Mechanism of photoinactivation and re-activation in the bioluminescence system of the ctenophore *Mnemiopsis*

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The bioluminescence of the ctenophore *Mnemiopsis* takes place when the photoprotein mnemiopsin in the photocytes reacts with Ca^{2+} . The luminescence is inhibited in sunlight and this photoinhibition is reversible by keeping the live specimens in the dark. The extracts of mnemiopsin are similarly photoinhibited, but the photoinhibition cannot be reversed in the dark. We have found that photoinhibited mnemiopsin can be re-activated in the dark by incubation with coelenterazine and O_2 only in solutions having a pH very close to 9.0. The re-activation *in vivo* probably takes place in the same manner, using the coelenterazine that is supplied from its abundant storage form. Various lines of experimental evidence suggest that the photoinactivation of mnemiopsin results in the dissociation of coelenterazine and oxygen from the molecule of photoprotein; the dissociated form of the former molecule is an inactive form of coelenterazine, not free coelenterazine.

The luminescent system of the ctenophore *Mnemiopsis leidyi* involves a photoprotein, mnemiopsin, which emits light in the presence of Ca^{2+} much in the same way as does aequorin (Ward & Seliger, 1974a,b; Girsch & Hastings, 1978). Mnemiopsin differs from aequorin, however, in that the former is easily inactivated by light (Peters, 1905; Moore, 1924, 1925; Harvey & Korr, 1938). Ward & Seliger (1976) have examined the characteristics of photoinhibition with purified mnemiopsin and found that inactivation occurred with a high quantum yield over a wide range of the energy spectrum, independently of the presence of oxygen. They have posulated that the photoprotein contains bound luciferin (i.e. coelenterazine) and oxygen, and that the bound luciferin is not the ultimate target of the photoinactivation, but 'active oxygen' dissociates from the photoprotein by absorption of light.

Girsch & Hastings (1973) reported that the photoinactivation of mnemiopsin was reversible in the presence of a reducing agent, but this result could not be substantiated by Ward & Seliger (1976).

The present work was initiated in an effort to elucidate the mechanism of photoinhibition of the *Mnemiopsis* luminescence system, mainly through

studies concerning the re-activation of photoinhibited photoprotein.

Materials and methods

Specimens of *Mnemiopsis leidyi* (marine animals like jellyfish with ciliate swimming organs) were collected at Woods Hole. Crude extracts were prepared by two methods. In the first method, the whole specimens of dark-adapted animals were squeezed, centrifuged, then extracted with 100 mM-Tris/HCl, pH 9.3, containing 20 mM-EDTA, according to the procedure described by Ward & Seliger (1974a). In the second method, crude extracts were prepared by excising two to four comb-plate rows with underlying meridional canals (wherein lie the photocytes), and immersing them in the dark in three changes of ice-cold Ca^{2+} -free artificial sea water containing 1 mM-EDTA, pH 6.5, for a total of 10 min. The tissues, ranging in volume from 1.5 to 2.0 ml, were then transferred into 1.5 ml of ice-cold 100 mM-Tris/HCl buffer, pH 9.0, containing 20 mM-EDTA and vortex-mixed. This preparation was next sonicated with a cell disruptor, vortex-mixed again, then fragments of cells and membranes were removed by centrifugation at 12000g and 0°C for 10 min. The pH of the supernatant was adjusted to 9.0 with NaOH and it was stored at 0°C in the dark until used. Extracts were diluted with the Tris/EDTA buffer as necessary to obtain roughly equal activities of

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mnemiopsin luminescence from one batch to another. As a rule, 200 μ l portions of the diluted extract were used to assay mnemiopsin by adding 200 μ l of 10mM-calcium acetate prepared in 100mM-Tris buffer, pH9.0. In calculating the amount of mnemiopsin from the emitted total light, a quantum yield value of 0.12 (Ward & Seliger, 1976) was used.

Luminescence was measured by recording peak intensities with an Aminco Chem-Glow photometer and Pharmacia chart recorder. Quantitative measurements of luminescence were done by recording integrated total light on a calibrated photomultiplier assembly (Shimomura *et al.*, 1972).

