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Biochemistry

ATP-dependent bioluminescence in the firefly squid, *Watasenia scintillans*

(cephalopod/membrane-bound system/coelenterazine)

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ABSTRACT **The Japanese firefly squid, *Watasenia scintillans*, emits intense flashes of light from three tiny luminous organs that are located at the tip of each of a pair of ventral arms. Light is also produced from hundreds of other minute organs that are scattered over the body. The luminescence is due to an ATP-dependent reaction, with an optimal pH of 8.80. The decay of light intensity follows first-order kinetics and the decay constant is independent of initial ATP concentration. The light emission also requires $MgCl_2$, a soluble component, and an insoluble component that is membrane bound. Squids represent a major group of organisms unrelated to fireflies in which ATP is required for bioluminescence.**

Luminescent squids are found in all oceans of the world, both in shallow water and in the deep sea. Among luminescent organisms, squids have developed one of the most complex organs known for light emission. Light is produced by one of three methods: (*i*) by harboring symbiotic luminous bacteria in luminous organs, (*ii*) by discharging a luminous secretion from a luminous organ into sea water, or (*iii*) by an intracellular chemical reaction that takes place within photogenic cells in the organ (1). The best-studied example chemically is a squid with luminous organs of the third type, *Watasenia scintillans*, famous for its beautiful and brilliant luminescence (Fig. 1). It is called "hotaru-ika" or "firefly squid" of Toyama, Japan. In the spring, this self-luminous squid comes inshore from the deep sea by the billions to breed. While swimming, it emits brilliant flashes of blue light from a cluster of three tiny black organs located at the tip of each of the fourth pair of arms; bright luminescence is also produced from hundreds of other small organs distributed throughout the body. From the arm organs, Goto and co-workers (2–5) isolated a sulfated form of the compound coelenterazine, 2-(*p*-hydroxybenzyl)-6-(*p*-hydroxyphenyl)-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one, which they called "luciferin," and from the liver, they isolated coelenterazine itself, which they called "preluciferin." They suggested that the unsulfated preluciferin is a precursor of luciferin used by the squid to produce light. Coelenterazine is employed as a luciferase substrate in the luminescence reactions in coelenterates and in the decapod shrimp *Oplophorus* (6–8). Although Goto and co-workers found that the coelenterazine produced light with a cold water extract (luciferase) of *Oplophorus*, thus confirming its identity, they were unable to demonstrate either a luciferin–luciferase or photoprotein reaction *in vitro*, and so the biochemistry of the light reaction in this and other squids has remained an unsolved problem.

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MATERIALS AND METHODS

Specimens of *Watasenia* (average wet weight, 8.9 g; average mantle length, 5.7 cm) were collected at Toyama Bay at night and maintained in a refrigerated holding tank in a semidarkened room at 7°C with continuous aeration. The squids swam actively and luminescence capability remained intact for about 48 hr.

Adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP), guanosine 5′-triphosphate (GTP), thymidine 5′-triphosphate (TTP), tetrasodium pyrophosphate ($Na_4P_2O_7$), creatine phosphate, phospho-L-arginine, β-NADH, β-NADPH, the tetrasodium salt of ethylenediaminetetraacetic acid (EDTA), and tris(hydroxymethyl)aminomethane (Tris) were obtained from Sigma. Cytidine 5′-triphosphate (CTP), uridine 5′-triphosphate (UTP), dithioerythritol, and protease (Pronase) were purchased from Calbiochem-Behring. The β,γ-difluoromethylene analogue of ATP (Ado*PP*[CF_2]*P*) (9) was a gift of Charles E. McKenna. Microconcentrator tubes (Centricon, M_r 10,000 and 30,000 cut-off) were purchased from Amicon. All other reagents were of the highest grade available. Deionized glass-distilled water was used throughout.

