Mechanism of the Enzyme-catalyzed Oxidation of Cypridina and Firefly Luciferins Studied by Means of $^{17}O_2$ and $H_2{}^{18}O$[1]

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SUMMARY. Mass spectral analyses of the CO_2 liberated in the Cypridina luciferin-luciferase and firefly luciferin-luciferase reactions run in the presence of $^{17}O_2$ and $H_2{}^{18}O$ show that the product is predominantly $C^{18}O^{16}O$ (mass 46) and not $C^{17}O^{16}O$ (mass 45). Incorporation of ^{18}O into medium CO_2 by exchange does not account for the observed results. These experiments provide evidence that the Cypridina and firefly bioluminescence reactions proceed via a linear peroxide mechanism rather than the dioxetane mechanism and suggest that a common mechanism may underly many bioluminescence reactions.

The oxygenases Cypridina luciferase and firefly luciferase catalyze the oxidation of their respective luciferins by molecular oxygen yielding as products oxyluciferin, CO_2, and light (1,2,3). The quantum yields are about 0.3 and 0.9, respectively. Based on results obtained by carrying out the reactions in $^{18}O_2$ and $H_2{}^{18}O$, two separate mechanisms have been proposed for the reactions (figs. 1 and 2). Thus, for Cypridina, one of the oxygens of CO_2 has been reported to originate from molecular oxygen and none from water (4,5,6,7), whereas in the firefly reaction, one of the oxygens of CO_2 has been found to arise from water and none from molecular oxygen (8,9,10). These results have been interpreted to mean that the pathway of oxygen during CO_2 production in Cypridina is via the dioxetane mechanism (fig. 1, pathway B), whereas in the firefly the pathway is by a linear peroxide mechanism (fig. 2, pathway A).

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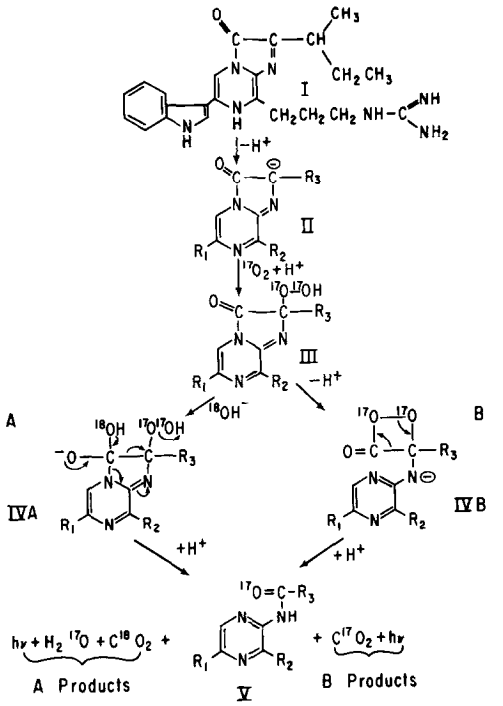

Fig. I: Proposed mechanisms for the enzymatic oxidation of Cypridina luciferin.

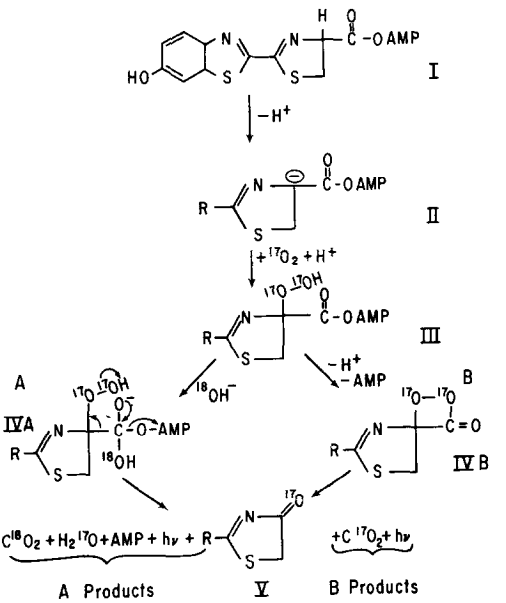

Fig. II: Proposed mechanism for the enzymatic oxidation of firefly luciferin.

This paper reports the results of experiments carried out in the presence of $^{17}O_2$ and $H_2{}^{18}O$. In both the Cypridina and firefly reactions, one of the oxygens of CO_2 was found to originate from water and only a small fraction from molecular oxygen. It is concluded therefore that the linear peroxide mechanism (figs. 1 and 2, pathway A) is the correct one for both bioluminescence reactions.

Materials and Methods

Cypridina luciferin was prepared as previously described (11), except that absolute methanol was used initially to extract luciferin. Further purification was achieved by chromatography on a powdered cellulose column (12) and by thick layer chromatography (Cellulose MN 300, 0.5 mm), using a mixture of ethyl acetate, ethanol, and water as the developing solvent (12). Cypridina luciferase was prepared by acrylamide gel electrophoresis (13). Crystalline firefly luciferin was synthesized by the method of Seto, et al. (14). Crystalline firefly luciferase was prepared as previously described (15). $^{17}O_2$ was purchased from Miles Laboratories, Elkhart, Indiana, and $H_2{}^{18}O$ from Monsanto Research Corp., Miamisburg, Ohio.