The free form of coelenterazine was assayed with *Oplophorus* luciferase (Shimomura *et al.*, 1978), by measuring the luminescence emitted when the luciferase (20 μ g), in 4 ml of 15mM-Tris/HCl, pH8.3, containing 50mM-NaCl and 2mM-EDTA, was added to 50–100 μ l of sample. In calculating the amount of coelenterazine, a quantum yield of 0.34 (Shimomura *et al.*, 1978) was used. The stabilized form of coelenterazine, possibly coelenterazine *enol*-sulphate (Inoue *et al.*, 1977), was first converted quantitatively into free coelenterazine by heating 10–100 μ l of sample with 100 μ l of 0.5M-HCl at 100°C for 1 min under argon gas, followed by rapid cooling and neutralization with NaHCO_3 ; free coelenterazine obtained was then assayed with *Oplophorus* luciferase, as described above. The amount obtained represents the sum of the amount of free coelenterazine that initially existed plus the amount of stabilized coelenterazine. A reversibly oxidized form of coelenterazine was converted into free coelenterazine in an unknown yield by incubating 50–100 μ l of sample with an equal volume of 2M-2-mercaptoethanol at room temperature for 30 min under argon gas, then free coelenterazine formed was assayed with *Oplophorus* luciferase, to obtain the sum of the amount of free coelenterazine that initially existed plus the amount of the oxidized form.

Results and discussion

The presence of coelenterazine moiety in mnemiopsin

The involvement of coelenterazine in the bioluminescence of mnemiopsin has been deduced by Ward & Seliger (1976), mainly on the basis of the cross-reaction between urea/mercaptoethanol-treated mnemiopsin and *Renilla* luciferase. We have positively confirmed the presence of a coelenterazine moiety in mnemiopsin, as follows.

A crude extract obtained from the whole bodies of 100 dark-adapted specimens was caused to luminesce by the addition of CaCl_2 , then extracted

with diethyl ether. Coelenteramide in the diethyl ether extract was purified and identified chiefly by t.l.c. and mass spectrometry as described by Shimomura *et al.* (1975). The yield of coelenteramide was approx. 10 μ g on the basis of A_{330} . Extracts not caused to luminesce with CaCl_2 , and extracts from photoinhibited specimens, both yielded only insignificant amounts of coelenteramide (less than 2 μ g) by the same procedure.

Re-activation of photoinhibited mnemiopsin

Crude extracts of meridional canals, maintained on ice and exposed to direct sunlight for 5 min, lost all detectable luminescent activity. Replacing the ice-cold extracts in the dark for 15 min failed to reactivate luminescence in response to the addition of Ca^{2+} . Luminescence activity was restored, however, when 5 μ l of a methanol solution containing 1 μ g of synthetic coelenterazine (courtesy of Dr. S. Inoue, Meijo University, Nagoya, Japan) was added to portions of photoinactivated extracts and the mixtures were left in the dark at 0°C for 15 min. Luminescence activity was not restored in a control experiment with methanol alone. The re-activation was highly pH-dependent. Only at a pH of 9.0, and in the presence of 20mM-EDTA or -EGTA, could 100% of the initial activity of the extracts be restored, extracts with a pH of 8.7 or 9.3 recovering only about 30% of their activity before light exposure. Similarly, lowering the concentration of EDTA or EGTA in the extracts from 20 to 2 mM at pH9.0 decreased the recovery from light exposure to 31–54% of the original activity. These observations indicate that bound coelenterazine is either dissociated from the photoprotein or otherwise de-activated after light exposure. It appears from the narrow pH range of re-activation that binding of coelenterazine to the protein is dependent on some specific ionizable group just as is the highly pH-sensitive binding of calcium to mnemiopsin (Girsch & Hastings, 1978), although the effect of EDTA concentration is not fully clear at present.

The necessity of O_2 in the re-activation

Harvey & Korr (1938) reported that light-exposed pieces of canals containing photocytes regained their capacity to luminesce in response to mechanical stimulation 5 min after being left in darkness, but failed to do so in an atmosphere of H_2 . However, the apparent O_2 -dependence *in vivo* could involve any of several cellular processes in the photocytes, not necessarily the photoprotein itself (Chase, 1941).

We have confirmed the necessity for O_2 in the re-activation of mnemiopsin as follows. A small amount (2 ml) of a crude extract of the meridional canals of *Mnemiopsis* was placed in one arm of a

glass vessel and exposed to direct sunlight for 5 min. An aqueous solution of synthetic coelenterazine (120 ng in 200 μ l) was introduced in the side arm of the same vessel. The vessel was flushed with argon, then evacuated with a vacuum pump for 10 min. The extract was mixed with the coelenterazine solution by tilting the vessel, and the mixture left in the dark for 15 min. The solutions were maintained on ice throughout the experiment. The vessel was opened, and 200 μ l portions of the mixture were then assayed with Ca^{2+} . Only 12% of the original activity was found when the mixture had been left in air for 40 s (the minimum delay between re-admission of air into the vessel and injection of calcium into the photometer vial). Air-exposed samples assayed 3, 5 and 10 min later possessed 24, 68 and 86% of the original activity respectively. Controls using the light-exposed photoprotein extract and coelenterazine solution under identical conditions, except that no vacuum was applied, gave the following results: 12% of the activity was recovered 40 s after addition of coelenterazine in the dark, and 32, 54 and 96% of the activity was recovered 3, 5 and 10 min later respectively. The results indicate that re-activation did not take place during 15 min of anaerobic incubation, consequently suggesting that O_2 is needed for the binding of coelenterazine to the photoprotein.

