Blue fluorescent protein from the calcium-sensitive photoprotein aequorin is a heat resistant enzyme, catalyzing the oxidation of coelenterazine

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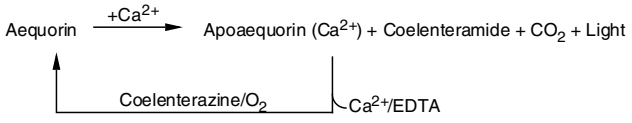
Abstract **Blue fluorescent protein from the calcium-sensitive photoprotein aequorin (BFP-aq) was prepared and determined to be a heat resistant enzyme, catalyzing the luminescent oxidation of coelenterazine (luciferin) with molecular oxygen as a general luciferase. After treatment with excess ethylenediaminetetraacetic acid to remove Ca^{2+} from BFP-aq, the blue fluorescence shifted to a greenish fluorescence. This greenish fluorescent protein (gFP-aq) was identified as a non-covalent complex of apoaequorin with coelenteramide (oxyluciferin) in a molar ratio of 1:1. By incubation with coelenterazine in the absence of reducing reagents, gFP-aq was converted to aequorin at 25 °C. BFP-aq and gFP-aq possessing both fluorescence and luminescence activities may work as novel reporter proteins.**

Keywords: Bioluminescence; Quantum yield; Luciferin; Enzyme–substrate complex; Refolding

1. Introduction

The photoprotein aequorin isolated from the bioluminescent jellyfish, *Aequorea aequorea* (synonyms *Aequorea victoria* or *Aequorea forskalea*), emits blue light by an intramolecular reaction when the protein binds Ca^{2+} [1] and decomposes into apoaequorin (Ca^{2+}), coelenteramide and CO_2 [2]:



The primary structure of apoaequorin has been determined by cDNA cloning [3] and consists of 189 amino acid residues. Recently, the crystal structure of aequorin was solved and revealed that three functional EF-hand structures and three free cysteine residues are present in the molecule. Further, the peroxide structure of coelenterazine at the C2 position was confirmed [4]. The cDNA encoding apoaequorin has been expressed in various eukaryotic and prokaryotic cells [5–7]. Apoaequorin expressed by these cells can be regenerated to aequorin in the presence of ethylenediaminetetraacetic acid (EDTA), a reducing reagent (i.e., 2-mercaptoethanol or dithiothreitol (DTT)), molecular oxygen (O_2) and coelenterazine [5,8]. Alternatively, semi-synthetic aequorins, in which the coelenterazine moiety has been replaced with various analogs of coelenterazine, have been produced and characterized [9–11].

Coelenterazine, a small organic compound (Mw = 423), is used as a luciferin for various luciferase reactions including *Renilla* luciferase [12], *Oplophorus* luciferase [13], *Periphylla* luciferase [14] and *Gaussia* luciferase [15] (Fig. 1) and also serves as the chromogenic compound of aequorin [4]. Although aequorin is not classified as a luciferase, the regeneration process combining with apoaequorin, coelenterazine and molecular oxygen to give aequorin is similar to the formation of an enzyme–substrate complex. Thus, aequorin (the non-covalent complex of apoaequorin with the hydroperoxide of coelenterazine) may be taken as comparable to an enzyme–substrate complex, as proposed by Hastings and Gibson in 1963 [16]. The product from the Ca^{2+}-triggered reaction of aequorin was named "blue fluorescent protein" (BFP-aq in this paper) [17] and the fluorescence spectrum was identical to that of the bioluminescence of aequorin [17–19]. BFP-aq, as a complex of Ca^{2+}-bound apoaequorin with coelenteramide, would then correspond to an enzyme–product complex.

In 1921, Harvey reported that squeezed extracts from the margin of the umbrella of *Aequorea* glowed blue for some hours, in one case for nine hours [20]. Shimomura and Johnson indicated that, after the Ca^{2+}-triggered luminescent reaction of native aequorin, the reaction mixture produced the continuous luminescence by incubation with coelenterazine [8]. Later in 1995, the conversion efficiency of BFP-aq from recombinant aequorin resulting from addition of excess Ca^{2+} was estimated to be 33% [21] and Shimomura proposed that the complex of Ca^{2+}-bound apoaequorin with coelenteramide might act as an enzyme, catalyzing the luminescent oxidation of coelenterazine [21]. However, the complex of Ca^{2+}-bound apoaequorin with coelenteramide or of apoaequorin with coelenteramide was not purified to homogeneity and not characterized in detail.

