Revision of the Structure of the Light-emitter in Aequorin Bioluminescence

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The structure of the excited light-emitter in aequorin bioluminescence was assigned not to an amide anion of coelenteramide but to a phenolate anion on the basis of the luminescence of regenerated aequorin and of the fluorescence of regenerated blue fluorescent protein with coelenteramide analogues including *N*-methyl derivatives.

Aequorin (AQ) is a calcium-binding protein (M_r = 21400) from the jellyfish, *Aequoria victoria*, which emits light (465 nm) by an intramolecular reaction when binding calcium ions.[1,2] AQ is composed of coelenterazine (an imidazopyrazine compound) and molecular oxygen bound to apoaequorin (apoprotein).[3–5] During light emission, coelenterazine is oxidized to coelenteramide and a blue fluorescent protein is formed which consists of coelenteramide non-covalently bound to apoaequorin. While the singlet excited state of coelenteramide is known to be the emitter in the reaction,[6] there has been uncertainty regarding the structure of the emitter. Chemiluminescence and fluorescence studies of imidazopyrazine derivatives of coelenterazine in basic aprotic solvents have indicated that the emitter is the amide anion.[7–12] We here report, based on studies of coelenterazine derivatives with recombinant apoaequorin, that the emitting species is a phenolate monoanion of coelenteramide. This finding has relevance to a wide range of animal systems in which coelenterazine serves as the luminescence substrate.[13]

The calcium-activated light reaction proceeds very rapidly with a pseudo-first-order rate constant of *ca.* 100 s^{-1}, whereas the regeneration reaction takes place over a period of hours (Fig. 1).[14–17] Semi-synthetic AQ was prepared by incubating recombinant apoaequorin with coelenterazine, EDTA, dissolved oxygen and dithiothreitol (Fig. 1 and 2).[17] The regenerated AQ gave a bioluminescence emission spectrum identical to the fluorescence emission spectrum of the spent reaction mixture (Fig. 3A),[18] the fluorescence of which is due to the formation of a blue fluorescent protein (BFP) (Fig. 1). Incubation of recombinant apoaequorin with coelenteramide

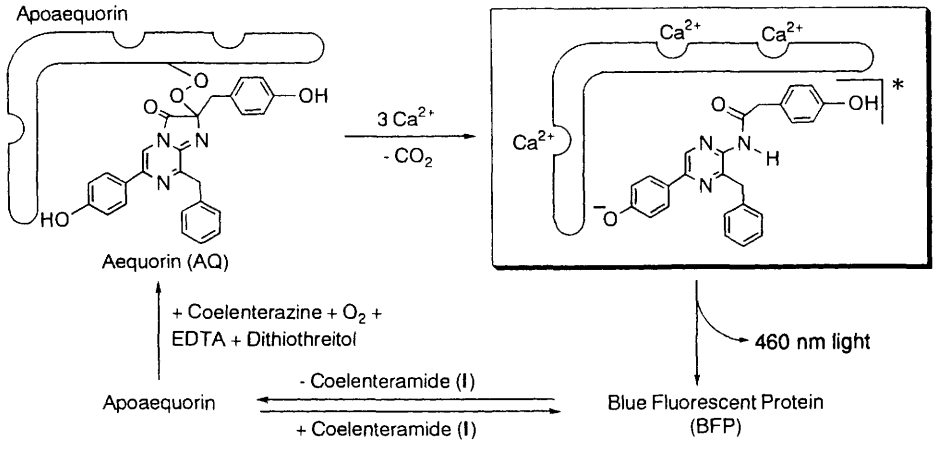

Fig. 1 Schematic diagram of the AQ regeneration and bioluminescence reactions

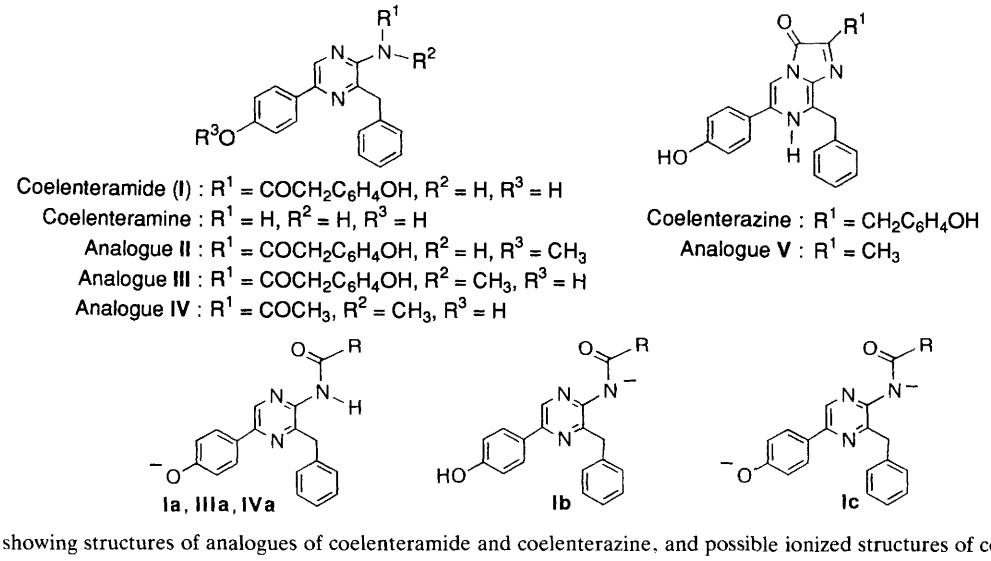

Fig. 2 Scheme showing structures of analogues of coelenteramide and coelenterazine, and possible ionized structures of coelenteramide