For performing experiments, extracts were prepared by homogenizing arm organs (each 1 mm or less in diameter and 1–2 mg in wet weight) from 10 specimens in an all-glass homogenizer in 2.0 ml of 1 mM $MgCl_2$ in an ice bath. Then 400 μl of the homogenate was mixed in a chloride meter vial with 44 μl of 1.0 M Tris·HCl, pH 8.26. The vial was placed in the holder of a Mitchell–Hastings photomultiplier photometer (10) and 200 μl of ATP or a test compound dissolved in 1 mM $MgCl_2$ plus 22 μl of 1.0 M Tris·HCl, pH 8.26, was injected into the mixture with a syringe. This procedure was followed unless described otherwise. The photometer was calibrated with a standard light source (11) and light intensity was recorded with a Soltec S-4201 strip chart recorder. Centrifugations were carried out in a Kubota KR-180 or Tomy Seiko RS-20-IV refrigerated centrifuge at 4°C. A Microelectrodes MI-410 pH probe was used to measure pH.

RESULTS

Requirement for ATP. Homogenates and cell-free extracts of the arm organs of *Watasenia* were either completely dark or gave off a weak luminescence. Hot- and cold-water extracts of the organs did not give the luciferin–luciferase reaction. Injections of 1 M KCl or NaCl into homogenates of the arm organs prepared in 50 mM Tris·HCl, pH 7.60, with or without added dithioerythritol, did not trigger light emission (12). However, injection of a solution of ATP into the

Abbreviation: Ado*PP*[CF_2]*P*, adenosine 5′-[β,γ-difluoromethylene]-triphosphate.

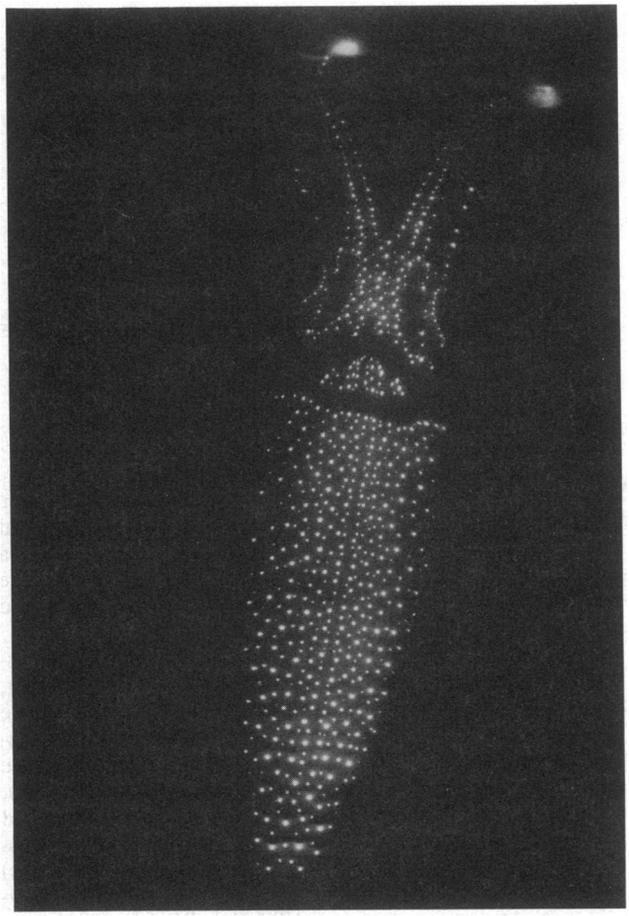

FIG. 1. Photograph of *Watasenia scintillans* taken by its own light, showing luminescing organs of arms and body (ventral view).