In this paper, the blue fluorescent protein (BFP-aq: a complex of Ca^{2+}-bound apoaequorin with coelenteramide) and the

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Abbreviations: BFP-aq, blue fluorescent protein from aequorin; gFP-aq, greenish fluorescent protein from aequorin; CTZ, coelenterazine; CTM, coelenteramide; CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; rlu, relative light units; TLC, thin-layer chromatography; SDS–PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis

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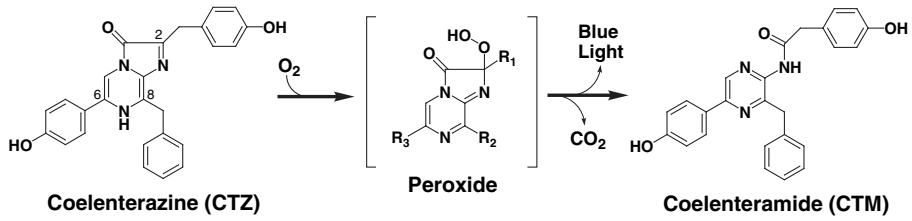

Fig. 1. Bioluminescence reaction of coelenterazine catalyzed by luciferase.

greenish fluorescent protein (gFP-aq: a complex of calcium-free apoaequorin and coelenteramide), both derived from aequorin, were prepared efficiently using slow interacting conditions with Ca^{2+} and characterized. We have shown that BFP-aq is a heat resistant protein that catalyzes the luminescence oxidation of coelenterazine and that gFP-aq can be regenerated to aequorin without reducing reagents.

2. Materials and methods

2.1. Materials

EDTA disodium salt, $CaCl_2 \cdot 2\ H_2O$, (±)-DTT, (−)-quinine sulfate dihydrate and 0.1 N H_2SO_4 (analytical grade) were obtained from Wako Pure Chemicals (Osaka, Japan). Coelenterazine was chemically synthesized. Coelenteramide as an authentic compound was kindly provided by Dr. O. Shimomura (Photoprotein Lab., Woods Hole) and coelenterazine analogs were kindly given by Dr. K. Teranishi (Mie Univ., Japan).

2.2. Preparation of recombinant aequorin

Recombinant apoaequorin and aequorin were prepared as previously reported [22,23]. Briefly, apoaequorin was expressed in the periplasmic space of *Escherichia coli* strain WA802 carrying piP-HE expression vector, followed by regeneration into aequorin and purification [23]. The purity of recombinant apoaequorin in aequorin on sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis was over 95% [24].

2.3. Preparation of BFP-aq and gFP-aq from aequorin

A solution of purified recombinant aequorin (8 mg) in 10 mM Tris–HCl, pH 7.6, containing 2 mM EDTA and 1.2 M $(NH_4)_2SO_4$ was concentrated to 0.1 ml using a centrifugal concentrator, Vivaspin 2 (10 000 MWCO; Sartorius AG, Germany), at $5000 \times g$ for 1 h at 4 °C. The concentrated solution of aequorin showed an orange color with a high viscosity. To prepare BFP-aq, 0.9 ml of 50 mM Tris–HCl, pH 7.6, containing 5 mM $CaCl_2$ and 2 mM DTT (Ca^{2+} solution) was layered on the aequorin solution in the centrifugal concentrator and allowed to stand at 4 °C. A blue luminescent band was observed at the interface between the aequorin solution and the Ca^{2+} solution over 24 h. After this continuous luminescence of aequorin was finished, the orange color of aequorin had disappeared completely and the blue fluorescence of BFP-aq could be detected under a long wave UV lamp ($\lambda_{max} = 366$ nm). The BFP-aq solution in the centrifugal concentrator was washed two times with 2 ml of the Ca^{2+} solution and concentrated by centrifugation using the same procedure. A quantitative yield of BFP-aq from aequorin was obtained. For preparation of gFP-aq from BFP-aq, 2 ml of 50 mM Tris–HCl, pH 7.6, containing 10 mM EDTA (EDTA solution) was added to 0.1 ml of the BFP-aq solution and washed by dilution and concentration three times with 2 ml of the EDTA solution. After removing Ca^{2+} ions from BFP-aq, gFP-aq having greenish fluorescence was obtained.

