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Review

Role of molecular oxygen in the bioluminescence of the firefly squid, *Watasenia scintillans*

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Abstract

The bioluminescence system in the "firefly squid," *Watasenia scintillans*, is described. The light-emitting components consist of luciferin (coelenterazine disulfate), a membrane-bound luciferase, ATP, Mg^{2+}, and molecular oxygen. A hypothetical scheme is proposed for the light-emitting reaction.

Keywords: Luciferin; Coelenterazine disulfate; Luciferase; Oxygenase; Enolization; ATP; Adenylation; Dioxetanone

Watasenia scintillans is a small (mantle length, ~6 cm; wet weight, ~9 g), luminescent deep-sea squid, indigenous to northern Japan. Females carrying fertilized eggs come inshore each spring by the hundreds of millions, even a billion, to lay eggs in Toyama Bay (max. depth, ~1200 m) and die, thereupon completing a 1-year life cycle [1].

Watasenia possesses numerous (~800), minute dermal light organs (photophores) on its ventral side. Other organs are scattered over the head, funnel, mantle, and arms, but none is found on its dorsal side. There are five prominent organs beneath the lower margin of each eye. They all emit a bluish light (Fig. 1). A cluster of three tiny black-colored organs (<1 mm diam) is located at the tip of each of the fourth pair of arms. They emit brilliant flashes of light which are clearly visible to the unaided eye even in a lighted room. Some of the flashes have a cadence resembling that of a terrestrial firefly flashing at night, and thus the squid is known in Japan as the "firefly squid" or "hotaru-ika."

The luminescence of *Watasenia* was first described 100 years ago by Watasé [2], after whom the squid is named. The light emitted was first thought to be due to the presence of symbiotic luminous bacteria in the light organs [3,4], but subsequent electron microscopic studies showed that the rod-like bodies in the organs were proteinaceous crystals [5]. Later, Goto and colleagues [6–9] isolated from the arm organs of 10,000 specimens a compound with a structure similar to that of coelenteramide [2-(*p*-hydroxyphenylacetyl)-amino-3-benzyl-5-(*p*-hydroxyphenyl)pyrazin], with the two phenolic hydroxyl groups esterified by sulfuric acid. The sulfated compound was presumed to be the oxidized product of *Watasenia* "luciferin" and was called *Watasenia* "oxyluciferin." Knowing that this structure was analogous to that of the oxyluciferin of *Cypridina hilgendorfii*, a luminescent marine ostracod, these workers were able to deduce a structure for *Watasenia* "luciferin." They then isolated and identified from the arm organs a compound which they called *Watasenia* "luciferin." The compound had the structure of coelenterazine disulfate [8-benzyl-3,7-dihydro-2-(4-hydroxysulfonyloxybenzyl)-6-(4-hydroxysulfonyloxyphenyl)-imidazo[1,2-*a*]pyrazin-3-one], identical to that proposed earlier for *Watasenia* "luciferin." The compound was synthesized, but it could not be tested for luciferin activity because a light-emitting extract could not be prepared from the light organs.

Subsequently, the *Watasenia* bioluminescence reaction was shown to be an ATP-dependent reaction [10]. The luminescence reaction requires a soluble component (luciferin), an insoluble membrane-bound component (luciferase),

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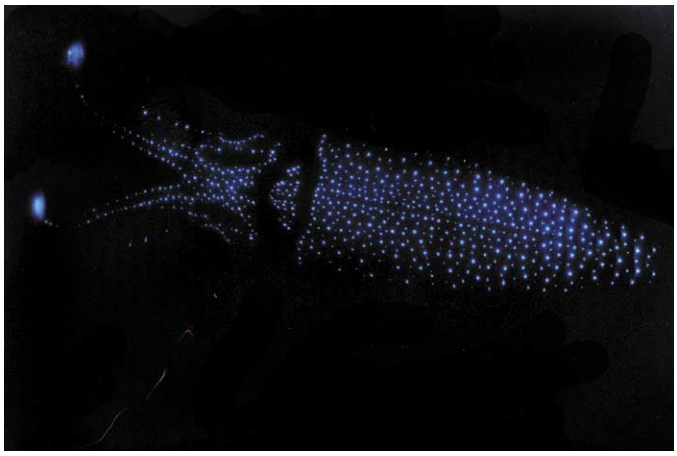

Fig. 1. Ventral view of luminescing *W. scintillans*. The brightest light is seen as flashes and glows emanating from a cluster of three tiny organs (<1 mm diam) located at the tip of each of the fourth pair of arms [10].