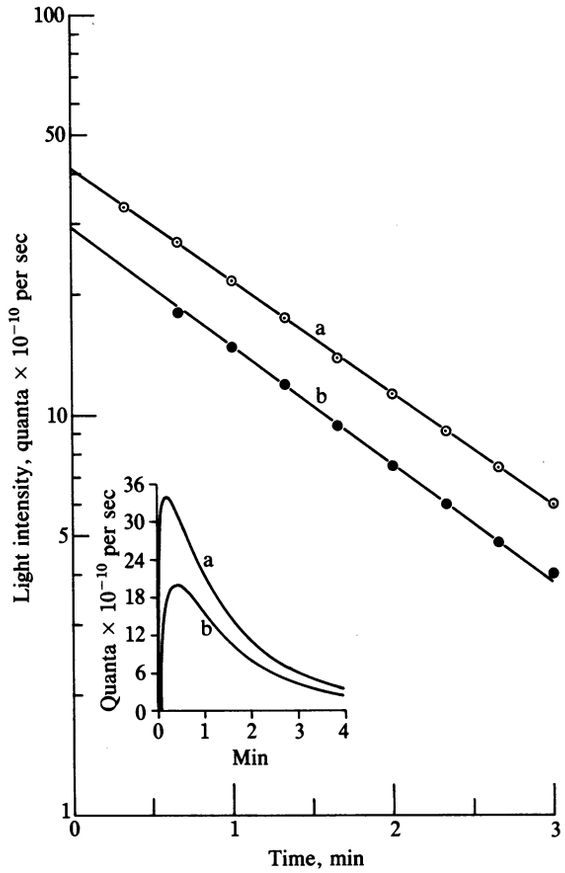

FIG. 2. Decay of light intensity after injection at zero minutes of 200 μl of ATP in 1 mM $MgCl_2$ plus 22 μl of 1.0 M Tris·HCl, pH 8.26, into a mixture of 400 μl of homogenate of arm organs plus 44 μl of 1.0 M Tris·HCl, pH 8.26. Initial concentration of ATP was 1.5 mM (curves a) or 0.5 mM (curves b). (*Inset*) Original data, with intensity plotted on a linear scale as a function of time. First-order decay constants were calculated from curves obtained when light intensities were replotted on a logarithmic scale: curve a, 0.64 min^{-1}; curve b, 0.68 min^{-1}.

homogenate caused an immediate and sustained luminescence that lasted for several minutes (Fig. 2 *Inset*). The light intensities measured at 1.5 mM and 0.5 mM ATP decayed according to first-order kinetics, with decay constants of 0.64 min^{-1} and 0.68 min^{-1}, respectively (Fig. 2). The initial maximal light intensity was the same for 1.5 mM and 2.0 mM ATP; it decreased to 62% of this value at 6.0 mM ATP. ADP also stimulated light emission, but the intensity was always less than that of an ATP control, and AMP and all other 5′-triphosphates tested gave either no or insignificant amounts of light (Table 1). The ATP analogue Ado*PP*[CF_2]*P* caused an 81% inhibition of ATP stimulation. Other potential stimulating compounds, such as $CaCl_2$, creatine phosphate, phospho-L-arginine, β-NADH, and β-NADPH did not produce any light.

Soluble and Insoluble Components. The clear, straw-colored supernatant, obtained by centrifuging an aliquot of a homogenate of the arm organs in 50 mM Tris·HCl, pH 7.60/1 mM $MgCl_2$, at 3000 × *g* for 20 min at 4°C, and the dark reddish pellet from the centrifugation, resuspended in 50 mM Tris·HCl, pH 7.60/1 mM $MgCl_2$, gave little light when injected with ATP. However, a reconstituted homogenate, prepared by homogenizing another pellet and supernatant, when injected with ATP gave complete restoration of luminescence activity in comparison to a control homogenate that was not centrifuged. Thus, light emission requires both a soluble and an insoluble component.

Supernatants prepared by centrifuging homogenates of the arm organs at 2000 × *g* for 20 min at 4°C and incubating with Pronase (2 mg/ml) for 30 min in an ice bath caused a 2-fold increase in light intensity when injected into an already luminescing mixture of ATP and homogenate. Both the increase and subsequent decrease in light intensity to near zero occurred within 10 sec. Supernatants without added Pronase injected similarly into a luminescing ATP/homogenate also produced a sharp increase in light intensity, but the subsequent decay lasted for several minutes. Injection of Pronase alone into a luminescing ATP/homogenate did not result in an increase, but only a sharp drop, in light intensity as in the first case. These results suggest that the soluble component contains a Pronase-resistant molecule that stimulates light emission and the sharp drop in light intensity is due to inactivation of other molecules essential for luminescence.

Aliquots of homogenates of the arm organs were incubated with Pronase (2 mg/ml) for 30 min in an ice bath and centrifuged to separate the pellet and supernatant. Such pellets were quickly homogenized in fresh supernatant and injected with ATP. They gave no light, but Pronase-treated supernatants similarly homogenized with fresh (untreated) pellets and injected with ATP gave light, albeit of a lower intensity (1.4×10^{10} quanta/sec) than the original homogenate (4.0×10^{11} quanta/sec). The above results suggest that the insoluble component contains a membrane-bound molecule that acts like an enzyme for the luminescence reaction and is inactivated by Pronase.

