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Biochimica et Biophysica Acta 1564 (2002) 189–197

BIOCHIMICA ET BIOPHYSICA ACTA
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Bioluminescence reaction catalyzed by membrane-bound luciferase in the "firefly squid," *Watasenia scintillans*

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Received 13 December 2001; received in revised form 11 March 2002; accepted 11 April 2002

Abstract

The small Japanese "firefly squid," *Watasenia scintillans*, emits a bluish luminescence from dermal photogenic organs distributed along the ventral aspects of the head, mantle, funnel, arms and eyes. The brightest light is emitted by a cluster of three tiny organs located at the tip of each of the fourth pair of arms. Studies of extracts of the arm organs show that the light is due to a luciferin–luciferase reaction in which the luciferase is membrane-bound. The other components of the reaction are coelenterazine disulfate (luciferin), ATP, Mg^{2+}, and molecular oxygen. Based on the results, a reaction scheme is proposed which involves a rapid base/luciferase-catalyzed enolization of the keto group of the C-3 carbon of luciferin, followed by an adenylation of the enol group by ATP. The AMP serves as a recognition moiety for docking the substrate molecule to a luciferase bound to membrane, after which AMP is cleaved and a four-membered dioxetanone intermediate is formed by the addition of molecular oxygen. The intermediate then spontaneously decomposes to yield CO_2 and coelenteramide disulfate (oxyluciferin) in the excited state, which serves as the light emitter in the reaction.

Keywords: Luciferin–luciferase; Coelenterazine disulfate; Base/enzyme-catalyzed enolization; ATP activation

1. Introduction

Each spring, in an out-of-the-way region of northern Japan, where the Japanese Alps run close along the coast of the Sea of Japan, females of the small, indigenous, deep-sea squid, *Watasenia scintillans* (mantle length ~ 5.7 cm; wet weight ~ 8.9 gm), come inshore to spawn [1,2]. They number in the hundreds of millions and even a billion when calculated on the basis of the number of tons caught annually by the local fishery. The squid has served as food from early times, as has, in Europe, the luminescent boring mollusc, *Pholas dactylus*, from which the terms "luciferine" and "luciferase" have their origin [2,3]. The migration takes place from the middle of March until the end of May. Virtually all (99.9%) of the squids caught by net during this period are females bearing fertilized eggs. The phenomenon of mass migration is most notable near the small maritime city of Namerikawa situated at the southern end of Toyama Bay (deepest depth ~ 1200 m) where the squids swim into shallow water, lay their eggs and die, thereupon completing a 1-year life cycle. The life cycle of the male is not known. *Watasenia* is famous in Japan from years past, but it is not known outside the country. The squid has some 800 minute dermal light organs scattered over its ventral side, five prominent organs distributed along the lower margin of each eye and a cluster of three tiny (<1 mm diameter) black pigmented organs located at the tip of each of the fourth pair of ventral arms. The arm organs, which discharge brilliant flashes of bluish light when the animal is mechanically or electrically stimulated, produce the brightest light. Except for a difference in color, the flashing of the arm organs resembles the flashing of a firefly, and thus the squid is commonly called "hotaru-ika" or the "firefly squid."

Watasenia luminescence has been studied for nearly 100 years by various workers [2,3], including Watasé [4], after whom the squid is named, and Harvey [3], but the mechanism

Abbreviations: Coelenteramide, 2-(*p*-hydroxyphenylacetyl)-amino-3-benzyl-5-(*p*-hydroxyphenyl)pyrazin; Coelenterazine, 2-(*p*-hydroxybenzyl)-6-(*p*-hydroxyphenyl)-3,7-dihydroimidazo-[1,2-a]pyrazin-one; Coelenterazine disulfate, 8-benzyl-3,7-dihydro-2-(4-hydroxysulfonyloxybenzyl)-6-(4-hydroxysulfonyloxyphenyl)-imidazo [1,2-a]pyrazin-3-one; ATP, adenosine 5′-triphosphate; AMP, adenosine 5′-monophosphate; $Na_4P_2O_7$, tetrasodium pyrophosphate; PAPS, adenosine 3′-phosphate 5′-phosphosulfate; APS, adenosine 5′-phosphosulfate; DTT, dithiothreitol; TTP, thymidine 5′-triphosphate; GTP, guanosine 5′-triphosphate; CTP, cytidine 5′-triphosphate; UTP, uridine 5′-triphosphate

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0005-2736/02/$ - see front matter
PII: S0005-2736(02)00447-9

of the luminescence reaction has remained unresolved. Some of the early workers believed that the light was due to symbiotic luminous bacteria inhabiting the light organs, but later electron microscopic studies demonstrated that the rod-like bodies in the light organs were proteinaceous crystals and not bacteria [5]. Chemical studies conducted by Goto et al. [6–9] have provided evidence that the light may be due to a luciferin–luciferase reaction, with the luciferin having an imidazopyrazine ring structure. The workers isolated from the arm organs of 10,000 *Watasenia* specimens a compound which they showed to be a sulfated form of coelenteramide and assumed it to be the oxidation product of the bioluminescence reaction. They then isolated a second compound and determined its structure to be coelenterazine disulfate, which they called *Watasenia* luciferin. They synthesized the compound but it was not tested for activity because an active luciferase extract could not be prepared from the arm organs. Tsuji [10] subsequently showed that *Watasenia* luminescence is due to an ATP-dependent reaction whose essential components are luciferin, a membrane-bound luciferase, ATP and Mg^{2+}, and whose optimum pH is 8.80. More recently, improved methods for the syntheses of coelenterazine disulfate and monosulfates of coelenterazine have been reported [11].