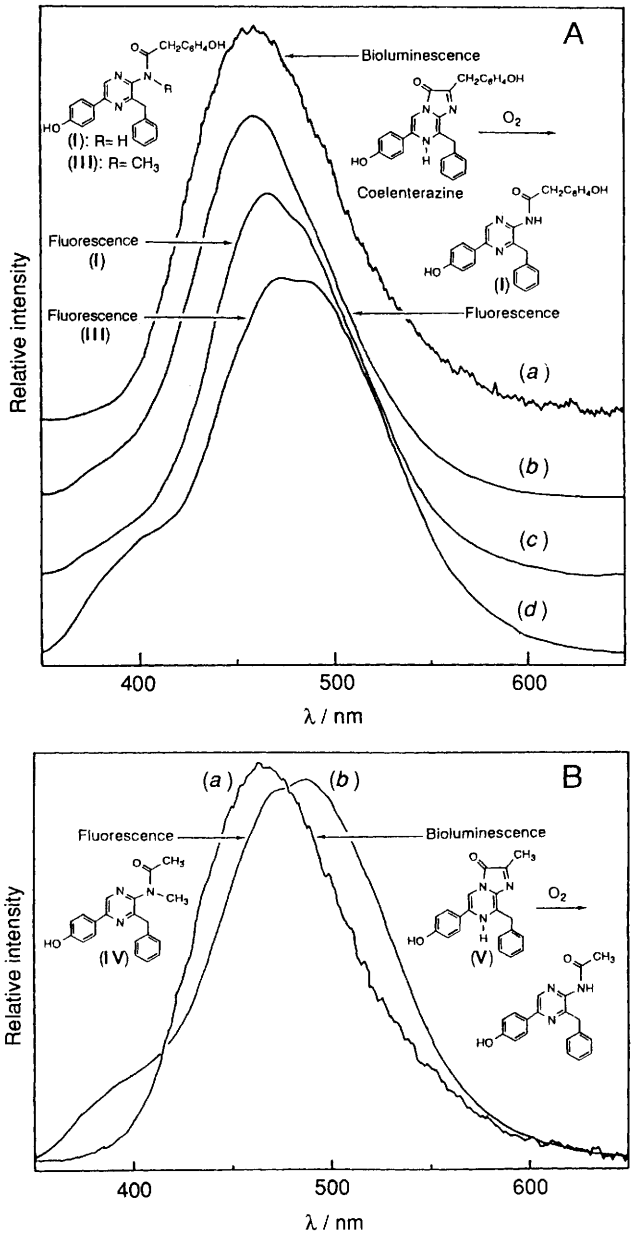

Fig. 3 Bioluminescence and fluorescence emission spectra of semi-synthetic AQs and BFPs prepared by incubating coelenterazine and its analogues with recombinant apoaequorin. A, Semi-synthetic AQ was regenerated by incubating recombinant apoaequorin[19] (4 μg) with coelenterazine[20,21] (160 μg) in 2.0 ml of 30 mmol l^{-1} Tris-HCl buffer (pH 7.6) containing 10 mmol l^{-1} EDTA and 17 mmol l^{-1} dithiothreitol (DTT) for 3 h in an ice bath.[17] A 500 μl aliquot of the AQ solution was injected into 1.0 ml of 30 mmol l^{-1} Tris-HCl buffer (pH 7.6) containing 30 mmnol l^{-1} $CaCl_2$ and the bioluminescence emission spectrum (*a*) was measured with a Hamamatsu PMA-10 image intensification-multiphoton counting system. The fluorescence emission spectrum (*b*) of the spent reaction mixture (BFP) was measured by excitation at 330 nm with a Hitachi F-4010 fluorescence spectrophotometer. The fluorescence emission spectra (*c*) and (*d*) of regenerated BFP were measured similarly after incubating coelenteramide **I**[21] and analogue **III**, respectively at a concentration of 8.0×10^{-5} mol l^{-1} with 80 μg of recombinant apoaequorin in 420 μl of 30 mmol l^{-1} Tris-HCl buffer (pH 7.6) containing 10 mmol l^{-1} EDTA and 2 mmol l^{-1} DTT for 24 h in an ice bath, followed by the addition of 1 μl of 30 mmol l^{-1} Tris-HCl buffer (pH 7.5) containing 1 mol l^{-1} $CaCl_2$. A nearly identical fluorescence spectrum was obtained when $CaCl_2$ was omitted. B, Semi-synthetic AQ was prepared by incubating recombinant apoaequorin (280 μg) with **V** (70 μg) in 700 μl of 30 mmol l^{-1} Tris-HCl buffer (pH 7.6) containing 10 mmol l^{-1} EDTA and 10 mmol l^{-1} dithiothreitol for 3 h in an ice bath.[17] After passage through a Pharmacia NAP-5 column to remove unreacted reagents, 750 μl of semi-synthetic aequorin was obtained, of which 50 μl was mixed with 250 μl of 30 mmol l^{-1} Tris-HCl buffer (pH 7.6) containing 30 mmol l^{-1} $CaCl_2$ and the emission spectrum (*a*) was measured with PMA-10. Fluorescence emission spectrum (*b*) of BFP was measured by excitation at 330 nm. BFP was prepared by incubating analogue **IV** at a concentration of 7.8×10^{-5} mol l^{-1} with 70 μg of recombinant apoaequorin in 368 μl of 30 mmol l^{-1} Tris-HCl buffer (pH 7.6) containing 10 mmol l^{-1} EDTA and 2 mmol l^{-1} dithiothreitol for 24 h in an ice bath, followed by the addition of 1 μl of 30 mmol l^{-1} Tris-HCl buffer (pH 7.5) containing 1 mol l^{-1} $CaCl_2$. a nearly identical fluorescence spectrum was obtained when $CaCl_2$ was not added.

