# A NEW TYPE OF ATP-ACTIVATED BIOLUMINESCENT SYSTEM IN THE MILLIPEDE *LUMINODESMUS SEQUOIAE*

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Received 13 April 1981

## 1. Introduction

The well-known bioluminescence system of fireflies requires 5 components for the emission of light, i.e., firefly luciferin, firefly luciferase, ATP, $Mg^{2+}$ and $O_2$ [1]. It is a useful system for assaying ATP, and has been widely employed in various fields of research for this purpose [2]. ATP is also required, though not conclusively, in the bioluminescence of the dipteran glow-worm *Arachnocampa* [3,4] which is taxonomically near to the coleopteran fireflies.

We now report a new type of ATP-activated bioluminescent system discovered in the millipede *Luminodesmus sequoiae* [5,6] which is biologically distantly related to the earlier examples cited above. The new system requires 4 components for the emission of light, i.e., a photoprotein, ATP, $Mg^{2+}$ and $O_2$. This system has a potential usefulness as a monitor of ATP in single cells, for which the firefly system would not work because of the easily diffusible nature of firefly luciferin.

## 2. Materials and methods

The specimens of *Luminodesmus sequoiae* (~200) were collected in the vicinity of the Belknap Campground near Camp Nelson (Tulare County CA) in May 1980. Only the most luminescent parts of the body, i.e., antennae, legs and terga, were used; those parts were cut off from specimens that were anesthetized with $CHCl_3$, quickly frozen with dry ice, then subsequently stored at −70°C for 3–4 weeks before extraction of the photoprotein active in light emission.

The activity of photoprotein was assayed by measuring the maximum intensity of emitted light when 10–100 μl sample solution containing photoprotein was mixed into 2 ml 10 mM Tris buffer (pH 8.5) containing 0.1 mM ATP (Sigma) and 1 mM $MgCl_2$ at 23°C, except as noted. After the mixing, the intensity of emitted light reached the maximum in a few seconds, followed by a gradually decreasing emission which continued over 15 min.

Extraction and purification of photoprotein were carried out at near 0°C, as described below. Between the steps of chromatography, the eluted photoprotein solutions were concentrated by precipitating the protein with 35% (w/v) of $(NH_4)_2SO_4$ followed by centrifugation. The precipitate was then dissolved in a small volume of buffer. The precipitated form of photoprotein could be stored at −70°C without a detectable loss of activity.

Frozen raw material obtained from 60 specimens of the millipede was ground in a mortar with 50 ml 50 mM sodium acetate buffer (pH 5.8) containing 10 mM EGTA (ethyleneglycol-*bis*(β-aminoethyl ether)*N*,*N*′-tetraacetic acid, Sigma) and 0.25 M NaCl, without noticeable light emission. The mixture was centrifuged (12 000 rev./min, 5 min), then the pellets were re-extracted with 50 ml of the same buffer. The supernatants were combined, photoprotein in this solution (100 ml) was precipitated with $(NH_4)_2SO_4$ (35 g), and the mixture was centrifuged. The pellets were dissolved in 10 ml 10 mM sodium phosphate buffer (pH 6.5) containing 5 mM EGTA and 0.2 M NaCl, and chromatographed on a column of Sephadex G-100 (Pharmacia; 2.6 × 55 cm) equilibrated with the same pH 6.5 buffer. Active fractions were combined, then concentrated by precipitating the photoprotein with $(NH_4)_2SO_4$.

Step 2 of chromatography was carried out on a column by Ultrogel AcA 34 (LKB; 1.6 × 50 cm) in

the same pH 6.5 buffer, with the sample dissolved in 5 ml buffer. Step 3 was done on a column of Sephadex A-50 (Pharmacia; 1.5 × 10 cm) equilibrated with 10 mM sodium phosphate buffer (pH 6.5) containing 5 mM EGTA and 0.12 M NaCl. The sample dissolved in 3 ml of the pH 6.5 buffer without NaCl was applied to the column, then elution was carried out with the equilibration buffer. The last step of chromatography was a repetition of the second step, with the sample dissolved in 2 ml of pH 6.5 buffer. The specific activity of photoprotein in the eluate fractions, i.e., the ratio of luminescence activity to absorbance at 280 nm, was found to be nearly constant in this last step. The final yield of photoprotein activity from 60 millipedes was $6 \times 10^{11}$ quanta/s (10% yield from the crude extract), which had a total light-emitting capacity of $5 \times 10^{13}$ quanta.

To estimate the molecular mass of the photoprotein, the column used for the last chromatography was calibrated with catalase, aldolase, ovalbumin, and chymotrypsinogen (all from Pharmacia).

## 3. Results and discussion

The active principle of luminescence in the millipede *Luminodesmus sequoiae* was extracted and purified, resulting in a photoprotein that was capable of emitting a greenish light in the presence of ATP and $Mg^{2+}$. The photoprotein was practically colorless (fig.1); a small absorption peak at 410 nm is probably

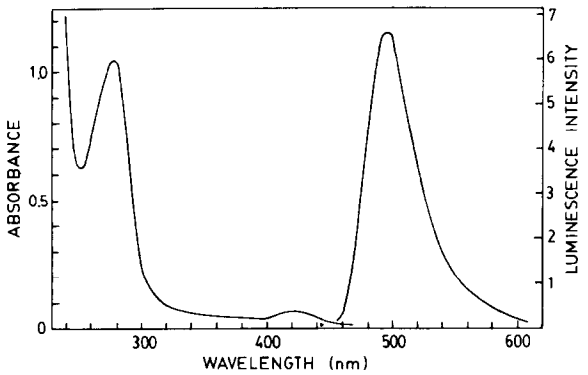


Fig.1. Absorption spectrum of a solution of *Luminodesmus* photoprotein having activity of $3.8 \times 10^{11}$ quanta . $s^{-1}$ . $ml^{-1}$ (left), and bioluminescence spectrum of a live specimen of *Luminodesmus sequoiae* anesthetized with $CHCl_3$ (right). The luminescence spectrum was recorded with a Perkin-Elmer fluorescence spectrophotometer model MPF-44B equipped with a corrected spectra unit.