Biochemical study of the mechanism of the *Watasenia* luminescence reaction has proved difficult because: (a) the squid is available only for a short time during spring, (b) the animal cannot be kept alive for more than 3–5 days in captivity, (c) the squid dies in about 10–20 min when taken out of the water with the photophores becoming irreversibly dark, (d) the physical vitality of the squid, including its ability to produce light, wanes as the temperature of the seawater rises with the oncoming summer, and (e) the luminescence system is highly unstable even when the light organ and extracts of the organ are kept in an ice-bath or frozen in dry-ice. The present paper reports the results of work carried out 3–4 weeks each spring over a period of 20 years.

2. Materials and methods

All of the experiments were carried out in Namerikawa at the Toyama Prefectural Fisheries Research Institute, which is largely concerned with the management of fishery stock and the monitoring of the marine environment in the region.

2.1. Materials

Specimens of *W. scintillans* were caught using trap nets set offshore in Toyama Bay. The nets were raised in early morning and the squids were quickly transferred to an 865-l plastic holding tank located in a darkened room. The tank was supplied continuously with rapidly flowing, aerated seawater pumped from a depth of 320 m in Toyama Bay. The temperature of the seawater in the tank ranged between 2.5 and 3.0 °C. About 30–40 specimens were kept in the tank at any given time in order to keep the mortality rate low and the animals were not fed. The squids remained quiescent at the bottom of the tank without luminescing. When netted, they flailed their arms and tentacles in the air and their arm organs emitted brilliant flashes of light that trailed off into long glows.

2.2. Chemicals

ATP (sodium salt), $Na_4P_2O_7$, α,β-methyleneadenosine 5′-triphosphate (lithium salt), β,γ-methyleneadenosine 5′-triphosphate (sodium salt), PAPS (lithium salt) and APS (sodium salt) were obtained from Sigma Chemical Co. The other chemicals were of analytical grade and were purchased from Wako Pure Chemicals, Osaka, Japan. Synthetic coelenterazine disulfate was a gift of Dr. Shoji Inoue; coelenterazine was chemically synthesized [7].

2.3. Measurement of light intensity

A Mitchell–Hastings photomultiplier photometer [12], calibrated with a carbon-14 light standard [13], was used to measure light intensity. The detector was a Hamamatsu 1P21 photomultiplier, selected for low noise, with an S-4 spectral response. The output from the amplifier was fed into a Panasonic VP-6712A chart recorder. Because Japanese law stipulates that all radioisotopes must be kept and used in a government approved radiological facility, the photometer was calibrated at the Osaka Bioscience Institute in Osaka and transported by rail to the Toyama Prefectural Fisheries Research Institute as required. When measurement of light intensity in absolute light units was not deemed essential, the photometer was used without calibration and the light intensity was recorded in relative light units (RLU).

2.4. Standard reaction mixture

Fifteen live specimens were used to prepare a homogenate. The cluster of three small black organs at the tip of each of the fourth pair of ventral arms was snipped off with a pair of ophthalmic scissors and homogenized in 5.0 ml of 0.001 M $MgCl_2$ in an all-glass homogenizer immersed in an ice-bath [10]. The homogenate was kept in the ice-bath throughout the experiment, which usually lasted for about 3.5 h, during which time the homogenate slowly lost luminescence activity. The standard reaction mixture consisted of 400 μl of homogenate + 44 μl of 1.0 M Tris–HCl, pH 8.26. The ATP solution consisted of 200 μl of 0.005 M ATP (dissolved in 0.001 M $MgCl_2$ and kept in an ice-bath throughout the experiment) + 22 μl of 1.0 M Tris–HCl, pH 8.26. Compounds to be tested were dissolved in 100 or 200 μl of 0.001 M $MgCl_2$ + 22 μl of 1.0 M Tris–HCl, pH 8.26, and added to the standard reaction mixture. Controls consisted of 0.001 M $MgCl_2$ without the test compound. To start the luminescence reaction, the ATP solution was injected into the standard

reaction mixture contained in a small reaction (chloride meter) vial placed in the sample compartment of a Mitchell–Hastings photomultiplier photometer [12]. The ATP solution was injected through a tiny hole in the lid of the sample compartment using a 1.0 ml syringe fitted with a long needle. The elapsed time for an assay—starting with the time it took to pipet the standard reaction mixture and the reading of the light intensity after ATP injection—was close to 3.0 min.