(Fig. 2, **I**) also produced BFP. The fluorescence intensity gradually increased with the incubation time and the spectrum (Fig. 3A) was red shifted *ca.* 5 nm, possibly due to imperfect alignment between coelenteramide and apoaequorin, but it was close to being the same as the bioluminescence and fluorescence emission spectra of semi-synthetic AQ (Fig. 3A). Since coelenteramide and apoaequorin are nearly non-fluorescent in aqueous solution, the fluorescence obtained by incubating the two is due to the formation of BFP.

As seen in Fig. 2, coelenteramide has three dissociable acidic protons located at the two phenolic hydroxyls and amide groups. The fact that the coelenterazine analogue in which $R^1 = CH_2C_6H_5$ is still very active with apoaequorin compared to coelenterazine with $R^1 = CH_2C_6H_4OH$ indicates that the hydroxy group is not an essential part of the structure of the emitter.[22,23] Earlier chemiluminescence studies[7–12] have shown that the emission at 465 nm is due to a monoanion (**Ib**) and those at 530 and 400 nm are due to a dianion (**Ic**) and a neutral species (**I**), respectively. Thus, **Ia** and **Ib** were considered as the possible emitter at 465 nm, as discussed in the case of two excited states induced by tryptophan modification of aequorin.[23]

To decide between the two structures, analogues **II**, **III** and **IV** were synthesized. **II** was prepared by treatment of *O*-methylcoelenteramine[21,22] with *p*-acetoxyphenylacetyl chloride. **III** was obtained by the coupling of *N*-methylcoelenteramine with *p*-acetoxyphenylacetic acid using BroP and **IV** by acetylation of *N*-methylcoelenteramine which was prepared by formylation of coelenteramine (Fig. 2) followed by lithium aluminium hydride reduction. The fluorescence emission spectrum of **II** in BFP could not be observed because **II** in Tris HCl buffer (pH 7.6) (excitation = 330 nm) produced an intense fluorescence (λ_{max} = 450 nm) which masked any emission at 465 nm. However, incubation of **III** with recombinant apoaequorin yielded a BFP with a fluorescence emission spectrum possessing a peak at 480 nm (Fig. 3A). This emission maximum is similar to the bioluminescence maximum of AQ, suggesting that the excited-state emitter in AQ could not be the amide anion but be the phenolate anion **Ia**. **IV** incubated with recombinant apoaequorin also showed a fluorescence spectrum of BFP at 485 nm (Fig. 3B). When **V** was synthesized[20] and incubated with recombinant apoaequorin, the regenerated semi-synthetic AQ had a bioluminescence emission spectrum (λ_{max} 465 nm) that was essentially the same as that of AQ regenerated with coelenterazine (Fig. 3B). Thus, the structures of the emitters, **III** and **IV**, at 480–485 nm would be **IIIa** and **IVa**, respectively, and that of coelenteramide at 465 nm would be **Ia** (Fig. 2), even though **Ia** had previously been reported to be unimportant in the emission.[12,24,25]

The assignment of the amide anion (**Ib**) by the previous workers as the emitter in AQ bioluminescence is based on spectral measurements of imidazopyrazine compounds in aprotic solvents.[7–12] Our finding that the phenolate anion (**Ia**) is the emitter in AQ bioluminescence indicates that excited-state formation in free solution differs from that taking place within the active site of the AQ molecule, where steric amino acid residues and microenvironment are presumably the controlling factors.[23] This assignment is not unreasonable since the chemiluminescence quantum yield of coelenterazine

and coelenterazine analogues in aprotic solvents is always significantly less than the quantum yield in AQ bioluminescence.[12,18,26]

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