The bioluminescence reaction was carried out in a vessel possessing two sidearms (16). Cypridina luciferase (M.W. = 68,000), dissolved in 2.0 ml of 0.02 M glycylglycine-NaOH buffer pH 7.8, containing 0.04 M NaCl and made up in either $H_2{}^{16}O$ or $H_2{}^{18}O$ (3.62 atom % excess) and three glass beads were placed in one sidearm. Cypridina luciferin dihydrochloride (M.W. = 478), dissolved in 0.1 ml of 90% methanol, and 0.7 ml of glycylglycine buffer made up in either $H_2{}^{16}O$ or $H_2{}^{18}O$ (3.62 atom % excess) and a volume of 0.1 N NaOH, prepared from carbonate-low saturated NaOH, just sufficient to neutralize the acid present in the luciferin was placed in the other sidearm. The solutions in the sidearms were degassed through three cycles of freezing and evacuating to less than 20 microns pressure, and thawing, using isopropanol-Dry Ice and liquid nitrogen to freeze the sidearms, respectively. Following the final evacuation, $^{17}O_2$ (77 atom % excess; contaminating $^{18}O_2$ = 0.8 atom % excess) was introduced into the reaction vessel from a container which had been immersed in liquid nitrogen for at least an hour to freeze out any contaminating CO_2. After thawing to about 22°, the solutions were mixed and continuously shaken until luminescence nearly ceased, 20-30 seconds. The reaction mixture was then quickly poured into one sidearm and the sidearm immersed in liquid nitrogen for 10 minutes to collect CO_2, after which remaining $^{17}O_2$ was pumped away. The sidearm was then immersed in isopropanol - Dry Ice. The released CO_2 was trapped with liquid nitrogen in a tube containing a small amount of degassed concentrated H_2SO_4. After thawing the H_2SO_4 was shaken to absorb any water and methanol present with the CO_2. The CO_2 was then collected in a small tube immersed in liquid nitrogen, the tube was then immersed in isopropanol - Dry Ice to release the CO_2, and the volume of CO_2 measured. After diluting with carrier CO_2, the atom % excess of ^{18}O was estimated from the ratio of mass 46/44 and the ^{17}O from mass 45/44 determined in a CEC Model 21-401 or Nuclide mass spectrometer (17). Standards (^{18}O-containing, mass 46 or ^{13}C-containing mass 45) served for instrument calibration and calculations.

The firefly experiments were carried out essentially as previously described (8). The luciferin and luciferase solutions were placed in

separate sidearms and degassed by evacuating slowly with only the luciferin exposed to freezing. After admitting the $^{17}O_2$, the solutions were mixed. Subsequent treatment was the same as for Cypridina.

The extent of exchange between $H_2{}^{18}O$ and bicarbonate in the reaction medium was measured by placing 0.3 ml of the medium, after completion of the enzymic experiment, in a tube and freezing in liquid nitrogen. After adding 150 mg $KHSO_4$, evacuating and thawing, the CO_2 released by the $KHSO_4$ was collected and treated to remove contaminating methanol (except for experiments with the firefly in which methanol was not used) and water as previously described. The CO_2 was then collected and measured directly in the mass spectrometer.

Results and Discussion

A different approach was used in this work from that used by earlier workers. Instead of running the reaction separately in $^{18}O_2$ and $H_2{}^{18}O$, the present experiments were carried out in the presence of both $^{17}O_2$ and $H_2{}^{18}O$. From examining the masses of the CO_2 produced, it was possible to decide whether the source of oxygen was $^{17}O_2$ or $H_2{}^{18}O$. The results summarized in Table 1 show unequivocally that close to one of the oxygens of the CO_2 produced in both the Cypridina and firefly reactions originated from water and that only a small fraction of the CO_2 contained oxygen arising from molecular oxygen. The latter may have been produced via a non-bioluminescent pathway.

One of the major questions raised about previous work has been the possibility that an exchange was taking place between the $H_2{}^{18}O$ of the reaction medium and the CO_2 released by the reaction. Such an exchange, if significant, could account for an apparent incorporation of ^{18}O from water into CO_2. Exchange may occur by the following reactions:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^- \quad (1)$$

$$CO_2 + {}^-OH \rightleftharpoons HCO_3^- \quad (2)$$

Because protonation occuring in reaction 1 is rapid compared to CO_2 hydration, exchange will result in incorporation of oxygens from medium bicarbonate into CO_2 released. Much more bicarbonate was present in the reaction medium (principally from bicarbonate in the buffers) than was produced in the luciferin reaction. Thus, even an exchange localized

Table 1

Incorporation of Oxygen into CO_2 Released During the Cypridina and Firefly Bioluminescence Reactions