ATP, magnesium ions, and molecular oxygen. The soluble and insoluble components may be prepared by homogenizing the arm organs of the squid in an all-glass homogenizer in 0.001 M $MgCl_2$ and centrifuging at 13,000*g*. The supernatant contains the soluble component (luciferin) and the pellet the insoluble component (membrane-bound luciferase). When an aliquot of the homogenate, diluted with Tris–HCl, pH 8.26, is injected with ATP/Mg^{2+}, a rapid rise in light intensity occurs, followed by a decay according to pseudo-first order kinetics. The decay constant is independent of initial ATP concentration. If, after the light intensity has decayed for several minutes, the reaction mixture is injected with 1.0 μg of synthetic coelenterazine disulfate, there is again a sharp increase in light intensity, followed by a gradual decay [11,12]. If a pellet is resuspended in a freshly prepared supernatant and an aliquot, diluted with Tris–HCl, pH 8.26, is injected with ATP/Mg^{2+}, a similar sharp increase in light intensity occurs, followed by a gradual decay. These and other findings strongly indicate that coelenterazine disulfate is the luciferin in the *Watasenia* reaction. The bioluminescence emission spectrum ranges from 400 to 580 nm, with a peak at 470 nm.

Using fresh pellets and ATP, it is therefore possible to assay for luciferin in the supernatant. By this assay, supernatants are found to contain relatively high concentrations of luciferin and the luciferin shows little loss of activity when kept at −40 °C for several years. In contrast, the membrane-bound luciferase is highly unstable and freshly prepared homogenates of arm organs rapidly lose activity in about 3 h in an ice-bath. When kept at −40 °C, homogenates lose ~35% of its activity in 24 h. At −80 °C, luminous organs lose all of their activity after several days of storage. Work with protease inhibitors and various detergents shows that loss in activity is not due to protease digestion.

Shoji [13] was the first to show that molecular oxygen (air) is required in *Watasenia* luminescence. The light from a piece of mantle tissue was extinguished when either hydrogen gas or carbon dioxide was passed through a glass vessel containing the tissue, and the tissue regained its luminescence when air was readmitted. Similar results may also be obtained with homogenates of the arm organs.

Fig. 2 shows the A-shaped, all-glass apparatus used for mixing components of the *Watasenia* luminescence reaction in the absence and presence of molecular oxygen [11]. The apparatus consists of two arms (tubes) connected to each other by a standard-taper joint. When the right arm is rotated upward, its content empties directly into the left arm. The apparatus has three stopcocks that are used for purging the assembled apparatus of oxygen (air), for sealing the apparatus from the outside atmosphere, and for admitting air into the apparatus. To carry out an experiment, 800 μl of 0.005 M ATP (sodium salt, dissolved in 0.001 M $MgCl_2$) + 88 μl of 1.0 M Tris–HCl, pH 8.26, are introduced into the right arm with a pipet. Then 1600 μl of a homogenate (prepared by homogenizing the arm organs of 15 specimens in 5.0 ml of 0.001 M $MgCl_2$ in an all-glass homogenizer in an ice-bath) + 176 μl of 1.0 M Tris–HCl, pH 8.26, are pipetted into the left arm through the opening at the top. After lubricating the standard-taper joint and the three stopcocks with silicone grease, the apparatus is assembled and held in an upright position with a clamp. The apparatus is flushed with 99.999% argon through the opening on the right arm for ~7 min to purge oxygen (air) present. Second, all stopcocks are closed. Third, after ~8 min from the start of flushing, the right arm is rotated upward, allowing the ATP/Mg^{2+} solution to flow into the left arm. Fourth, at ~9.5 min, all stopcocks are opened to the atmosphere and oxygen (air) is admitted at a slight positive pressure into the apparatus through the open stopcock on the left arm (arrow), causing the mixture to be aerated and mixed. The light intensity in the bottom of the left arm is monitored with a photomultiplier photometer [14], previously calibrated with a light standard [15], with the window of the photomultiplier nearly

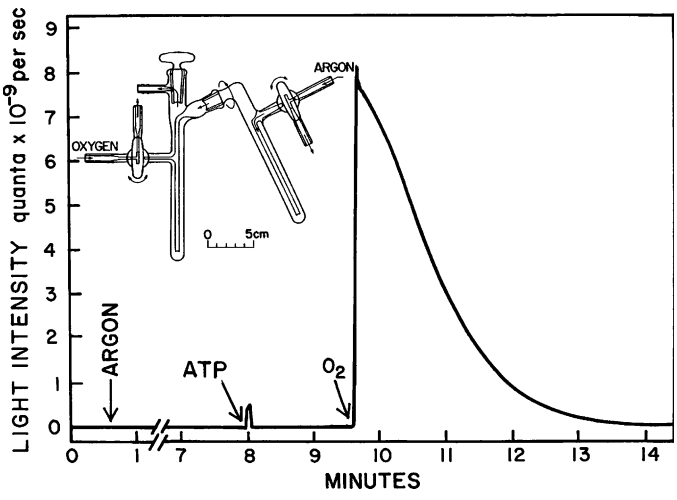

Fig. 2. Apparatus used for mixing *Watasenia* luciferin and membrane-bound luciferase in the absence of molecular oxygen (air) and the sharp stimulation in light emission following injection of molecular oxygen [11].