Aliquots (1 ml) of a homogenate of arm organs were centrifuged at 2000 × *g* for 5 min. The supernatants were

Table 1. Stimulation of light emission from homogenates of arm organs by ATP and related compounds

Homogenate	Compound	Initial conc., mM	Initial maximal light intensity, quanta × 10^{-9}/sec
1*	ATP	1.5	930
	ADP	1.5	57
	AMP	1.5	0.1
	$Na_4P_2O_7$	1.5	0
2*	ATP	1.5	610
	UTP	1.5	0.33
	CTP	1.5	0.25
	GTP	1.5	0
	TTP	0.5	0
3†	ATP	1.2	580
	ATP + Ado*PP*[CF_2]*P*	1.2	110

*In 1 mM $MgCl_2$/0.1 M Tris·HCl, pH 8.26.
†Homogenates, with and without added Ado*PP*[CF_2]*P*, were allowed to incubate for 15 min before ATP was injected. Initial concentration of Ado*PP*[CF_2]*P* was 0.0162 mM. The mixture contained 1 mM $MgCl_2$ and 0.1 M Tris·HCl, pH 8.26.

placed in Centricon tubes (M_r 10,000 and 30,000 cut-off) and centrifuged for 30 min at 2000 × *g*. The pellets were homogenized separately in 1.0 ml of 1 mM $MgCl_2$, and a 400-μl aliquot was mixed with 44 μl of 1.0 M Tris·HCl, pH 8.26, and injected with 200 μl of 5 mM ATP/1 mM $MgCl_2$ plus 22 μl of 1.0 M Tris·HCl, pH 8.26, initiating a weak luminescence (3.0×10^{10} quanta/sec versus 1.2×10^{12} quanta/sec for the original homogenate). Injection of the filtrates from the M_r 10,000 and 30,000 cut-off tubes into the weakly luminescing mixtures caused a 3- and 6-fold stimulation of light intensity, respectively, and a subsequent injection of the unfiltered supernatant remaining in the Centricon tubes caused a further 2-fold increase in light intensity in both cases. These results indicate that the molecular weight of the active, soluble component is less than 10,000.

Effect of pH. The pH/activity curve had a sharp peak, with an optimal pH close to 8.80 (Fig. 3).

Effect of EDTA and Metal Ions. EDTA completely inhibited the stimulation of light emission by ATP (Table 2) and Mg^{2+} reversed the inhibition. Of the cations tested, Mg^{2+} gave the greatest stimulation with ATP, and certain ions, such as Fe^{2+} and Cu^{2+}, had an inhibitory effect on light emission, whereas other ions, such as Ca^{2+}, K^+, and Mn^{2+}, had practically no effect compared to controls. Thus, Mg^{2+} appears to be essential for the ATP-dependent reaction.

Stability of the ATP-Stimulable Activity of Homogenates. Homogenates of the arm organs kept in an ice bath gradually lost activity over a period of 3–4 hr. Dithioerythritol neither increased nor stabilized the activity. Freshly excised luminous organs stored at −40°C and homogenized 24 hr later gave activities equal to about 35% of those observed in initial controls.

DISCUSSION

The requirement for ATP by the *Watasenia* bioluminescence reaction is supported by the following observations: (*i*) ATP strongly stimulated light emission from homogenates of the arm organs; (*ii*) the ATP analogue Ado*PP*[CF_2]*P* markedly inhibited the stimulation of light emission by ATP; (*iii*) the decay of light intensity followed first-order kinetics and the decay constant was independent of initial ATP concentration; and (*iv*) other 5′-triphosphates did not significantly stimulate light emission in comparison to ATP. The stimulation observed with ADP was probably due to the presence of adenylate kinase, forming ATP. Besides ATP, the