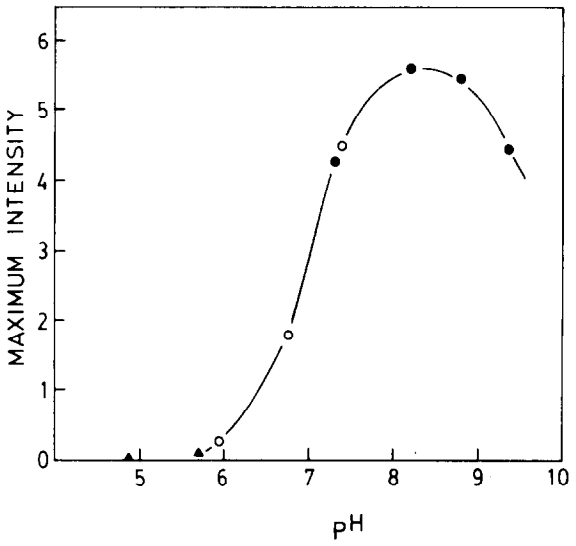


Fig.2. Influence of pH on the intensity of luminescence (in arbitrary units) when 0.5 ml of a solution of *Luminodesmus* photoprotein was added to 2 ml 10 mM sodium acetate buffers (▲), 10 mM sodium phosphate buffers (○) or 10 mM Tris–HCl buffers (●), each containing 1 mM $MgCl_2$ and 0.05 mM ATP.

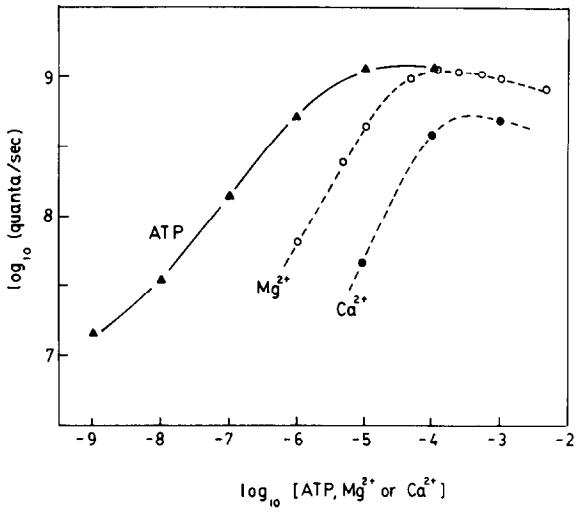


Fig.3. Influence of the concentration of ATP (▲), $Mg^{2+}$ (○) or $Ca^{2+}$ (●) on the maximum intensity of *Luminodesmus* luminescence, when 10 µl photoprotein solution ($A_{280}$ = 0.3) was added to 2 ml 10 mM Tris–HCl buffer (pH 8.3) containing either 1 mM $MgCl_2$ plus various [ATP], or 0.05 mM ATP plus various [$Mg^{2+}$] or [$Ca^{2+}$]. For the data at zero concentration of ATP, $Mg^{2+}$ or $Ca^{2+}$, the intensity values on the ordinate were 7.0, 7.2 and 7.2, respectively.

due to the chromophore of this photoprotein, although the possibility that this absorption is due to a trace of protein-bound porphyrin impurities cannot be excluded at present. The molecular mass of the photoprotein estimated by gel-filtration on Ultrogel AcA 34 was 60 000. A solution of this photoprotein did not exhibit any prominent fluorescence before or after the luminescence reaction. The spectrum of luminescence from a live specimen (fig.1) had a peak at 496 nm, coinciding almost exactly with the data in [6]. The luminescence spectrum of the purified photoprotein could not be accurately measured due to the small amount of material obtained, although it appeared to be practically identical to the spectrum of live specimens.

The quantum yield of the photoprotein was only 0.3% based on 60 000 $M_r$, the data in section 2 and fig.1, assuming the absorbance of a 0.1% photoprotein solution to be 1.0. This unexpectedly low value is probably due to the presence of a large percentage of inactivated photoprotein. In fact, the final yield of photoprotein activity was only 10% of the activity of the initial crude extract despite the fact that only 10% or less of activity was eliminated together with impurities in each step of chromatography, thus indicating a considerable extent of inactivation during chromatography.

The effect of pH, which indicated an optimum pH of 8.3 for this luminescence system (fig.2) and the activation of luminescence by ATP and $Mg^{2+}$ (fig.3) were both consistent, at least qualitatively, with the observation made on crude luminescing extracts [6]. In regard to the activation effect of ATP, we found a 100-fold activation in contrast to their observation of only 10–30% activation. From the data of fig.3, the minimum concentrations of ATP and $Mg^{2+}$ detectable with the *Luminodesmus* photoprotein were estimated to be $10^{-9}$ and $10^{-6.5}$ M, respectively. The role of $Mg^{2+}$ could be partially substituted by $Ca^{2+}$ which, however, gave considerably less activation (fig.3).

## Acknowledgements

I thank Dr Demorest Davenport for the information on collecting *Luminodesmus* specimens, and Dr Frank H. Johnson for reading the manuscript. This work was supported by grants from the National Science Foundation (PCM78-22959) and the National Institutes of Health (GM-25093).

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