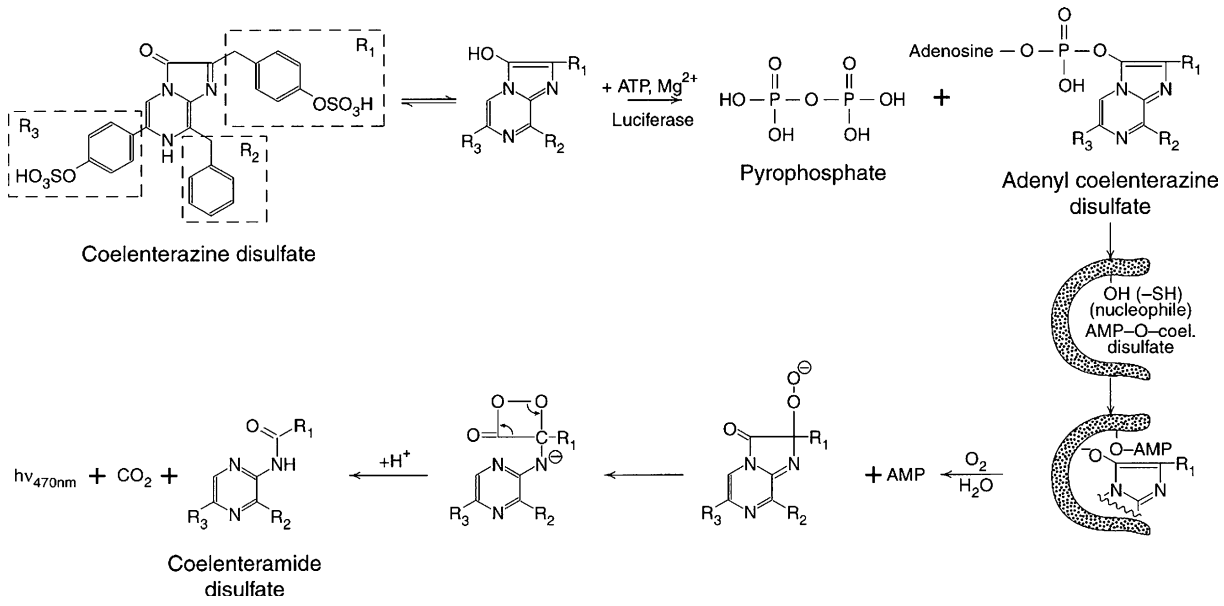

Fig. 3. Hypothetical scheme for the *Watasenia* luminescence reaction catalyzed by membrane-bound luciferase [11].

touching the bottom of the left arm (the arm is taped with black electrical tape, exposing only the bottom). In the figure, a small amount of light is seen when ATP/Mg^{2+} is added to the homogenate. This light is due to a trace of oxygen (air) remaining in the apparatus. The admission of oxygen (air) into the homogenate mixture in the left arm is seen to result in a rapid rise in light intensity, followed by a gradual decay. From these results we conclude that the *Watasenia* reaction requires molecular oxygen.

Besides requiring luciferin (coelenterazine disulfate), ATP, magnesium ions, membrane-bound luciferase, and molecular oxygen, the *Watasenia* luminescence reaction also requires an alkaline pH, ranging in pH from ~7.50 to ~9.50, with an optimum at 8.80 [10]. The light intensity produced by injecting ATP/Mg^{2+} into a mixture of homogenate and Tris–HCl, pH 8.26, is highly dependent on hydroxyl ion concentration [11]. The light intensity is maximum when the luciferin and luciferase (e.g., homogenate) are in Tris–HCl, pH 8.26, whereas it is negligible or significantly reduced if only luciferin (supernatant) or luciferase (pellet) is in Tris–HCl, pH 8.26, before injection of ATP/Mg^{2+}. These results suggest that in the luminescence reaction luciferin (coelenterazine disulfate) is activated by hydroxyl ions and luciferase acting in concert.

A hypothetical scheme for the *Watasenia* luminescence reaction is presented in Fig. 3 [11]. The scheme is consistent with all of the observed results. First, a rapid base/enzyme-catalyzed enolization of the keto oxygen of the C-3 carbon of coelenterazine disulfate (luciferin) takes place. Second, the enol group is adenylated by ATP, forming the compound adenyl coelenterazine disulfate. The AMP group serves as a recognition moiety for docking the molecule to the membrane-bound luciferase. Third, the AMP group is removed and molecular oxygen is added to the C-2 carbon, forming an unstable dioxetanone intermediate. Fourth, the intermediate spontaneously decomposes to yield CO_2, coelenteramide disulfate, and 60 kcal/mol required for the blue light emission (470 nm). During cleavage of the dioxetanone ring, one of the oxygens of molecular oxygen is incorporated into CO_2 and the other into coelenteramide. The light emitter is the amide anion in the singlet excited state bound to luciferase [8,16,17].

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