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Mechanism of Oxidative Carbon Dioxide Production during *Renilla reniformis* Bioluminescence

(sea pansy luciferin/firefly)

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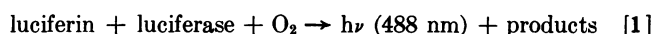
ABSTRACT The oxidation of luciferin catalyzed by sea pansy luciferase results in the emission of light. Molecular oxygen is required and carbon dioxide is produced. When the reaction occurs in the presence of $H_2{}^{18}O$, both of the oxygens of the carbon dioxide are labeled. One of the oxygens arises from the nonenzymic exchange of the ketone group of the substrate; the other oxygen is incorporated during the enzymic oxidation of the luciferin. When the reaction is carried out in the presence of $^{18}O_2$, neither of the oxygens of the carbon dioxide is labeled. Thus the source of oxygen in the carbon dioxide is water. A mechanism for the oxidative reaction is proposed.

The light-emitting reaction of the sea pansy, *Renilla reniformis*, is illustrated by the following reaction:



luciferin + luciferase + $O_2 \rightarrow h\nu$ (488 nm) + products [1]

Luciferase, an energy conversion protein of low molecular weight, has been purified to homogeneity by Karkhanis and Cormier (1), and methods for obtaining pure luciferin have been described (2). Reaction 1 gives rise to two products in addition to light. One of these has been shown to be the emitter of the *in vitro* reaction since its fluorescence emission is essentially identical with that of the bioluminescence emission *in vitro* (3).

We show here that the other product of reaction 1 is CO_2. Similar findings have recently been reported for the firefly and *Cypridina* bioluminescent reactions (4, 5). Using ^{18}O to study the mechanism of firefly luminescence, DeLuca and Dempsey established (6) that one of the oxygen atoms of the liberated CO_2 arise from water and that neither of the oxygens arises from molecular oxygen. The experiments reported here demonstrate that for the luminescent reaction of the sea pansy the source of oxygens in the liberated CO_2 is also water.

MATERIALS AND METHODS

Renilla reniformis luciferin and luciferase were prepared as described (1, 2). A solution of luciferin (39 nmol) in methanol was placed in one sidearm of a reaction vessel (Fig. 1) and evaporated to dryness under nitrogen. Luciferase (2 mg, 80 nmol) in 3.7 ml of phosphate buffer (0.1 M, pH 7.2) was placed in the other sidearm of the vessel along with several glass beads. The vessel was attached to a high-vacuum line and completely evacuated. $^{18}O_2$ or $^{16}O_2$ (CO_2-free) was admitted to the vessel. The vessel was removed from the vacuum line, the luciferase and luciferin were mixed rapidly and deposited in one sidearm, and the other sidearm was then plunged into liquid nitrogen. This acted as a trap for CO_2 as it was released during the reaction period. When light emission ceased (after about 40 min), the chamber containing the enzyme and substrate was also submerged in liquid nitrogen. The vessel was again attached to the vacuum line and CO_2 was collected as described previously (7). Control experiments to determine the level of nonenzymic oxygen exchange were conducted similarly, except that CO_2 was bubbled through the medium by means of the apparatus shown in Fig. 1. The atom per cent of excess ^{18}O was calculated from the mass ratio 44/46 as described previously (7).

RESULTS AND DISCUSSION

The experiments (Table 1) demonstrate that CO_2 is a product of the luciferase-catalyzed bioluminescent oxidation of sea pansy luciferin, and suggest striking similarities to the chemical mechanism of firefly light emission. The level of

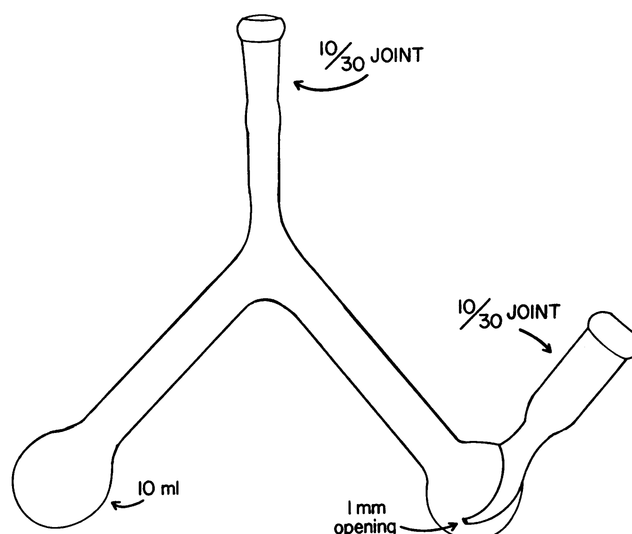


FIG. 1. Reaction vessel used for the nonenzymic experiments (Table 1, *b* and *d*). The same type of vessel was used for the enzymic experiments (Table 1, *a* and *c*), except without the device for CO_2 admission on the right-hand sidearm.