2.4. Preparation of Ca^{2+}-bound apoaequorin

The complex of Ca^{2+}-bound apoaequorin was prepared from reduced apoaequorin with $CaCl_2$. Thus, Ca^{2+}-free apoaequorin was purified by reversed-phase HPLC as previously described [22,23]. The solution of Ca^{2+}-free apoaequorin (1 mg/ml) was incubated in 50 mM Tris–HCl (pH 7.6) containing 1 mM EDTA and 10 mM DTT at 4 °C. After incubation for 18 h, $CaCl_2$ was added to a final concentration of 10 mM and the complex of Ca^{2+}-bound apoaequorin for assay was obtained.

2.5. Measurements of absorption and fluorescence spectra

Absorption spectra of BFP-aq and gFP-aq were measured in 50 mM Tris–HCl (pH 7.6) containing 10 mM $CaCl_2$ or 10 mM EDTA with a Jasco (Tokyo, Japan) V-560 spectrophotometer (band path 1.0 nm; response, Medium; scan speed, 200 nm/min) at 22–25 °C using a quartz cuvette (10 mm light path). The fluorescence spectra were measured with a Jasco FP-777W fluorescence spectrophotometer (emission and excitation band path, 1.5 nm; response, 0.5 s; scan speed, 60 nm/min) and were corrected according to the manufacturer's instructions. Fluorescence quantum yields were determined relative to quinine sulfate (quantum yield = 0.55, excited at 355 nm) in 0.1 M H_2SO_4 as a standard.

2.6. Measurement of bioluminescent spectra

Luminescence spectra of recombinant aequorin with Ca^{2+} and BFP-aq with coelenterazine were measured on a Jasco FP-777W fluorescence spectrophotometer (scan speed, 60 nm/min) at 22–25 °C with the excitation light source turned off.

2.7. Measurement of circular dichroism (CD) spectra

CD spectra were measured on a Jasco J-820 spectrometer at 20 °C or 45 °C using a quartz cuvette (2 mm light path, scan speed, 50 nm/min). The protein solutions (0.22 mg/ml) in 10 mM MES (pH 6.6), 100 mM KCl and 1 mM DTT were measured in the presence or absence of 1 mM $CaCl_2$.

2.8. Identification of coelenteramide as a prosthetic group for fluorescence in BFP-aq and gFP-aq

The BFP-aq or gFP-aq protein (0.26 mg/0.1 ml) was added to 0.9 ml of methanol (HPLC grade) and the solution was heated at 95 °C for 3 min in a heating block. After cooling on ice, the supernatant was obtained by centrifugation at $12\,000 \times g$ for 5 min at room temperature. The supernatant was analyzed by thin-layer chromatography (TLC) employing a silica gel 60 F-254 (Merck, Germany) and ethyl acetate/chloroform (2:1) as a solvent phase. The migration of the coelenteramide was monitored under UV light ($\lambda_{max} = 366$ nm), comparing with synthetic coelenteramide as an authentic compound ($R_f = 0.50$). The absorption and the fluorescence spectra of the methanol extract showed the absorption peaks at 278, 294 and 333 nm, and the emission peak at 428 nm (on excitation at 335 nm), respectively. The concentration of coelenteramide was determined spectrophotometrically using the absorption coefficient of 16.0×10^3 $M^{-1}\,cm^{-1}$ at 335 nm.

2.9. Protein analysis

Protein concentration was determined by the dye-binding method of Bradford [25] using a commercially available kit (Bio-Rad, Richmond, CA) and bovine serum albumin as a standard (Pierce, Rockford, IL). SDS–PAGE analysis was carried out under reducing conditions using a 12% separation gel (TEFCO, Tokyo, Japan), as described by Laemmli [24].

2.10. Mass spectrometry

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was measured on a Voyager DE Pro mass spectrometer (PerSeptive Biosystems Inc., Framingham, MA) as previously described [26].

2.11. Regeneration of aequorin from gFP-aq

The gFP-aq solution without DTT was prepared as follows: the gFP-aq solution (100 μl) was further washed three times with 2 ml of the EDTA solution using a centrifugal concentrator, Vivaspin 2. For regeneration, the reaction mixtures (100 μl) containing 20 μg of gFP-aq

and 2 μg of coelenterazine in the presence or absence of 1 mM DTT were incubated at various temperatures. The initial intensity of luminescence from regenerated aequorin was measured.