2.5. Measurement of luminescence emission spectrum

The bioluminescence emission spectrum was measured using a Shimadzu (Kyoto, Japan) RF-540 spectrofluorophotometer with the detector turned "on" and the excitation lamp "off." The emission monochromator was set at a bandwidth of 2 nm and scan speed was 254 nm/min. A Shimadzu DR-3 data recorder was used to record the results. To initiate the luminescence reaction, a mixture of 400 μl of 0.005 M ATP in 0.001 M $MgCl_2$ + 44 μl of 1.0 M Tris–HCl, pH 8.26, was injected into a mixture of 800 μl of homogenate + 88 μl of 1.0 M Tris–HCl, pH 8.26, contained in a small glass cuvette placed in the sample compartment of the spectrofluorophotometer.

2.6. Determination of requirement for molecular oxygen

A small A-shaped glass apparatus, consisting of two tubes serving as arms, joined at the apex by a standard taper joint, was used to add ATP solution, contained in the right arm, into the standard reaction mixture in the left arm (Fig. 2) [14]. To start the experiment, the standard reaction mixture and ATP solution were each pipeted into their respective arms and the left arm was clamped upright in the sample compartment of the Mitchell–Hastings photometer so that the bottom of the left arm nearly touched the window of the photomultiplier tube, which faced upwards. Prior to starting the reaction, the contents of both arms were flushed with 99.999% argon (Taiyo Oxygen, Atsugi, Japan) for 7 min through the inlet tube on the right arm. The argon passed through the ATP solution and into the standard reaction mixture in the left arm before exiting through openings in two stopcocks on the left arm. The regulator valve of the argon tank was closed and the apparatus was sealed from the outside by closing the three stopcocks. At the eighth minute, the right arm was quickly rotated upwards, causing the ATP solution to flow into the standard reaction mixture in the left arm. At 1.5 min post-addition, all three stopcocks were opened and air blown by mouth into the standard reaction mixture through the T-stopcock on the left arm. A Panasonic VP-6712A chart recorder was used to record the results.

2.7. Measurement of decay of luminescence activity in homogenates of arm organs

Two sets of reaction vials were prepared. Each vial contained 400 μl of homogenate + 44 μl of 1.0 M Tris–HCl, pH 8.26. To the vials of one set, DTT in 0.001 M $MgCl_2$ was added to give a final concentration of 2 mM DTT, while to the vials in the other set the same volume of 0.001 M $MgCl_2$ was added. The vials were kept in an ice-bath and at times 0, 30, 60, 90, 120 and 180 min, each of the vials in the two sets was removed and injected with 200 μl of 0.005 M ATP + 22 μl of 1.0 M Tris–HCl, pH 8.26. The initial maximal light intensities for each set of vials were plotted against time to obtain the decay curves.

3. Results

3.1. The luciferin–luciferase reaction

After dark-adapting the eyes for 20 min in a darkroom, freshly excised arm organs were homogenized separately in an all-glass homogenizer in 5.0 ml of 0.001 M $MgCl_2$ in one case and in 5.0 ml. of 0.001 M $MgCl_2$ + 22 μl of 1.0 M Tris–HCl, pH 8.26, in another. With the start of grinding, the only light detected, if any, was a very dim, transient flash. However, when a standard reaction mixture was injected with a mixture of 200 μl of 0.005 M ATP in 0.001 M $MgCl_2$ + 22 μl of 1.0 M Tris–HCl, pH 8.26, there was a sharp increase in light intensity, which was followed by a pseudo-first-order decay [10]. When ATP solution was injected into a still luminescing mixture that had decayed for 10 min to 8.8% of its initial intensity, there was an immediate 9.7% step-wise increase in light intensity, followed by a decay. When a luminescing mixture was quickly centrifuged and the supernatant placed in the photometer, no light was detected. Centrifuging a standard reaction mixture at 13,000 × *g* for 3 min yielded a clear straw-colored supernatant and a small black pellet. When the pellet was resuspended in 400 μl of 0.001 M $MgCl_2$ + 44 μl of 1.0 M Tris–HCl, pH 8.26, and injected with 200 μl of 0.005 M ATP in 0.001 M $MgCl_2$ + 22 μl of 1.0 M Tris–HCl, pH 8.26, no light was detected, nor was light detected when fresh ATP solution was injected into the clear supernatant. Thus, the luminescence reaction requires both a soluble component present in the supernatant and an insoluble component associated with the pellet. The fact that Pronase rapidly inactivates the insoluble component, but not the soluble component in the supernatant, indicates that the pellet contains the luciferase and the supernatant the luciferin [10].

3.2. Luminescence emission spectrum of the reaction

Fig. 1 shows the uncorrected bioluminescence emission spectrum of the reaction run in 0.1 M Tris–HCl, pH 8.26. The spectral distribution ranged from about 400 to 580 nm, with a peak at 470 nm and a half-height bandwidth of 67 nm. The emission spectrum was not corrected for the spectral sensitivity of the detector.

