Vol. 81, No. 3, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1978 Pages 980-986

MECHANISM OF THE ENZYME-CATALYZED BIOLUMINESCENT OXIDATION OF COELENTERATE-TYPE LUCIFERIN

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Received February 27, 1978

SUMMARY: The use of a fully active, synthetic analogue of coelenterate-type luciferin labeled in the carbonyl position with ^{14}C and ^{18}O was used to probe the mechanism of the Renilla luciferase catalyzed oxidative decarboxylation of this compound. In the presence of $^{17}O_2$, the CO_2 produced in this oxidation can be shown to contain approximately one ^{17}O atom per CO_2 molecule. This result is consistent with a cyclic peroxide or dioxetanone-type mechanism. In the presence of luciferase, the oxygen in the luciferin carbonyl group is rapidly exchanged with solvent water prior to the production of CO_2. Thus, the reaction CO_2 contains considerable oxygen derived from water, via exchange with the carbonyl group, and about one oxygen from O_2 via a cyclic peroxide.

Renilla reniformis luciferase catalyzes the oxidative decarboxylation of luciferin, in the presence of oxygen, to give CO_2, oxyluciferin (III) and light (1). The mechanism of this oxidation has been studied using isotopically labeled oxygen and water (2,3). In these studies the pattern of incorporation of oxygen atoms into the CO_2 produced indicated a "linear" mechanism for the reaction (Figure 1). Similar mechanistic studies on other bioluminescent systems, Cypridina and firefly, have yielded conflicting results (3-7) which could support either a "linear" or "dioxetanone" mechanism.

In an attempt to clarify the mechanism of the bioluminescent reaction in Renilla we have synthesized a fully active analogue of the native luciferin (8,9) which is isotopically labeled in the 3-position (I, Figure 1) with both ^{18}O and ^{14}C. Preliminary results on the mechanism of oxidation using the [3-^{18}O]luciferin have been reported (10). These results indicated that either the simple mechanisms as outlined in Figure 1 did not apply, or the reaction CO_2 was diluted with medium CO_2. To test this latter possibility we have synthesized

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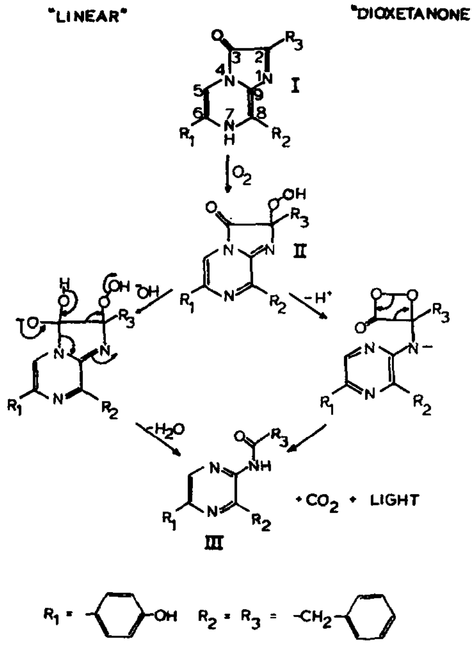

Figure 1

Proposed Mechanism for the Enzymatic Oxidation of Coelenterate-type Luciferin.

the [3-^{14}C]luciferin and used it to determine the dilution of the reaction CO_2.

The results presented here demonstrate that one oxygen in the reaction CO_2 is derived from O_2 as predicted by the "dioxetanone" mechanism (Figure 1). In addition, the [3-^{18}O]luciferin has allowed us to show that the carbonyl oxygen, which was previously assumed to be incorporated into the product CO_2, is almost completely exchanged with oxygen from H_2O prior to CO_2 production.

MATERIALS & METHODS

Renilla luciferase was prepared as previously described (11). The pure enzyme, diluted with bovine serum albumin was lyophilized in ampoules and stored at -80°.

[3-^{18}O]luciferin[†] (I) (3,7-Dihydro-2-benzyl-6-(p-hydroxyphenyl)-8-benzylimidazo-[1,2-a]-pyrazin-3-one) was prepared by a modification of the method of Hori, et al. (1), by heating diethoxybenzyl glyoxal and the methyl ether of etioluciferin in ethanolic, acidic $H^{18}OH$ (Monsanto Research Corp., Miamisburg, Ohio).

[†] Full procedures will be published elsewhere on the synthesis of both luciferins.

The methyl group was cleaved off as previously described (1). The incorporation of ^{18}O into the 3 position of [3-^{18}O]luciferin was determined by mass spectrometry.

[3-^{14}C]luciferin† was prepared by a similar route, by conversion of [2-^{14}C] glyoxylic acid (Amersham-Searle) to the diethoxybenzyl glyoxal, followed by reaction as described above. The specific activity of the [3-^{14}C]luciferin was determined by oxidation of a sample in dimethyl sulfoxide with subsequent collection of the released CO_2 in Oxyfluor-CO_2 (New England Nuclear). A second CO_2 trap containing an equal volume of Oxyfluor-CO_2 contained less than 2% of the liberated $^{14}CO_2$. The specific activity was calculated, after gravimetric addition of standard [^{14}C]hexadecane (Amersham-Searle), as 26,600 dpm per μmole.