2.12. Assay for luminescence activity

The luminescence activity was measured using an Atto (Tokyo, Japan) AB2200 luminometer equipped with a Hamamatsu R4220P photomultiplier. The initial intensity (I_{max}) of 1 ng of the purified recombinant aequorin showed 6.4×10^4 relative light units. (i) Assay for aequorin: Following injection of 100 μl of 50 mM $CaCl_2$ in 50 mM Tris–HCl (pH 7.6) into the regenerated aequorin solution, the initial intensity of the luminescence was recorded. (ii) Assay for luciferase activity: The total reaction mixture (100 μl) contained coelenterazine (1 μg/μl dissolved in methanol) and 1 mM DTT in 50 mM Tris–HCl, pH 7.6. The reaction was started by addition of protein (2 μg) and the luminescence produced was recorded for 1 min with an AB2200 luminometer. The K_m value for coelenterazine was determined at concentration from 0.1 to 25 μM of coelenterazine by the method of Lineweaver–Burk plots.

3. Results and discussion

3.1. Preparation of BFP-aq and gFP-aq from aequorin

To avoid confusion, the abbreviations BFP-aq and gFP-aq have been introduced in this paper for the blue and greenish fluorescent proteins derived from aequorin. We have used these abbreviations because the name of green fluorescent protein (GFP) from the same jellyfish, *A. aequorea*, is familiar and the Y66H mutant of GFP having blue fluorescence has been called BFP [27].

During the purification of recombinant aequorin on a gram scale, an aequorin fraction with blue-greenish fluorescence was observed accidentally. This observation suggests that the slow interaction of Ca^{2+} with high concentrations of aequorin may give a stable complex of Ca^{2+}-bound apoaequorin with coelenteramide (BFP-aq). Following the procedures described in Section 2, aequorin has been converted to the fluorescent proteins, BFP-aq and gFP-aq (Fig. 2a). To confirm the presence of coelenteramide as a chromophore, the ethanol extracts from BFP-aq and gFP-aq were analyzed by spectral analyses (UV spectra, fluorescence emission/excitation), TLC analysis and mass spectral analyses, in comparison with the synthetic coelenteramide. The fluorescence chromophore in both gFP-aq and BFP-aq is coelenteramide. Calculations based on the absorption coefficient of coelenteramide indicate that better than 95% of coelenteramide was retained in BFP-aq. After treatment with excess EDTA to give gFP-aq from BFP-aq, coelenteramide in gFP-aq was also retained without loss. The conversion from gFP-aq to BFP-aq was reversible without loss of fluorescent intensity. By MALDI-TOF-MS analyses of BFP-aq and gFP-aq, the mass values gave *m/z* 21635.1 and 21631.5, respectively. These values were in good agreement with that of recombinant aequorin (average mass = 21632.2) [26]. These results suggest that no modification of amino acid residues such as decarboxylation and dehydration occurs in apoaequorin molecule and that coelenteramide is non-covalently bound to apoaequorin in BFP-aq and gFP-aq.

3.2. Spectral analyses of BFP-aq and gFP-aq

The absorption spectra and fluorescence emission spectra of BFP-aq and gFP-aq, and the luminescence spectrum of aequorin with Ca^{2+} are shown (Fig. 2b). The spectroscopic data of BFP-aq and gFP-aq including fluorescence quantum yield are

Table 1
Absorption, fluorescence and bioluminescence spectra of BFP-aq and gFP-aq

Spectroscopy	BFP-aq	gFP-aq
Absorbance		
λ_{max} (nm)	281, 336	282, 336
0.1% soln. at 280 nm	2.82	2.80
0.1% soln. at 335 nm	0.56	0.65
Fluorescence		
λ_{max} (nm) ex. at 335 nm	459	467
WHMF (nm)[a]	84	89
Quantum yield	0.079	0.073
Bioluminescence		
λ_{max} (nm)	460	459[c]
WHML (nm)[b]	84	84[c]

[a] WHMF, width at half-maximum of fluorescence band.
[c] Aequorin regenerated from gFP-aq.
[b] WHML, width at half-maximum of luminescence band.