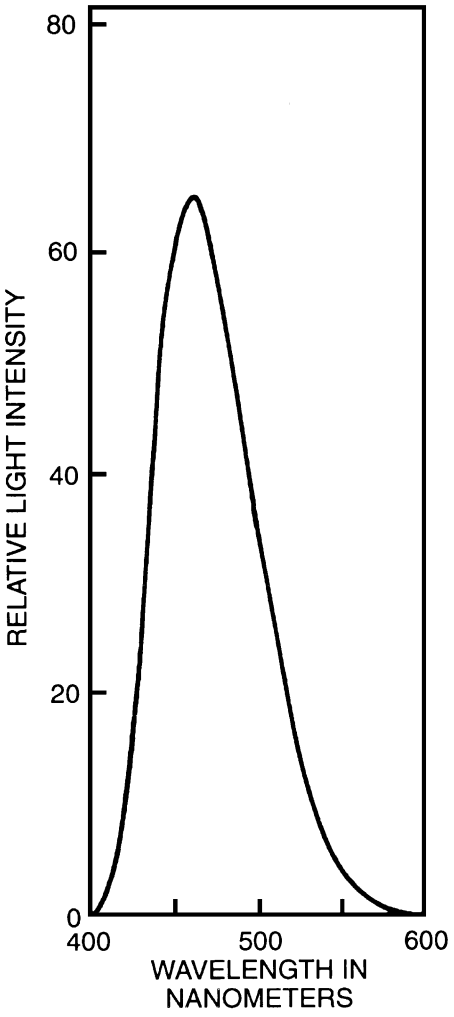

Fig. 1. Bioluminescence emission spectrum (uncorrected) of the *Watasenia* reaction measured in 0.1 M Tris–HCl buffer, pH 8.26. Light intensity is expressed in RLU; the wavelength is in nanometers; and the peak intensity is at 470 nm. The initial concentration of ATP was 1.5 mM.

3.3. Dependence on molecular oxygen

Fig. 2 shows the result of allowing ATP and homogenate to mix in the absence of molecular oxygen, which was then followed by the introduction of air into the mixture. It is seen that the *Watasenia* reaction has an absolute requirement for molecular oxygen. The small rise in light intensity after the mixing of ATP and homogenate is presumably due to a trace amount of air remaining in the apparatus.

3.4. Stimulation of light intensity by synthetic coelenterazine disulfate and identity of luciferin

The injection of 10.0 μg of synthetic coelenterazine disulfate, but not coelenterazine, into a partially spent standard reaction mixture (previously injected with ATP and allowed to decay for ~ 1.5 min) resulted in a sharp, off-scale rise in light intensity, followed by a rapid decay (Fig. 3). When the experiment was repeated with the phototube set at a lower gain, the injection of 1.0 μg of synthetic coelenterazine disulfate into a partially spent reaction mixture (previously injected with ATP and allowed to decay for ~ 3.5 min from an initial maximal light intensity of 10×10^{11} quanta/s) caused an immediate increase in light intensity having an initial maximal light intensity of 26×10^{11} quanta/s. Injection of 1.0 μg of synthetic coelenterazine disulfate (dissolved in 200 μl of 0.001 M $MgCl_2$) into a standard reaction mixture did not produce any light, but when the injection was followed immediately with an injection of ATP, there was a sharp increase in initial maximal light intensity which was 50% greater than that obtained by injecting ATP into another standard reaction mixture. Thus, the evidence indicates that coelenterazine disulfate is the luciferin in the *Watasenia* reaction and that there is a relatively large amount of coelenterazine disulfate present in an homogenate of the arm organs, but no ATP (since light would otherwise be seen). An adjunct experiment conducted at the same time showed that eggs of *Watasenia* contain a relatively large amount of coelenterazine disulfate (as determined by the marked stimulation of light emission obtained by injecting extracts of the eggs into a partially spent reaction mixture), but little coelenterazine (as determined by incubating extracts of the eggs with recombinant apoaequorin and testing with calcium for regenerated aequorin [15]).

3.5. Inhibition of luminescence activity by analogues of ATP

In a vial containing the standard reaction mixture, 200 μl of 0.005 M α,β-methyleneadenosine 5′-triphosphate in 0.001 M $MgCl_2$ was added. After 3 min of incubation, the homogenate was injected with 200 μl of 0.005 M ATP in 0.001 M $MgCl_2$ + 22 μl of 1.0 M Tris–HCl, pH 8.26. Similarly, 200 μl of 0.005 M β,γ-methyleneadenosine 5′-triphosphate in 0.001 M $MgCl_2$ was added to a standard reaction mixture. After 3 min of incubation, ATP solution was injected. The initial maximal light intensities were 26% and 33%, respectively, of controls. Previously, an 81% inhibition was found with another ATP analogue [10].

3.6. Inhibition of luminescence by pyrophosphate

When $Na_4P_2O_7$ was added to a standard reaction mixture at an initial concentration of 1.5 mM and injected with ATP solution, a 70% decrease in initial maximal light intensity was recorded, compared to a 100% inhibition previously [10].

