emission is almost instantaneous on adding Ca^{2+} (pseudo-first-order rate constant ~ 100 s^{-1}; half rise-time ~ 6 ms) [9] and since coelenterazine chemiluminesces spontaneously in hydrophobic solvents [28,29], coelenterazine and molecular oxygen, for mechanistic reasons, must lie close together in a hydrophobic, putative catalytic site. One such scheme based on NMR evidence would be to have one of the oxygens of molecular oxygen bound to the C-2 carbon of coelenterazine [4] and to have the other oxygen, in order to stabilize the complex and prevent spontaneous luminescence, coordinated to an amino acid residue. The finding that mutant aequorins retain activity even when the 3 cysteine [14] and 6 tryptophan [15] residues are replaced with other amino acids, that luminescence activity is abolished or practically abolished when His^{169}, but not when His^{16}, His^{18}, His^{27} and His^{58}, is modified, and that luminescence activity is completely lost without change in secondary structure when apoaequorin is treated with DEPC, indicates that His^{169} is the site of binding of molecular oxygen in aequorin.

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