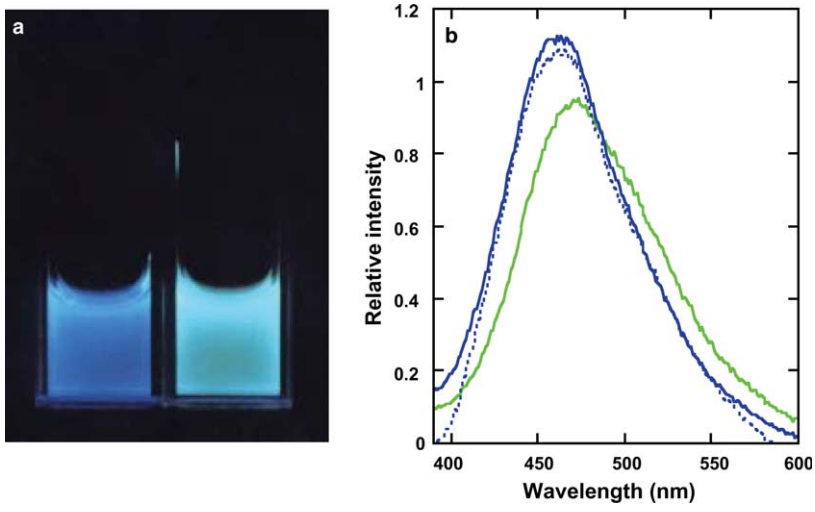

Fig. 2. BFP-aq and gFP-aq from aequorin. (a) Photograph of BFP-aq (left) and gFP-aq (right) under a long wave UV lamp (366 nm). Both protein concentrations were 2.6 mg/ml in 50 mM Tris–HCl (pH 7.6) containing 10 mM $CaCl_2$ or 10 mM EDTA. (b) Fluorescence emission spectra of BFP-aq (blue line) and gFP-aq (green line), and bioluminescence spectrum of aequorin with the slow interaction with Ca^{2+} (dashed blue line). Protein concentration for fluorescence spectra was 0.026 mg/ml in 50 mM Tris–HCl (pH 7.6) containing 10 mM $CaCl_2$ or 10 mM EDTA.

summarized in Table 1. The fluorescence spectrum of BFP-aq was identical to the bioluminescent spectra of aequorin triggered by Ca^{2+} and of BFP-aq with coelenterazine. As previously reported, the light emitter of aequorin is the amide anion of coelenteramide [28]. The species of the light emitter in both fluorescence of BFP-aq and the luminescence of BFP-aq with coelenterazine is presumably the amide anion of coelenteramide. In gFP-aq, the emitter species of coelenteramide is not clear. The fluorescence spectral change from blue to green suggests that the binding environment of coelenteramide in the protein was affected by Ca^{2+} release from BFP-aq. Fluorescence quantum yields of BFP-aq and gFP-aq showed approx. one-tenth the value of GFP quantum yield [27]. The spectroscopic properties of BFP-aq and gFP-aq are distinct from the slow discharged fluorescent protein from aequorin (emission $\lambda_{max} = 435$ nm) [29].

3.3. Fluorescence stability of BFP-aq and gFP-aq

Aequorin, a non-fluorescent protein, is heat labile and the luminescence is inactivated completely by incubation at 95 °C for 3 min. The heat stabilities of BFP-aq and gFP-aq prepared from aequorin were examined using fluorescence measurements. When BFP-aq was heated at 95 °C for 3 min, the blue fluorescence of BFP-aq disappeared in a second. However, the fluorescence recovered to over 93% after incubating for a further 20 min at 24 °C (Fig. 3), showing that BFP-aq is a heat resistant protein. Under the same conditions, the fluorescence recovery of gFP-aq reached only to 31%. On incubation at 45 °C for 10 min, the fluorescence of BFP-aq also disappeared but was recovered completely on ice in a minute. The studies on BFP-aq and gFP-aq by CD spectrometry showed that no significant spectral changes occurred in BFP-aq between 20 and 45 °C, but that spectral changes occurred in gFP-aq. At 45 °C, Ca^{2+}-bound apoaequorin without coelenteramide also gave no significant spectral changes, similar to the case of BFP-aq. These results suggest that Ca^{2+} binding to the EF-