3.7. Test of ATP in mediating transfer of sulfate groups to the p-hydroxybenzyl and p-hydroxyphenyl groups of coelenterazine

In a vial containing standard reaction mixture, a solution of 200 μl of 0.005 M PAPS in 0.001 M $MgCl_2$ + 9 μg of coelenterazine was added and injected with ATP solution. No stimulation of light emission was observed. Similarly, injec-

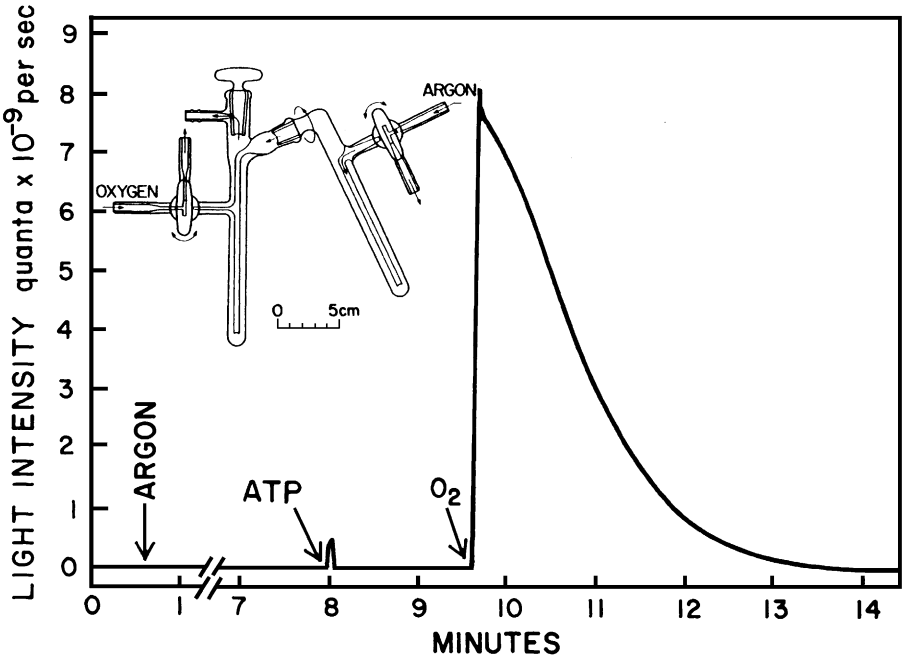

Fig. 2. Dependence of the *Watasenia* reaction on molecular oxygen. The right arm contained 800 μl of 0.005 M ATP in 0.001 M $MgCl_2$ + 88 μl of 1.0 M Tris–HCl, pH 8.26, and the left arm contained 1600 μl of homogenate + 176 μl of 1.0 M Tris–HCl, pH 8.26. The "O_2" marks the point at which air was admitted into the mixing apparatus. After going off-scale, the gain of the photomultiplier tube was quickly decreased to bring the light intensity within range of the recorder. Initial concentration of ATP, 1.5 mM.

tion of ATP into a standard reaction mixture containing 200 μl 0.005 M APS + 9 μg of coelenterazine did not show any increase in light intensity. Adding the sulfates of cesium, lithium, potassium, sodium, ammonium and magnesium to the standard reaction mixture did not affect the light intensity when compared to the chloride controls of the same cations.

3.8. Decay of luminescence activity in homogenates of arm organs

The initial maximal light intensities plotted against time (0, 30, 60, 90, 120, 180 min) for the two sets of vials (with and without DTT) gave decay curves that were linear and nearly identical. DTT neither increased nor stabilized the activity of the homogenate, as previously reported [10]. In both sets, the loss in activity was 24.5% over a 2.5-h period. Arm organs frozen overnight at − 20 °C did not give active homogenates the next day, neither did arm organs frozen in dry ice. Washing pellets to remove proteases that might be responsible for the inactivation did not prevent the pellet from losing activity. Addition of protease inhibitors (cocktails) to homogenates also did not affect the decline in activity. Attempts to solubilize luciferase by homogenizing pellets with a wide variety of ionic and non-ionic detergents, at and above the critical micelle concentration, followed by detergent removal, yielded solutions that were only slightly active and these, too, soon lost activity. In contrast, the luciferin in the supernatant was stable and retained activity for over 3 years when stored at − 20 °C and assayed with fresh pellet.

3.9. Activation of luciferase by ATP

A solution of 200 μl of 0.005 M ATP in 0.001 M $MgCl_2$ + 22 μl of 1.0 M Tris–HCl, pH 8.26, was injected into a standard reaction mixture and the light allowed to decay for 3 min. Simultaneously, an identical standard reaction mixture (without added ATP) was allowed to stand for 3 min. The two mixtures were centrifuged at 13,000 × *g* for 3 min. and the pellet from the first reaction mixture was quickly resuspended in 400 μl of 0.001 M $MgCl_2$ + 44 μl of 1.0 M Tris–HCl, pH 8.26, and injected with 200 μl of supernatant from the second mixture. No light was detected, suggesting that luciferase was not activated by ATP or the lifetime of the activated luciferase is very brief. When 200 μl of supernatant from the first reaction mixture was injected into a suspension of the second pellet suspended in 400 μl of 0.001 M $MgCl_2$ + 44 μl of 1.0 M Tris–HCl, pH 8.26, light, albeit of a low intensity (caused in part by the attendant dilution) was obtained, which was increased four-fold when fresh ATP was subsequently injected.