The bioluminescent reactions were carried out as previously described (5,10) with the following modifications. Luciferin (0.25 μmole), dissolved in methanol was added to one sidearm of the reaction vessel, and to this 6 ml of buffer (0.5 M NaCl, 0.1 M K_2HPO_4, 1 mM Na_2EDTA, 0.6 mM NaN_3, pH 7.6) was added. The lyophilized enzyme (0.2 mg luciferase) was added to the other sidearm and maintained at 0° during degassing by two freeze-thaw cycles of the buffer solution. $^{17}O_2$ was admitted to 0.2 atmosphere pressure and the buffer was then warmed to room temperature. The luciferin/buffer solution was poured onto the luciferase and mixed continuously by shaking during the reaction. Reactions were allowed to proceed for 4 minutes, followed by freezing in dry ice/isopropanol and collection of the first batch of CO_2 by bleeding through a coil immersed in liquid N_2. The reaction mixture was then thawed quickly and immediately refrozen in dry ice/isopropanol to yield a second collection of CO_2. Up to three collections of CO_2 were obtained. One ml of the spent reaction mixture was frozen and then acidified with 5 grams $KHSO_4$ in an appropriately evacuated vessel and the liberated CO_2 collected. The ratios of mass 45/44 and 46/44 for the liberated CO_2 were measured with a mass ratio spectrometer. The volume of the remaining sample was remeasured on a calibrated manometer and the CO_2 transferred to 0.5 ml of 0.2 M NaOH. This solution was transferred to scintillation mixture, with washing with 0.5 ml water. The $^{14}CO_2$ was determined in a liquid scintillation counter using standard $Na_2{}^{14}CO_3$ to determine the specific activity.

RESULTS & DISCUSSION

The present study on the mechanism of bioluminescence of coelenterate-type luciferin is the most detailed to date. The experiment described here involves oxidation of both [3-^{14}C] and [3-^{18}O]luciferin with Renilla luciferase in the presence of $H^{16}OH$ and $^{17}O_2$. The use of [3-^{18}O]luciferin allows us to determine whether the carbonyl oxygen of luciferin is incorporated into the reaction CO_2. Furthermore the use of [3-^{14}C]luciferin allows us to determine the extent of dilution of the reaction CO_2. Previous studies have assumed that the trapped CO_2 is due only to the CO_2 formed during the bioluminescent reaction. The correction for dilution by medium CO_2 enables accurate determination of the incorporation of all sources of oxygen into the reaction CO_2.

The results from such an experiment are shown in Tables 1 & 2, the data presented being representative of a number of experiments, all of which lead essentially to the same mechanistic conclusion. Table 1 shows the calculation

Table 1

Calculation of Dilutions of Reaction CO_2 by CO_2 Derived from the Medium

CO_2	Amount[a]	Specific Activity of CO_2	Ratio of Specific Activities: Luciferin/CO_2
	μmole	dpm per μmole	
1st Collection	0.39	1860 ± 360[c]	7 ± 2[c]
2nd Collection	0.24	842 ± 214	16 ± 4
3rd Collection	0.15	820 ± 330	16 ± 7
Acidification	0.89	48 ± 12	277 ± 75

[a] Measured on calibrated manometer.

[b] Specific activity of the [3-^{14}C]luciferin: [3-^{18}O]**luciferin:** mixture = 13,300 dpm per μmole.

[c] "±" values represent estimates of cumulative errors

of the dilutions of the reaction CO_2 by CO_2 derived from the medium. The trapped CO_2, after measurement of the ratios of $^{18}O/^{16}O$ and $^{17}O/^{16}O$, was then counted for ^{14}C. After adjustment for counting efficiency the specific activity was calculated. The dilution of the reaction CO_2 by CO_2 derived from the medium was calculated from the specific activities of the luciferin and trapped CO_2. The decrease in the measured specific activity of the CO_2, when compared to the starting luciferin, is a measure of dilution by non-reaction CO_2. Thus in the 1st collection of CO_2 only 14% of the 0.39 μmoles CO_2 trapped comes from the bioluminescent reaction. The amount of trapped CO_2 decreases with subsequent collections. The results show that there is extensive dilution of the reaction CO_2 by CO_2 derived from the reaction mixture. The final acidification indicated that there was still over 5 μmoles of bicarbonate plus CO_2 dissolved in the 6 ml buffer, although the CO_2 liberated contains only low levels of reaction CO_2.