Various coelenterazine derivatives in Mnemiopsis

Crude extracts obtained from dark-adapted whole specimens contained at least four different forms of coelenterazine, namely: (1) as a moiety of mnemiopsin; (2) free coelenterazine; (3) a stabilized form, probably the *enol*-sulphate; and (4) an oxidized form of coelenterazine, having an unknown structure, that yields free coelenterazine on treatment with 1 M-2-mercaptoethanol. They were found in a molar ratio of approx. 5:4:200:20. The presence of a large amount of the stabilized form, compared with the amount of mnemiopsin, suggests that the stabilized form is probably the store that supplies free coelenterazine as needed for use in the re-activation of mnemiopsin.

Coelenterazine moiety of mnemiopsin after photoinactivation

According to Ward & Seliger (1976), the photoinactivation of mnemiopsin causes primarily the release of the bound form of oxygen, leaving bound coelenterazine intact. If the oxygen has been released, but coelenterazine is still bound to the photoprotein molecule, then there should be no obstacle in detecting the coelenterazine moiety, unlike the case of native mnemiopsin, or aequorin, in which bound oxygen reacts with coelenterazine under various conditions to denature the photo-

protein (Shimomura & Johnson, 1976). In our experiments, however, coelenterazine could not be detected in the photoinactivated samples of mnemiopsin (see below), even after treating the samples with urea, methanol or boiling 0.1 M-HCl. Considering also that the re-activation of photoinactivated mnemiopsin requires the presence of coelenterazine and molecular oxygen at the same time, it seems more reasonable to assume that the photoinactivation of mnemiopsin causes splitting of both moieties of coelenterazine and oxygen from the photoprotein, the former moiety in an altered, inactive form.

The following experiments were carried out in an effort to identify the released form of coelenterazine. When mnemiopsin was precipitated from crude extracts by saturation with $(\text{NH}_4)_2\text{SO}_4$, 95% of free coelenterazine, 90% of its stabilized form and 60% of the oxidized form were found in the supernatant, indicating that the precipitate was still contaminated with coelenterazine and its derivatives which would obscure the results. Gel filtration of the photoprotein precipitate through a column (1 cm $\times$ 15 cm) of Sephadex G-25 prepared with 20 mM-EDTA/100 mM-Tris/HCl, pH 9.0, gave an eluate fraction of mnemiopsin that was almost free of the other forms of coelenterazine derivatives. Photoinactivation of this photoprotein sample yielded neither free coelenterazine, the stabilized form (coelenterazine sulphate) nor the reversibly oxidized form. In the photoinactivated sample, 2-hydroxycoelenterazine [the yellow compound described by Shimomura & Johnson (1978)] was also not detected by *Oplophorus* luciferase after dithionite reduction, and, according to Ward & Seliger (1976), coelenteramide should not be present. Thus the identity of the coelenterazine moiety of photoinactivated mnemiopsin still remains unknown.

The absence of the reversibly oxidized form of coelenterazine in a sample of photoinactivated mnemiopsin, described above, appears to contradict the results reported by Ward & Seliger (1976) that the urea/mercaptoethanol treatment of photoinactivated mnemiopsin yielded free coelenterazine, but no explanation for it can be offered at present.

Re-activation of mnemiopsin in vivo

The failure to observe the re-activation of mnemiopsin by simply keeping the photoinactivated samples of crude extract in the dark must be due to the great dilution of the reactants, i.e. mnemiopsin and the free form of coelenterazine. Assuming that all of the mnemiopsin and free coelenterazine are packaged in photocytes of microscopic size, and if the extraction resulted in 1000-fold dilution of the reactants, then the rate of

re-activation in the extract is expected to become 1 million times slower than in the original, undisturbed photocytes. We postulate that the re-activation *in vivo* is the same as the reaction *in vitro* that we describe in the present paper.

Under conditions of continuous light there may be some mechanism working *in vivo* to prevent the re-activation of photoinactivated mnemiopsin, thereby preventing continuous consumption of coelenterazine and its storage form. For example, the re-activation in sunlight might be controlled by limiting the supply of free coelenterazine, or possibly by slightly offsetting the pH inside the photocytes from the optimum pH of 9.0.

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