3.10. Effects of pre-exposure of luciferin and luciferase to Tris–HCl, pH 8.26

Table 1 shows the composition of Mixture 1 and Mixture 2, with the Tris–HCl, pH 8.26, apportioned between the two mixtures. The luminescence reaction was initiated by injecting Mixture 1 containing ATP into Mixture 2. The results show that exposing luciferin and luciferase to Tris–HCl, pH 8.26, markedly increases the light intensity. The

Table 1
Initial maximal light intensities (RLUs) of the *Watasenia* reaction under various conditions in Tris–HCl buffer, pH 8.26

Experiment no.	Mixture 1	Mixture 2	RLU
1	100 μl of 0.01 M ATP/0.001 M $MgCl_2$ 66 μl of 1.0 M Tris–HC1, pH 8.26 400 μl of supernatant Tris–HCl concentration = 0.12 M	pellet homogenized in 100 μl of 0.001 M $MgCl_2$	0.5
2	200 μl of 0.005 M ATP/0.001 M $MgCl_2$ 66 μl of 1.0 M Tris–HC1, pH 8.26 Tris–HCl concentration = 0.25 M	400 μl of homogenate	2.0
3	200 μl of 0.005 M ATP/0.001 M $MgCl_2$	400 μl of homogenate 66 μl of 1.0 M Tris–HC1, pH 8.26 Tris–HCl concentration = 0.14 M	81.0
4	200 μl of 0.005 M ATP/0.001 M $MgCl_2$ 22 μl of 1.0 M Tris–HC1, pH 8.26 Tris–HCl concentration = 0.1 M	400 μl of homogenate 44 μl of 1.0 M Tris–HC1, pH 8.26 Tris–HCl concentration = 0.1 M	48.0
5	400 μl of supernatant 100 μl of 0.01 M ATP/0.001 M $MgCl_2$	pellet homogenized in 100 μl of 0.001 M $MgCl_2$ 66 μl of 1.0 M Tris–HC1, pH 8.26 Tris–HCl concentration = 0.4 M	24.0

initial maximal light intensities were recorded in RLU. In all experiments, the initial concentration of ATP was 1.5 mM and the final concentration of Tris–HCl, pH 8.26, was 0.1 M.

4. Discussion

4.1. Watasenia bioluminescence emission spectrum

The emission spectrum of the *Watasenia* reaction (Fig. 1) is seen to have a λ_{max} at 470 nm. Other workers have observed a small scattering of dermal organs on the ventral side emitting a greenish-yellow light instead of blue light [16,17]. When the organs were irradiated with 360 nm light, the dermal organs fluoresced in the same color as their bioluminescence. We attempted to obtain spectra of light emitted by extracts of luminous tissue of the ventral side and eye organs injected with ATP, but the emitted light was too weak to be measured. For *Watasenia*, living in a changing photic environment that exists in the deep sea, the ability to emit two different colors of light may have survival value. Studies with live luminescent deep-sea squid have provided strong evidence that squids use their ventral light to countershade for ambient light in order to obliterate their silhouettes from a predator below [18].

4.2. Watasenia bioluminescence requires molecular oxygen

Like all bioluminescence reactions studied to date, the *Watasenia* reaction also requires molecular oxygen, as shown in Fig. 2; the oxygen is required to oxidize the substrate, luciferin, to form the product (oxyluciferin) excited state.

4.3. Coelenterazine disulfate is Watasenia luciferin

The sharp off-scale increase in light intensity upon the injection of 10.0 μg of synthetic coelenterazine disulfate (Fig. 3) into a partially spent reaction mixture of the homogenate provides strong evidence that the luciferin in the *Watasenia* luminescence reaction is coelenterazine disulfate, as suggested by Goto et al. [6–9]. Considering that the