Table 2

Incorporation of Oxygen into the Reaction CO_2 During Bioluminescence

		^{18}O			^{17}O		
CO_2	Dilution Factor[a]	Measured a.p.e.	Reaction[b] a.p.e.	Atoms[c] Incorporated	Measured a.p.e.	Reaction[b] a.p.e.	Atoms[d] Incorporated
1st Collection	7	0.229	1.6 ± 0.4[e]	0.09 ± 0.03[e]	0.606	4.3 ± 1.2[e]	0.6 ± 0.2[e]
2nd Collection	16	0.078	1.25± 0.3	0.07 ± 0.02	0.200	3.2 ± 0.8	0.4 ± 0.1
3rd Collection	16	0.058	0.9 ± 0.4	0.05 ± 0.02	0.074	1.2 ± 0.5	0.2 ± 0.1
Acidification	277	0.003	0.8 ± 0.2	0.03 ± 0.01	0.001	0.3 ± 0.1	0.04± 0.01

[a] From Table 1.

[b] Reaction a.p.e. = Measured a.p.e. x dilution factor

[c] Obtained by dividing a.p.e. of reaction CO_2 by a.p.e. of [3-^{18}O] luciferin (17.5).

[d] Obtained by dividing a.p.e. of reaction CO_2 by a.p.e. of $^{17}O_2$ (7.39).

[e] "±" values represent estimates of cumulative errors

The only possible sources of ^{18}O and ^{17}O found in the trapped CO_2 were from the [3-^{18}O]luciferin or $^{17}O_2$, respectively. The measured incorporations were adjusted for the fraction of the total CO_2 that was derived from the medium. Table 2 shows this adjustment for both the ^{17}O and ^{18}O incorporations. Taking this into account one can calculate the atom percent excess (a.p.e.) for ^{18}O or ^{17}O in the reaction CO_2. These a.p.e.'s are then divided by the a.p.e. of the starting [3-^{18}O]luciferin or $^{17}O_2$ to give the number of atoms of each incorporated into the reaction CO_2.

It can be seen from Table 2 that 0.6 atom of oxygen derived from $^{17}O_2$ was present in the reaction CO_2. The level of ^{17}O in the reaction CO_2 declines with subsequent collection, indicating a relatively rapid exchange of the oxygen in the reaction CO_2 with solvent water. The incorporation of ^{18}O into the reaction CO_2 from the carbonyl group of luciferin was very low, with only about 0.1 atom of oxygen from the luciferin being retained in the reaction CO_2. The data on

^{17}O incorporation indicates that about 40% of the reaction CO_2 was exchanged with solvent H_2O before it was trapped. Correcting the incorporation of ^{18}O for this "washout" of label shows that only 0.15 atom of oxygen was incorporated into the reaction CO_2 from luciferin. Therefore there must be a rapid exchange of the carbonyl group with solvent water prior to luminescence.

This exchange of the carbonyl oxygen of luciferin with water, prior to luminescence, has been shown not to occur in the absence of luciferase under a variety of conditions (12). Therefore this exchange occurs at some intermediate stage during the reaction, before the final concerted bond cleavage to produce CO_2 and light. This exchange may occur by a mechanism similar to the one postulated by McCapra et al. (13) in 1972, which involves an attack on the luciferin hydroperoxide (II) by a sulfhydryl group from the enzyme. There is evidence that a sulfhydryl group is located in or near the active center of Renilla luciferase (14).

The results presented here can be summarized as follows: The luciferin carbonyl oxygen is exchanged rapidly on the enzyme with oxygen from water prior to incorporation of an oxygen atom from O_2 via a "dioxetanone" pathway. The resultant reaction CO_2 also exchanges its oxygens rapidly with oxygen from water.

These results suggest reasons for the conflicting data on the labeling studies previously carried out. Some precautions were taken throughout the experiments described here to minimize contaminating CO_2. Even after several "freeze-thaw cycles" there is enough bicarbonate and CO_2 remaining in the medium, to subsequently cause substantial dilution of the reaction CO_2. Further we have shown a rapid exchange of the oxygens of the reaction CO_2 with oxygen from water. Thus the previous report (2) of up to 1.9 atoms of oxygen from H_2O incorporated into the CO_2 is probably due to a combination of diluting the reaction CO_2 by medium CO_2, rapid exchange of the oxygens of the medium CO_2 with oxygen from water, and exchange of the luciferin carbonyl prior to CO_2 formation.

We have shown that the amount of CO_2 derived from bicarbonate in our buffers will give rise to approximately 0.25 - 0.5 μmole CO_2 in the first CO_2

collection. Therefore, with the Renilla bioluminescent system, meaningful labeling patterns can only be obtained if (i) the reaction CO_2 is produced in excess of the 0.25 - 0.5 μmole medium CO_2, (ii) a ^{13}C or preferably, ^{14}C labeled luciferin is included to quantitate the dilution of the reaction CO_2, or, (iii) close to bicarbonate and CO_2-free conditions are obtained and maintained.

In summary our results are consistent with a "dioxetanone" mechanism for the luminescent oxidation of Renilla luciferin in solution (15).

Acknowledgements. The authors would like to thank Richard McCann and Dr. John Matthews for supplying pure luciferase, and NSF for financial support through grants BMS 74-06914 and BMS 72-02462.

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