This is paper X of a series entitled "Studies on the Bioluminescence of *Renilla reniformis*."

* Requests for reprints should be sent to this address.

TABLE 1. *Source of oxygen in CO_2 released during light emission by sea pansy luciferase.*

Expt.* Conditions	Atom per cent excess ^{18}O in CO_2†	Oxygens incorporated
a. $H_2{}^{18}O$; $^{16}O_2$ 40 min	1.05	1.9
b. $H_2{}^{18}O$; $C^{16}O_2$ 15 min	0.081	0.1
20 min	0.101	0.2
40 min	0.140	0.2
c. $H_2{}^{16}O$; $^{18}O_2$ 40 min	0.182	<0.1
d. $C^{18}O_2$, $(CH_3)_2SO$	0.140	<0.1

* Expt. *a:* Reaction medium was prepared in $H_2{}^{18}O$ (Miles Laboratories, Elkhart, Ind.) distilled prior to use; enrichment of complete medium during the enzymic reaction was 1.10 atom % excess. CO_2 released by the enzyme was immediately trapped in liquid nitrogen during the reaction period.

b: Control. Reaction medium contained only phosphate buffer, pH 7.2, prepared in $H_2{}^{18}O$ (1.35 atom % excess). $C^{16}O_2$ was bubbled through the medium, by means of the apparatus depicted in Fig. 1, and immediately trapped in liquid nitrogen during the reaction period.

c: Reaction medium was prepared in $H_2{}^{16}O$; $^{18}O_2$ (92.1 atom % excess; Miles Laboratories, Elkhart, Ind.) was admitted after complete evacuation of the medium. CO_2 released by the enzyme was immediately trapped in liquid nitrogen during the reaction period.

d: Reaction medium contained dry dimethyl sulfoxide (3 ml) and a solution of 0.3 ml of potassium *t*-butoxide (0.01 M) in dry *t*-butanol. $C^{18}O_2$ (0.147 atom % excess) was bubbled through the medium, by means of the apparatus depicted in Fig. 1, and immediately trapped in liquid nitrogen.

† Mass ratio 46/44 was measured with a Hitachi-Perkin-Elmer RMU-6D mass spectrometer; CO_2 was admitted to the instrument via an all-glass inlet system. Results are the mean of duplicate or triplicate experiments differing by less than 5%.

CO_2 released was in the range expected from the substrate levels used, which shows that approximately 1 mole of CO_2 was produced per mole of luciferin oxidized. The data in Table 1 further demonstrate that the oxygen in the CO_2 released during light emission arose from water and not from molecular oxygen. When light emission proceeded in an $^{18}O_2$ atmosphere there was negligible incorporation of ^{18}O into CO_2. However, when the reaction was carried out in the presence of $H_2{}^{18}O$, both oxygens in the CO_2 released were labeled. Control experiments using a device (Fig. 1) which simulated the conditions of the enzymic experiments showed that even after 40 min the nonenzymic exchange of water and CO_2 oxygens was very low. The fact that the partial pressure of CO_2 was kept very low by use of a liquid nitrogen trap, conditions which do not favor solution in the medium of appreciable quantities of CO_2, is the most probable explanation for the low level of exchange of water and CO_2 in the nonenzymic experiment. Thus, the rapid hydration and dehydration of CO_2 known to occur at neutral pH was minimized (8–11). Additional control experiments with either enzyme alone or substrate alone showed that neither component released CO_2 in the absence of the other, nor did they singly catalyze exchange of CO_2 and water oxygens.