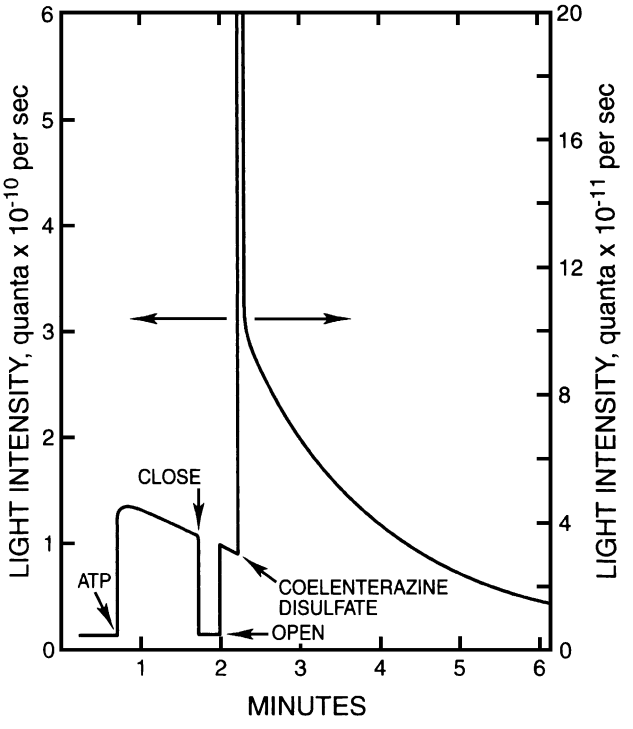

Fig. 3. Effect of injecting synthetic coelenterazine disulfate into a luminescing mixture of *Watasenia* homogenate. Composition of reaction mixtures: 200 μl of 0.005 M ATP in 0.001 M $MgCl_2$ + 22 μl of 1.0 M Tris–HCl, pH 8.26, injected into 400 μl of homogenate + 44 μl of 1.0 M Tris–HCl, pH 8.26, followed by the injection of 200 μl of 0.001 M $MgCl_2$ containing 10.0 μg of coelenterazine disulfate. Initial concentration of ATP, 1.5 mM. "Close" and "open" refer to closing and opening of shutter of photomultiplier photometer while syringe was replaced. ATP was injected through a small hole in the lid of the sample compartment using a 1.0 ml syringe fitted with a long needle, which was exchanged for a new syringe and needle when coelenterazine disulfate was injected.

three light organs at the tip of each arm are miniscule and that from 15 specimens a total of 90 organs ($3 \times 2 \times 15$) were removed and homogenized in 5.0 ml of 0.001 M $MgCl_2$, which calculates to 7.2 organs per 400 μl of homogenate, the light intensity obtained (10^{11} quanta/s) (Fig. 3) on injecting 10.0 μg of synthetic coelenterazine disulfate would appear to be extremely bright. Light in the range of 10^{11} quanta/s is readily visible to the dark-adapted eye. The light intensity in Fig. 3 is seen to decay very rapidly and this may be due to depletion of molecular oxygen or ATP, or both, suggesting that they are consumed in the reaction.

4.4. ATP is required in the Watasenia reaction

The inhibition by ATP analogues agrees with the results previously reported, as does the result with sodium pyrophosphate ($Na_4P_2O_7$), which inhibited the light reaction very strongly [10]. The fact that UTP, CTP, GTP and TTP, which when injected into the standard reaction mixture have little, if any, stimulatory effect indicates that none is an essential component of the luminescence reaction [10].

4.5. Origin of Watasenia luciferin

We do not yet know whether the coelenterazine disulfate is synthesized de novo or comes from the diet. The most reasonable explanation would be for the **C-2** *p*-hydroxybenzyl and **C-6** *p*-hydroxyphenyl groups of coelenterazine to be sulfated by a transfer of sulfate group from PAPS, catalyzed by a putative "luciferin sulfotransferase," possibly in the liver. However, both APS, a precursor of PAPS, and PAPS did not stimulate light emission. Further, the large amount of coelenterazine disulfate found in extracts of the arm organs and the virtual absence of light when arm organs are homogenized in the dark can only mean that ATP is the trigger which sets off the flash in *Watasenia*. It is conceivable that a mechanism exists whereby light emission is coupled to a source of ATP synthesis, such as the mitochondria.

4.6. Watasenia reaction is greatly enhanced by high alkaline pH

Previously, we reported that the *Watasenia* reaction has a sharp pH optimum of 8.80 [10]. From the results of the Tris–HCl, pH 8.26, experiments, it is apparent that the highest light intensities were obtained when the homogenate (luciferin + luciferase) was in brief contact (< 60 s) with Tris–HCl, pH 8.26 (see the third and fourth experiments, Table 1). In both cases, exposure of the homogenate to Tris–HCl yielded the highest light intensities. In contrast, exposure of the supernatant and ATP (first experiment), and exposure of the pellet (fifth experiment), as well as non-exposure of the homogenate (second experiment), to Tris–HCl gave the lowest intensities. These results are interpreted to mean that the *Watasenia* reaction begins with a rapid base/enzyme-catalyzed enolization [19] of the keto oxygen of the C-3 carbon of coelenterazine disulfate at pH 8.26. The rapidity with which the light emission proceeds following coelenterazine disulfate injection (Fig. 3) suggests that it is not a simple base-catalyzed enolization, but an enolization involving both base and luciferase.