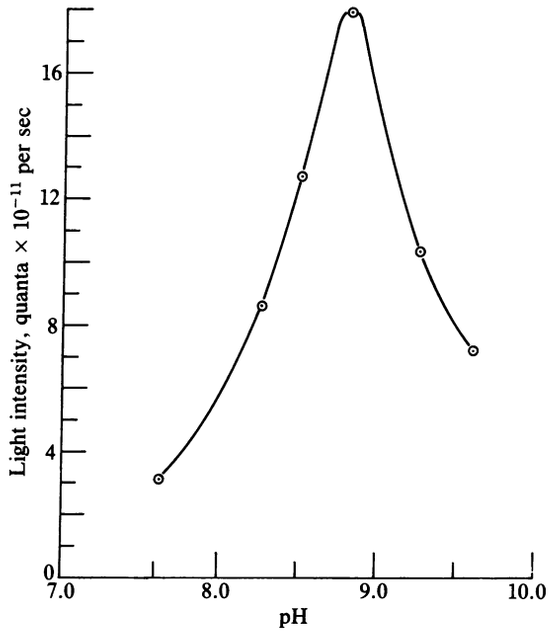

FIG. 3. Relationship between pH and initial maximal light intensity of ATP-stimulated luminescence in homogenates of arm organs. Initial maximal light intensities were obtained by injecting 200 μl of 5 mM ATP/1 mM $MgCl_2$ plus 22 μl of 1.0 M Tris·HCl of the appropriate pH into a mixture of 400 μl of homogenate plus 44 μl of 1.0 M Tris·HCl of the same pH. Optimal pH, 8.80.

Watasenia reaction required at least three other components: an insoluble component (pellet), a soluble component (supernatant), and Mg^{2+}. While the insoluble component was readily inactivated by Pronase, the soluble component consisted of a Pronase-resistant molecule that stimulated light emission and possibly others that were difficult to detect because of the rapidity with which Pronase inactivated the ATP-stimulated light emission. Molecular oxygen has also been shown to be essential in *Watasenia* bioluminescence (13).

In contrast to *Watasenia*, homogenates of the luminous organ of the self-luminous squid *Symplectoteuthis* are stimulated to emit light only by high concentrations of KCl and

Table 2. Effects of EDTA and metal ions on the ATP stimulation of light emission from homogenates of arm organs

Homogenate	Addition	Initial conc., mM	Initial maximal light intensity, quanta × 10^{-9}/sec
1*	None	—	360
	EDTA	4.3	0
2†	None	—	250
	$MgCl_2$	1.0	400
	$CaCl_2$	1.0	290
	KCl	1.0	270
	$MnSO_4$	1.0	260
	$FeCl_2$	1.0	45
	$CuCl_2$	1.0	5
	$ZnCl_2$	1.0	0

*Arm organs were homogenized in 1 mM $MgCl_2$ and aliquots, with and without added EDTA, were injected with ATP. Initial concentrations: ATP, 1.44 mM; Tris·HCl, pH 8.26, 0.1 M.
†Arm organs were homogenized in distilled water and aliquots, with added Tris·HCl and cation solutions, were injected with ATP. Initial concentrations: ATP, 1.5 mM; Tris·HCl, pH 8.26, 0.1 M.

other monovalent cations (12). Further, EDTA and dithioerythritol protect homogenates of *Symplectoteuthis* from loss of activity, whereas *Watasenia* homogenates were unaffected by dithioerythritol and reversibly inactivated by EDTA. Thus, there appears to be differences between the two systems, even though membrane-bound components are used in both squids for light emission.

Aside from the dipteran glowworm *Arachnocampa luminosa* (14, 15) and the millipede *Luminodesmus sequoiae* (16–18), which are, respectively, closely and distantly related taxonomically to fireflies, *Watasenia* is the second example in which ATP has been found to be essential for bioluminescence. Although ATP is used to form luciferyl adenylate in the firefly reaction (19), present evidence on the structure of *Watasenia* luciferin and oxyluciferin (2–5) suggests that ATP is used in *Watasenia* to phosphorylate adenylsulfate to give 3′-phosphoadenosine 5′-phosphosulfate, which then serves as a sulfuryl donor to form the putative sulfated coelenterazine by a sulfotransferase reaction (20). These roles are reasonable since sulfotransferase reactions have been found to be widespread in the sulfation of phenolic and tyrosyl residues (present in coelenterazine) (21, 22) and because 3′-phosphoadenosine 5′-phosphosulfate is also involved in the removal of a sulfuryl group from *Renilla* luciferyl sulfate (23).

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