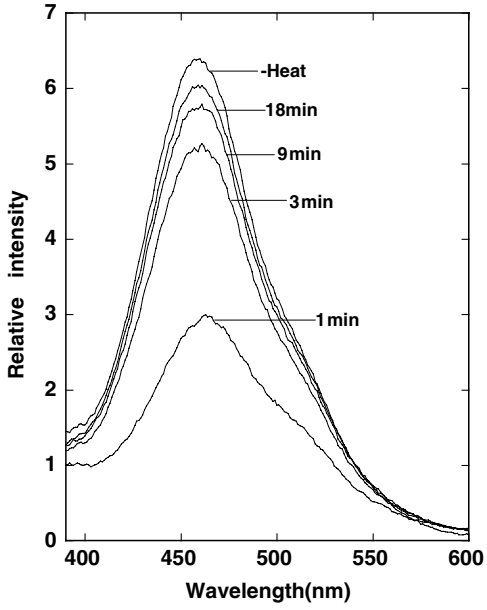

Fig. 3. Fluorescence recovery of BFP-aq after heat treatment. Protein concentration was 0.26 mg/ml in 50 mM Tris–HCl (pH 7.6) containing 1 mM DTT and 1 mM $CaCl_2$. The recovery of fluorescence was monitored from 1 to 18 min at 24 °C, after heating at 95 °C for 3 min.

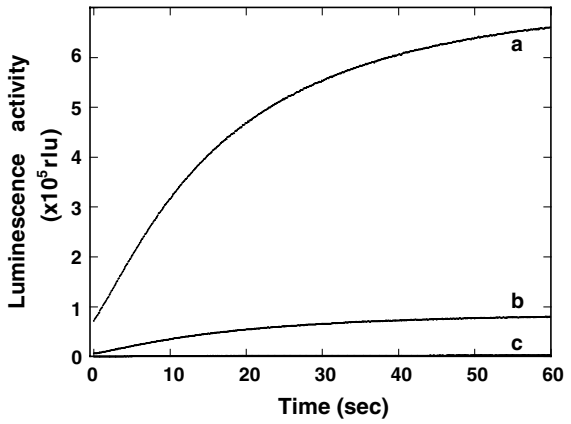

Fig. 4. Luminescence catalyzed by BFP-aq (a), Ca^{2+}-bounded apoaequorin (b) and apoaequorin (c) with coelenterazine in the presence of 1 mM DTT.

Table 2
Luciferase activity of aequorin, apoaequorin, apoaequorin-Ca^{2+} complex and apoaequorin-Ca^{2+}-coelenteramide (BFP-aq)

Conditions	Relative activity (%)
Reaction mixture[a]	>0.001
+Aequorin	>0.001
+Apoaequorin	0.5
+Apoaequorin-Ca^{2+} complex	12.1
+Apoaequorin-Ca^{2+}-coelenteramide (BFP-aq)	100

[a] Mixture (100 μl) contains 1 μg of CTZ in 50 mM Tris–HCl (pH 7.6)/1 mM DTT.

Table 3
Effect of DTT and heat treatment on luciferase activity of BFP-aq

Conditions		Relative activity (%)
DTT addition[a]	Heat treatment[b]	
+	–	100
+	+	93
–	+	63
–	–	92

[a] 1 mM DTT.
[b] 95 °C, 3 min.

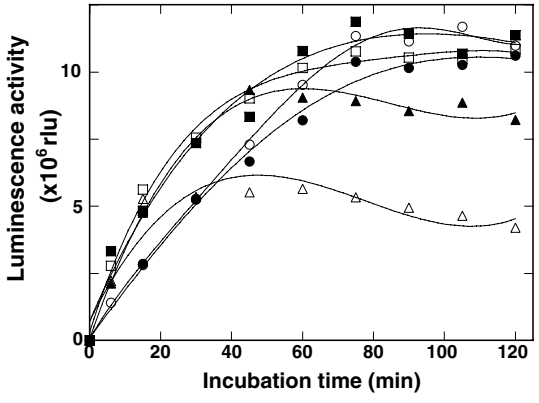

Fig. 5. Regeneration to aequorin from gFP-aq in the presence or absence of reducing reagent at various temperatures. Incubation at 4 °C with DTT (●) and without DTT (○), at 25 °C with DTT (■) and without DTT (□), at 37 °C with DTT (▲) and without DTT (△).

