Vol. 81, No. 3, 1978
April 14, 1978

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 987-992

USE OF ^{14}C-CARBOXYL-LUCIFERIN IN DETERMINING THE MECHANISM OF THE FIREFLY LUCIFERASE CATALYZED REACTIONS

Jon Wannlund[1], Marlene DeLuca[1], Kerstin Stempel[2], and Paul D. Boyer[2]

[1]Department of Chemistry, University of California, San Diego, La Jolla, California 92093

[2]Department of Chemistry and Molecular Biology Institute, University of California, Los Angeles, California 90024

Received February 27, 1978

SUMMARY: The use of ^{14}C-carboxyl-labelled luciferin as a substrate for the firefly luciferase catalyzed reaction produces $^{14}CO_2$ as a product. We have studied this reaction in the presence of $^{17}O_2$ and $H^{18}OH$, using an excess of luciferin over luciferase. The initial collection of CO_2 contained close to one oxygen from $^{17}O_2$ for each molecule of $^{14}CO_2$ derived from luciferin, which is consistent with a cyclic peroxide mechanism. About half of the $^{14}CO_2$ remained bound to the enzyme and was collected after acidification of the medium. This CO_2 contained less than 0.1 of an atom of oxygen from $^{17}O_2$ for each molecule of $^{14}CO_2$ derived from luciferin. Exchange of medium CO_2-HCO_3^- with water was not sufficiently great to account for the loss of any ^{17}O previously incorporated. The most likely explanation appears to be a preferential exchange of oxygens of enzyme-bound CO_2 with water oxygens. Such exchange, and dilution of CO_2 from luciferin by medium CO_2, may explain previous results in which little incorporation of atmospheric oxygen was noted.

Firefly luciferase in the presence of Mg-ATP and molecular oxygen catalyzes the oxidation of D-luciferin with the production of CO_2 and light. The source of the oxygen atoms in the CO_2 has been controversial (1-5). DeLuca and Dempsey originally reported the incorporation of oxygen from $H^{18}OH$ and with negligible incorporation from $^{18}O_2$ (1,2). Recently, Shimomura et al. reported oxygen incorporation from $^{18}O_2$ as well as from $H^{18}OH$ (3). One factor that may have contributed to lack of observable incorporation of atmospheric oxygen in the experiments of DeLuca and Dempsey (1,2) is dilution of enzymically produced CO_2 by CO_2 derived from the medium CO_2 and HCO_3^-. Another is possible exchange of CO_2 with water oxygens at the enzyme site. DeLuca and Dempsey (1,2) used a larger excess of enzyme over substrate than did Shimomura et al. (3), conditions where the quantum yield is 0.88 (6). This could have favored exchange of

0006-291X/78/0813-0987$01,00/0

CO_2 oxygens with water oxygens. In an effort to resolve apparent discrepancies we have repeated experiments using ^{14}C-carboxyl-labelled luciferin, which allows quantitation of the $^{14}CO_2$ produced from the bioluminescent reaction, and with excess luciferin over luciferase. Also, we have measured the extent of exchange between medium CO_2 and HCO_3^- with HOH during the experiment. Results of these experiments demonstrate incorporation of both ^{17}O from $^{17}O_2$ and ^{18}O from $H^{18}OH$ into CO_2 released from the luciferase and an apparent capacity of luciferase to bind CO_2 from luciferin in a manner that promotes its exchange with water.

MATERIALS AND METHODS

$^{17}O_2$ gas, 92 atom % excess (a.p.e.) was obtained from Miles Laboratories, Elkhart, Ind. and its ^{17}O content verified by conversion to CO_2 and measurement of the mass 45/44 ratio.

Crystalline firefly luciferase was prepared as described previously (7).

2-cyano-6-hydroxybenzothiazole was synthesized according to the method of Seto et al. (8). ^{14}C-carboxyl-labelled DL cystine was purchased from New England Nuclear Corp.

The ^{14}C-cysteine was prepared by the electrolytic reduction of the ^{14}C-cystine as described by Dohon and Woodward (9). ^{14}C-carboxyl labelled DL-luciferin was synthesized by adding ^{14}C-carboxyl labelled DL cysteine to 2-cyano-6-hydroxybenzothiazole. 17 µmoles of 2-cyano-6-hydroxybenzothiazole in 1 ml of CH_3OH was added to 10 µmoles ^{14}C-cysteine in 1 ml of 0.23 M K_2CO_3, pH 6.5. Both solutions were bubbled with nitrogen prior to mixing and the reaction was allowed to proceed for 30 min in the dark in a nitrogen atmosphere. The pH was adjusted to 4.5 and the ^{14}C-DL-luciferin was purified by chromatography on a 1 x 40 cm column of G-25 Sephadex (fine). The column was eluted with 0.04 M NaCl and 0.01 M NaAc, pH 4.5. The fractions containing ^{14}C-DL-luciferin were combined and extracted with 1 volume of ethyl acetate. The ethyl acetate was removed by vacuum and the ^{14}C-DL-luciferin was dissolved in water. This luciferin gave the same amount of total light in the bioluminescent assay as a comparable amount of cold synthetic DL-luciferin and all of the counts migrated with luciferin on TLC using the solvent C_2H_5OH:1 M ammonium acetate (7:3). The specific activity of the purified luciferin was 1600 cpm/nmole.