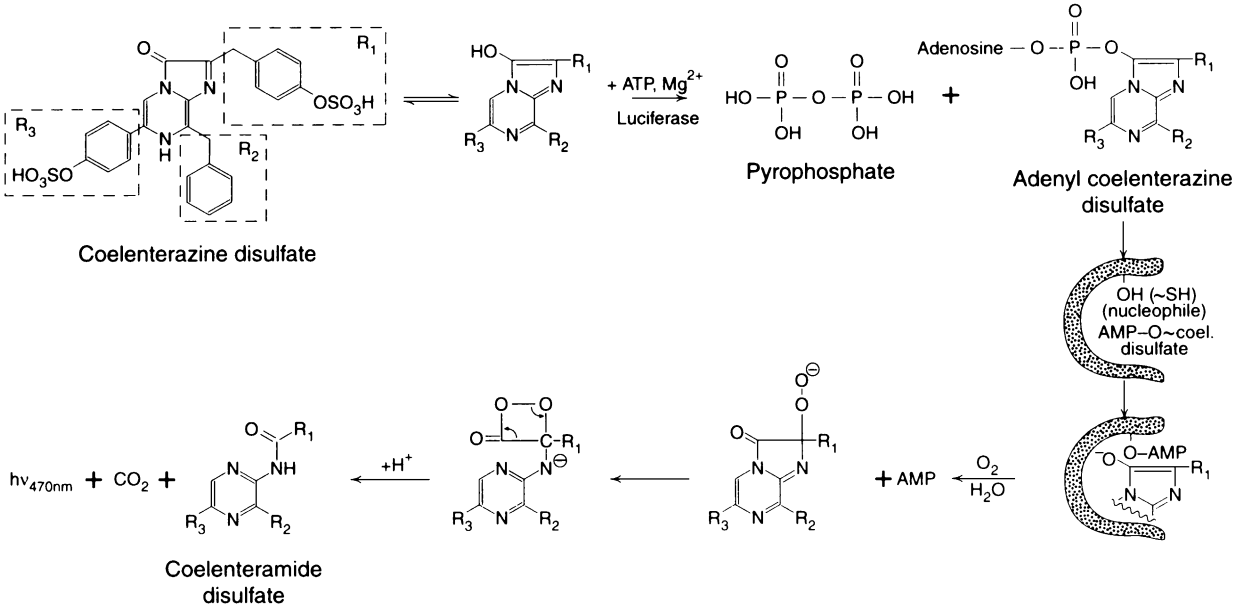

Fig. 4. Proposed scheme for the *Watasenia* luminescence reaction catalyzed by membrane-bound luciferase. The chemical structures of coelenteramide disulfate (oxyluciferin) and coelenterazine disulfate (luciferin) are the same as those reported by Goto et al. [6,8].

4.7. Proposed scheme for the Watasenia reaction

In summary, we find that *Watasenia* luminescence is due to a luciferin–luciferase reaction, the luciferin is coelenterazine disulfate, a high pH is required for maximum light emission, there is an absolute requirement for molecular oxygen, the reaction is strongly inhibited by pyrophosphate and ATP analogues and the luciferase is attached to membrane in such a way as to be resistant to extraction by detergent. Evidence was not found for ATP playing a role in the phosphorylation of either luciferin or luciferase as is sometimes seen with the hydroxyl of serine, tyrosine and threonine residues in proteins, nor was sulfation of coelenterazine or adenylation of luciferase detected (except, see Fig. 4). To account for the above findings, a scheme for the *Watasenia* reaction is proposed (Fig. 4). First, because light intensity is greatly enhanced when homogenate is exposed to alkaline pH, there is a strong likelihood that a rapid base/luciferase-catalyzed enolization of the C-3 keto oxygen of coelenterazine disulfate takes place. Second, since the reaction requires ATP and is strongly inhibited by pyrophosphate and AMP [10], an adenylation reaction takes place in which ATP is used to adenylate the enol group, forming the intermediate, adenyl coelenterazine disulfate. It is noteworthy that in the bioluminescence reaction of the terrestrial firefly, ATP is used to adenylate the carboxyl group of luciferin, forming luciferyl adenylate and inorganic pyrophosphate [20,21]. Third, since there is an absolute requirement for molecular oxygen, the reaction proceeds with the removal of AMP and the addition of molecular oxygen to the C-2 carbon forming a dioxetanone intermediate. The dioxetanone intermediate then spontaneously decomposes to yield CO_2, coelenteramide disulfate and 60 kcal/mol required for the blue light emission. While not shown in the figure, the amide anion bound to luciferase in the excited state would be the probable light emitter in the reaction [22]. We also postulate that the AMP of the adenyl coelenterazine disulfate serves as a recognition moiety for proper docking of the molecule to luciferase so that the reaction proceeds to the next step. In view of the finding of coelenteramide disulfate (oxyluciferin) and coelenterazine disulfate (luciferin) in the arm organs of *Watasenia* by Goto et al. [6,8] and because of similarities of the proposed *Watasenia* reaction scheme with those of other luminescence reactions in which molecular oxygen, acting through a dioxetanone mechanism, is used to oxidize substrate [8,23], it is not unreasonable to think that the scheme shown in Fig. 4 is the correct one for the *Watasenia* reaction.

Acknowledgements

For providing assistance in various ways during the long course of the research, the author is deeply indebted to the following individuals: Mr. T. Sawada, Mayor of Namerikawa City; Mr. N. Nakura, Mr. A. Imamura, Dr. S. Hayashi, Mr. H. Wakabayashi, Mr. T. Miyazaki and Dr. D. Fujita, Toyama Prefectural Fisheries Research Institute, Namerikawa; Dr. K. Kawasaki, Toyama Food Research Institute, Toyama; Dr. S. Inoue, Meijo University, Nagoya; Dr. M. Ohashi, Kanagawa University, Kanagawa and Dr. H. Niwa, The University of Electro-Communications, Chofu. The author is also very grateful to the Namerikawa Hotaruika Museum and WAVE Namerikawa for providing partial financial support.

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