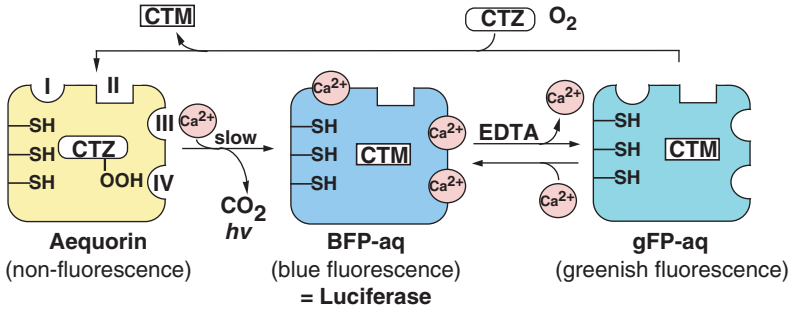

Fig. 6. Proposed mechanism for the BFP-aq and gFP-aq formation from aequorin, and the regeneration to aequorin.

hand domains of apoaequorin stabilizes the secondary structure and coelenteramide in Ca^{2+}-bound apoaequorin retains during heat treatment. Thus, the fluorescence activity of BFP-aq can be restored after heat treatment.

3.4. Catalytic properties of BFP-aq as a luciferase

When excess coelenterazine as a substrate was added to a BFP-aq solution in the presence of DTT, continuous luminescence was observed over 1 h (Fig. 4a). In the absence of DTT, the luminescence activity of BFP-aq slowly decreased (half period = 8.3 min). This phenomenon may be explained by disulfide-bond formation among three free cysteine residues in apoaequorin [3,5,8] during the oxidation of coelenterazine. On the other hand, Ca^{2+}-bound apoaequorin showed weak luminescence with coelenterazine and Ca^{2+}-free apoaequorin could not give significant luminescence (Fig. 4, lines b and c, respectively). Thus, BFP-aq and Ca^{2+}-bound apoaequorin are recognized as a luciferase. However, BFP-aq acts as a luciferase more efficiently than Ca^{2+}-bound apoaequorin (Table 2). Although coelenteramide is not an essential component to catalyze oxidation of coelenterazine, the role of the coelenteramide in BFP-aq as an enzyme appears to be to maintain an efficient conformation for catalysis. For this reason, the catalytic site for oxidation of coelenterazine may differ from the binding site of coelenteramide in BFP-aq and coelenteramide in BFP-aq retains during the luminescence reaction.

After heat treatment at 95 °C for 3 min in the presence of DTT and then cooling on ice, over 93% of the luminescence activity of BFP-aq with coelenterazine was recovered (Table 3). This result is consistent with the fluorescence recovery of BFP-aq (Fig. 3) and BFP-aq is identified as a heat resistant protein with blue fluorescence and luciferase activities. BFP-aq and gFP-aq did not show significant loss of their fluorescence and luciferase activities on prolonged storage, even after one year at 4 or −80 °C. As an enzyme, the K_m value of BFP-aq for coelenterazine was 13.3 μM.Substrate specificity was also examined using several coelenterazine analogs (final concentration; 250 μM) [30]. The relative luminescence activity was as follows: coelenterazine (100%), *h*-coelenterazine (175%), *e*-coelenterazine (0.3%), bisdeoxycoelenterazine (0.15%), and C6-methoxyphenylcoelenterazine (0.06%).

3.5. Regeneration of aequorin from gFP-aq without reducing reagents

Reducing reagents such as DTT or 2-mercaptoethanol are necessary to regenerate aequorin from apoaequorin with coelenterazine and molecular oxygen at 4 °C [5,8]. Interestingly, gFP-aq possessing coelenteramide could convert to aequorin without a reducing reagent and at 25 °C rather than 4 °C (Fig. 5). Based on the luminescence activity, the conversion efficiency to aequorin from gFP-aq was over 90% in 1 h at 25 °C. After regeneration to aequorin from gFP-aq, the greenish fluorescence of gFP-aq disappeared completely. This evidence strongly suggests that coelenteramide in gFP-aq was replaced by the peroxide of coelenterazine in the aequorin regeneration process (Fig. 6).

In *Aequorea* jellyfishes, high concentration of aequorin is present in photocyte of the outer margin of the umbrella [1]. After the luminescence reaction is triggered by an increase in free Ca^{2+}, aequorin in the photocyte is converted to BFP-aq. As free calcium levels in the photocyte fall, the bound Ca^{2+} is released from BFP-aq to give gFP-aq. When coelenterazine is present in the photocyte, gFP-aq can be regenerated to aequorin without reducing reagents. To account for our findings, the schematic representations of BFP-aq and gFP-aq formation from aequorin and the regeneration to aequorin from gFP-aq are summarized in Fig. 6. These two fluorescent proteins that have luciferase activity or Ca^{2+}-sensitive luminescence activity could play useful reporter proteins in various assay systems.

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