The bioluminescent reaction was carried out as described previously (5) with the following modifications. After the first collection of CO_2, the reaction mixture was thawed and refrozen in Dry Ice-isopropanol and a second collection of CO_2 was made. This procedure was repeated and a third collection of CO_2 was obtained. The total CO_2 collection at each stage was measured in a calibrated manometer and when necessary for analysis it was dosed with a known amount of carrier CO_2. Following the third collection 1 ml of reaction mixture was transferred to a 2-armed vessel, frozen at Dry Ice temperature, the vessel evacuated, and the contents thawed and acidified with 5 g of $KHSO_4$. The large amount of $KHSO_4$ reduced exchange of medium CO_2 with water, presumably by the increase in surface area promoting CO_2 escape. The CO_2 liberated was collected

TABLE I. QUANTITATION OF $^{14}CO_2$ PRODUCED DURING BIOLUMINESCENCE[a]

Experiment	Collection	nmoles CO_2 from $[^{14}C]$-LH_2[b]	% of Total $^{14}CO_2$ Recovered[b]
1	1	15.2	18.7
	2	9.9	12.1
	3	5.1	6.2
	Acidification	51.0	62.9
2	1	27.8	33.1
	2	13.3	15.8
	3	5.4	6.4
	Acidification	37.5	44.6

[a]Reaction was carried out in 0.025 M glycylglycine buffer pH 7.8. There was a four-fold molar excess of DL-luciferin (324 nmoles) over enzyme (82 nmoles). After mixing the reaction was shaken vigorously for 1-1/2 min prior to freezing. $H^{18}OH$ was present at 2.8 a.p.e. in both experiments. In experiment 1, $^{17}O_2$ was 8.7 a.p.e., in experiment 2, $^{17}O_2$ was 75.9 a.p.e.

[b]The nmoles of $^{14}CO_2$ derived from ^{14}C-LH_2 were calculated from the known specific activity of the ^{14}C-LH_2 and the total counts obtained from the $^{14}CO_2$ by trapping and counting in Oxyfluor. (See Methods.)

and analyzed. This provided a measure of the maximum amount of exchange between medium bicarbonate and $H^{18}OH$.

The ^{14}C-luciferin lost from the medium was determined by taking an aliquot of the reaction mix before and after the bioluminescence and counting it in Oxifluor-CO_2, obtained from New England Nuclear Corp. After mass analysis the remaining CO_2 was recovered from the mass spectrometer by freezing out in a liquid N_2 trap; from the amount of CO_2 and its ^{14}C content the fraction of CO_2 used for mass analysis that was derived from ^{14}C-luciferin was calculated.

Mass spectrometer measurements were made as previously described (5).[1]

RESULTS AND DISCUSSION

The data from two representative experiments carried out in the presence of $^{17}O_2$ and $H^{18}OH$ are given in Table I and II. Table I reports appearance of

[1]A minor point about calculation of the atom fraction of ^{18}O in CO_2, designated C, from mass 46/44 ratios, designated R, needs to be clarified. With regard to our previous paper (5), Shimomura et al. (3) state, "It should be pointed out here that the calculation used in previous reports i.e. $C = R/(2 + R)$, results in an error that increases as the value of R increases." This statement is incorrect. From simple statistical considerations the probability of mass 48 is C^2, of mass 46 is $2C(1-C)$, and of mass 44 is $(1-C)^2$. Thus $R = 2C(1-C)/(1-C)^2$, from which $C = R/(2 + R)$.

TABLE II. INCORPORATION OF ^{17}O INTO $^{14}CO_2$ PRODUCED DURING BIOLUMINESCENCE[a]

Experiment	CO_2 Collection	Mass 45/44 Observed	45/44 Theoretical	Atoms O from $^{17}O_2$
1	1	0.252	0.269	0.93
	2	0.095	0.092	1.03
	Acidification	0.027	0.261	0.07
2	1	2.13	2.37	0.89
	2	0.534	0.775	0.689
	Acidification	0.214	2.45	0.087

[a]Conditions are the same as described in Table I. The theoretical number for mass 45/44 was calculated from the amount of $^{14}CO_2$ collected and the enrichment of the $^{17}O_2$, assuming incorporation of 1 oxygen from $^{17}O_2$.

^{14}C in CO_2 collected after the bioluminescent reaction. As can be seen, even with three collections of CO_2 by the freezing-evacuation-thawing cycle a large amount of $^{14}CO_2$ produced by bioluminescence remains in the medium. This CO_2 is released upon acidification. As noted later in this paper all or most of this residual CO_2 appears to be found to the enzyme. In both experiments reported in Table I the amount of $^{14}CO_2$ remaining in the medium is less than the amount of enzyme present.