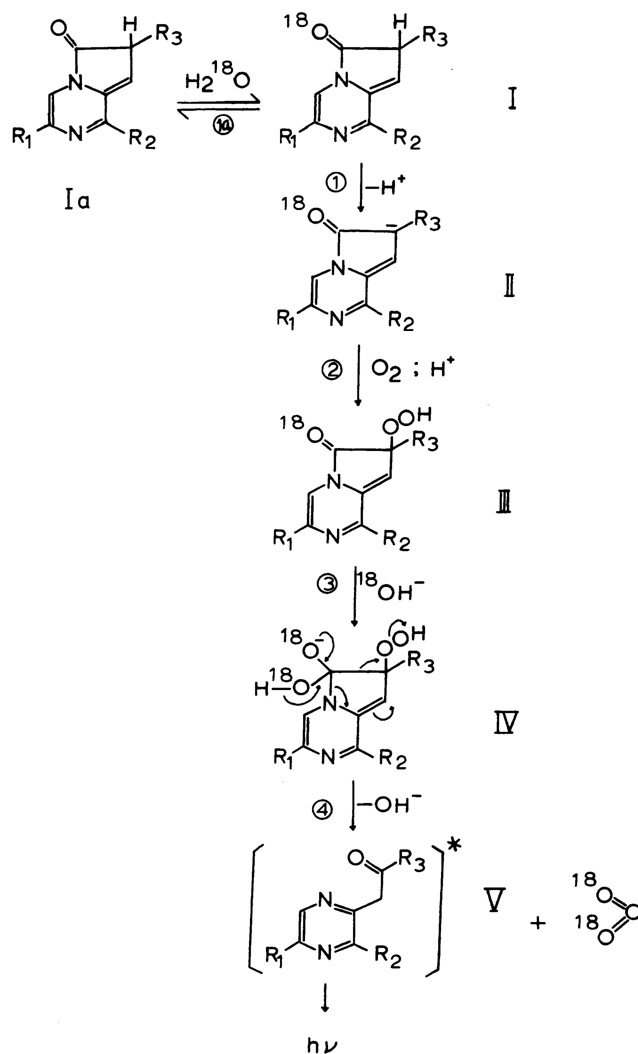


FIG. 2. Proposed mechanism for oxidative CO_2 production during *Renilla reniformis* bioluminescence.

The mechanism presented in Fig. 2 predicts that CO_2 released during light emission will contain two oxygens derived from water. One of the two oxygens found experimentally (Table 1) is probably contributed by the nonenzymic exchange of water oxygen with the ketone oxygen of luciferin, the group that finally gives rise to CO_2 (Fig. 2, Reaction 1*a*) (12). Such exchange would be expected during the time required for these experiments.

Mechanisms that have been suggested for certain chemiluminescent reactions (13–15) involve the intermediate formation of a four-membered oxygen-containing ring (dioxetane) which decomposes in a concerted reaction to yield the observed luminescent products. Such a mechanism, if applied to bioluminescence, would predict that the source of oxygen in the liberated CO_2 would be molecular oxygen. This is not consistent with the results presented in Table 1. Since conditions for optimal quantum yields during these chemiluminescent reactions require a nonpolar, highly basic environment, it seems reasonable to assume that the catalytic sites of the various luciferases provide a similar environment if we are to account for the high quantum yields observed during bioluminescence. It is possible that in such an environment an exchange may occur between the oxygens of CO_2 and the

trace amounts of water present. The control (*d*) shown in Table 1 shows that this is not the case. Thus it is unlikely that a rapid nonenzymic exchange of CO_2 and water is occurring within the catalytic site of the enzyme. Even in the presence of high concentrations of water, such an exchange would be unlikely at high pH, since these conditions would result only in the hydration of CO_2.

A detailed mechanism for the bioluminescent oxidation of luciferin catalyzed by the sea pansy luciferase is shown in Fig. 2. The structure of the major portion of the sea pansy luciferin molecule has recently been established (Hori, K., M. J. Cormier, and R. Lovins, unpublished results.); R_1 is a 3-substituted indole. Some uncertainties remain regarding groups R_2 and R_3. Reaction **1a** is the reversible hydration–dehydration of the ketone. This would account for the incorporation of one ^{18}O atom in the CO_2 when the reaction is carried out in $H_2{}^{18}O$. Reactions **1** and **2** represent the formation of a carbanion followed by the addition of oxygen to form a linear peroxide, III. In reaction **3**, hydroxyl ion adds at the carbonyl carbon to form compound IV. In reaction **4**, compound IV then rearranges to compound V; this results in the liberation of CO_2 and the formation of an electronically excited state of compound V. This compound returns to the ground state with the emission of light. When the reaction medium contains $H_2{}^{18}O$, the incorporation of ^{18}O occurs at steps **1a** and **3**, resulting in the production of CO_2 containing two ^{18}O atoms, as was experimentally observed.

When the reaction is carried out in the presence of $^{18}O_2$ our mechanism predicts that there would be no significant incorporation of ^{18}O into the CO_2, a prediction that is consistent with the results (Table 1).

For the two bioluminescent systems examined thus far, firefly and sea pansy, the oxygen atoms incorporated into CO_2 arise from water. Thus, the mechanism presented here may be rather widespread in bioluminescent reactions. Certainly these results cannot be explained by the mechanisms suggested (13–15) for chemiluminescence of *Cypridina* and firefly luciferin derivatives in which an intermediate dioxetane ring has been proposed. It would be of interest to repeat these experiments using the enzyme from *Cypridina*, since the luciferin structure is similar to that of sea pansy luciferin and it has recently been shown that CO_2 is a product of that bioluminescent reaction.

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