Luciferin	Luciferase	Atom of O Incorporated per Each CO_2 formed: From $H_2{}^{18}O$ Total	From $H_2{}^{18}O$ By Exchange[a]	From $^{17}O_2$ Total
Cypridina:				
1) 4.4 µmoles + $H_2{}^{18}O$	0.04 µmoles + $H_2{}^{18}O$	1.09[b]	0.04	0.06
2) 6.3 µmoles + $H_2{}^{18}O$	0.04 µmoles + $H_2{}^{16}O$	0.49[c]	0.06	0.06
3) 4.8 µmoles + $H_2{}^{16}O$	0.04 µmoles + $H_2{}^{18}O$	1.11[d]	0.09	0.06
Firefly:				
4) 33 mµmoles + $H_2{}^{18}O$	64 mµmoles + $H_2{}^{18}O$	0.85[e]	0.06	0.08
5) 33 mµmoles + $H_2{}^{18}O$	64 mµmoles + $H_2{}^{18}O$	0.92[f]	0.04	0.05

(a) Represents ^{18}O present in bicarbonate of the reaction medium after collection of the experimental CO_2. This gives the maximal possible amount of ^{18}O from water incorporated into medium bicarbonate by exchange.

(b) Enrichment of ^{18}O in medium during enzymic reaction was 3.5 atom % excess; reaction time, 22 seconds; pH of spent reaction mixture, 7.9.

(c) Enrichment of ^{18}O in medium during enzymic reaction was 0.9 atom % excess; reaction time, 20 seconds; pH of spent reaction mixture, 7.9.

(d) Enrichment of ^{18}O in medium during enzymic reaction was 2.6 atom % excess; reaction time, 30 seconds; pH of spent reaction mixture, 7.9.

(e) Enrichment of ^{18}O in medium during enzymic reaction was 3.32 atom % excess; reaction time, 32 seconds; pH of spent reaction mixture, 7.8.

(f) Enrichment of ^{18}O is the same as for (e), reaction time was 59 seconds.

near the enzyme catalytic site would be expected to introduce medium bicarbonate oxygens into the CO_2 produced. The results presented in Table 1 show that medium bicarbonate exchanged only slightly with water under conditions of the experiment, and further, that the liberated CO_2 had much more ^{18}O present than did medium bicarbonate. Thus, the liberated CO_2 did not acquire ^{18}O by exchange.

These results allow conclusion that the linear peroxide mechanism (figs. 1 and 2, pathways A) (8,9,10) is the source of one of the oxygens of CO_2 released in both bioluminescence reactions, and not the dioxetane mechanism (4,5,6,7,18,19). A linear peroxide mechanism will yield some 100 kilocalories, which is more than adequate for light emission (20).

Table 1 also reports the observation that in the Cypridina reaction, about one of the oxygens of CO_2 originates from water when the luciferase is dissolved in $H_2{}^{18}O$, but the incorporation is reduced to about 0.5 when the enzyme is dissolved in $H_2{}^{16}O$ and the luciferin in $H_2{}^{18}O$. The data suggest that exchange of water of the medium and water present in a hydrophobic environment of the catalytic site of luciferase may not be complete during the time required for luciferin to penetrate to the site and CO_2 to be released.

A number of precautions were taken to avoid potential sources of error. Relatively large amounts of CO_2 were used for mass analyses, with concomitant checking against standards. The presence of ^{17}O in the O_2 gas used at levels approximately as claimed by supplier was confirmed by conversion of oxygen to CO_2 by exposure to hot carbon. In preliminary experiments, methanol was found to contaminate CO_2 produced. Methanol was not removed by exposure to P_2O_5, but could be absorbed completely with concentrated H_2SO_4 without absorbing CO_2. The rate of autoxidation of Cypridina luciferin in 0.02 M glycylglycine-NaOH buffer, pH 7.8, containing 0.04 M NaCl, was also examined by following the spectral changes at 435 nm and 360 nm and found to be negligible over a period of 3-5 minutes, the time interval that

luciferin was exposed to $^{17}O_2$ prior to mixing. The Cypridina luciferin solution in the sidearm showed some turbidity before mixing due to the concentration being high, but this did not appear to affect the light emission. The volume of CO_2 released by the reaction was measured on a Pirani gauge in a known volume and was diluted with exactly equivalent volumes at the same pressure. Samples were diluted until the total CO_2 was large enough to be accurately read in the mass spectrometer. The yield of CO_2 in the Cypridina reaction ranged from 0.4 - 0.5 μmole.

The finding that one of the atoms of oxygen of the CO_2 molecule produced by the Cypridina reaction is derived from water is consistent with the results reported earlier for the Renilla bioluminescence reaction in which one of the atoms was found to originate from water and the second from a non-enzymatic exchange of the carbonyl oxygen of luciferin with water (16). The structure of Cypridina (crustacean) luciferin is similar to that of Renilla (coelenterate) luciferin, but not to that of firefly (insect) luciferin. The results show that a common bioluminescence mechanism may be responsible for light emission among members of such diverse phyla.

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