Table II shows the caluclated incorporation of oxygen from $^{17}O_2$ into the $^{14}CO_2$ from luciferin. In experiment I close to one atom of ^{17}O was incorporated into each $^{14}CO_2$ derived from luciferin in the first collection. The third collection of CO_2 did not produce enouth gas to measure accurately. In contrast to the $^{14}CO_2$ of the first collection, the $^{14}CO_2$ that is released upon acidification contained less than 0.1 of an atom of 17-oxygen per mole of $^{14}CO_2$. In experiment II similarly there is incorporation of ^{17}O in both of the first two samples of $^{14}CO_2$ collected and again less than 0.1 of an oxygen from $^{17}O_2$ into each $^{14}CO_2$ derived from luciferin and liberated by acid from the remaining solution.

In these experiments no attempt was made to have the initial reaction

medium free of CO_2 and HCO_3^-. The amount of CO_2 in collected samples arising from the initial medium CO_2-HCO_3^- was 30 to 100 times as great as that derived from the [^{14}C]luciferin. Exchange between water and medium CO_2 and HCO_3^- was small in these experiments. The amount of ^{18}O in the CO_2 collected by acidification of the reaction mixtures was only about 4% of that expected assuming 1 oxygen from water had exchanged with the medium CO_2-HCO_3^-. Thus any $^{14}CO_2$ that appears as dissolved CO_2 or as HCO_3^- in the medium would not be expected to have undergone significant oxygen exchange.

The amount of ^{17}O in the $^{14}CO_2$ derived from luciferin decreased from about 1 in the first collection to <0.1 in the CO_2 collected after acidification. As noted above, exchange of medium CO_2-HCO_3^- with water is small. Thus, if ^{17}O was lost by exchange, such exchange must have occurred with CO_2-HCO_3^- localized on the enzyme, very likely at the catalytic site. That luciferase does retain a tightly-bound CO_2 has been shown in separate experiments in which the $^{14}CO_2$-enzyme complex was isolated after passage through Sephadex. Experiments are in progress to further characterize the nature of the enzyme bound CO_2.

The present results appear to clarify uncertainties from previous experiments (1-3). Use of $^{17}O_2$ allowed sensitive detection of low levels of atmospheric oxygen in the CO_2 collected and, more importantly, use of ^{14}C-luciferin allowed quantitation of the number of atmospheric oxygens appearing in each CO_2 derived from luciferin. This approached 1 in the first collection, and as noted by Shimomura et al. (3), favors a reaction sequence involving a cyclic peroxide. A particularly interesting observation is the large fraction of total $^{14}CO_2$ from luciferin that is retained on the enzyme, and the apparent exchange of oxygens of this CO_2 with water oxygens. Such oxygen exchange and the unknown fraction of total CO_2 used for analysis that was derived from luciferin, as well as the sensitivity of the detection procedures used, could readily account for the lack of detection of incorporation of atmospheric oxygen into CO_2 in the experiments of DeLuca and Dempsey (1,2). As mentioned previously, they used higher ratios of luciferase to luciferin than in the

experiments of Shimomura et al. (3) or those reported here.

The above interpretations are based on the resonable assumption that the enzyme bound $^{14}CO_2$ from luciferin originally contained oxygen from the $^{17}O_2$ and lost this oxygen by exchange. Present data, however, do not allow elimination of the possibility that this CO_2 originally derived some water oxygens as part of the bioluminescent reaction. In this connection, the behavior of cypridina luciferase (5) should be reexamined with ^{14}C-labelled substrate. A considerable amount is yet to be learned about the products produced from luciferin in bioluminescence.

References

1. DeLuca, M. and Dempsey, M. E., Biochem. Biophys. Res. Comm. 40, 117-122 (1970).
2. DeLuca, M., Dempsey, M. E., in Chemiluminescence and Bioluminescence, Cormier, M. S., Hercules, D. M., and Lee, J., eds., Plenum Press, New York, pp. 345-355 (1973).
3. Shimomura, O., Goto, T., and Johnson, F. H., Proc. Nat. Acad. Sci., USA 74, 2799-2802 (1977).
4. McCapra, F., and Chang, Y. C., J. Chem. Soc. Comm. 1011 (1967).
5. Tsuji, F. I., DeLuca, M., Boyer, P. D., Endo, S., and Akutagawa, M., Biochem. Biophys. Res. Comm. 74, 606-613 (1977).
6. Seliger, H. H., and McElroy, W. D., Arch. Biochem. Biophys. 88, 136-140 (1960).
7. Green, A. A., and McElroy, W. S., Biochim. Biophys. Acta 20, 170-176 (1956).
8. Seto, S., Ogura, K., and Nishiyama, Y., Bull. Chem. Soc., Japan 36, 331-333 (1963).
9. Dohan, J. S., and Woodward, G. E., J. Biol. Chem. 129, 393-397 (1939).