MECHANISM OF THE LUMINESCENT OXIDATION OF CYPRIDINA LUCIFERIN[1]

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SUMMARY. By analysis of the products, including CO_2 labelled with ^{18}O, the aerobic oxidation of Cypridina luciferin with luciferase is shown to proceed mostly via an intermediate having a four membered peroxide ring, yielding equimolar amounts of oxyluciferin and carbon dioxide, whereas oxidation of the same luciferin in organic solvents proceeds predominantly via a different pathway, yielding almost equimolar amounts of etioluciferin and -keto- - methyl-n-valeric acid.

INTRODUCTION

In an aqueous medium with Cypridina luciferase, or in an aprotic solvent without luciferase, the aerobic oxidation of Cypridina luciferin is accompanied by emission of light (1,2,3), which for convenience we will call "bioluminescence" and "chemiluminescence", respectively. The products of the bioluminescent reaction have been reported as oxyluciferin and CO_2 (4,5).

On the basis of reactions of analogs, a mechanism of chemiluminescence of both Cypridina and firefly luciferins, involving the formation and decomposition of a four membered peroxide ring, has been suggested (6,7,8); the same mechanism has been thought to apply to bioluminescence of both systems (9,10). In regard to bioluminescence, however, the hypothesis has become questionable through the discovery by DeLuca and Dempsey (11) that molecular ^{18}O does not become incorporated into the CO_2 product of firefly luciferin oxidation. Moreover, in the chemiluminescence of Cypridina luciferin, we have obtained evidence which also contradicts the hypothesis, through the formation of etioluciferin (12) possibly accompanied by α-keto-β-methyl-n-valeric acid. The present study was undertaken to clarify these matters.

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MATERIALS AND METHODS

All organic solvents were "Spectroquality", from Matheson, Coleman and Bell. Diglyme (Bis(2-methoxyethyl)ether) was used immediately after filtration through alumina. The Cypridina luciferin used was the dihydrobromide, 3 times recrystallized, and almost colorless. Cypridina luciferase was of the same purity as used previously (13). The $^{18}O_2$ (89 atom % excess) was obtained from Isomet Corporation, Oakland, N. J., and $H_2{}^{18}O$ (4.23 atom % excess) from Miles Laboratories, Elkhart, Indiana. Quantum yields were measured, using Cypridina bioluminescence as the secondary standard (12, 14), by a photomultiplier, Hamamatsu R-136, of which the spectral distribution of sensitivity had been calibrated.

The contents of oxyluciferin and etioluciferin in reaction mixtures were calculated from UV absorbance measured in ethanolic 0.1 N HCl at two wavelengths, 310 nm and 333 nm, using the molecular extinction coefficients of pure oxyluciferin dihydrochloride (M.W. 478); 13,600 (310 nm) and 14,900 (333 nm), pure etioluciferin dihydrochloride (M.W. 382); 22,500 (310 nm) and 7,450 (333 nm). Some error would be expected due to possible presence of minor by-products, however, such error is expected to be relatively small and should not affect the conclusion of the present study.

The content of α-keto acid was estimated by the 2,4-dinitrophenylhydrazine colorimetric method modified by Rohrbough (15), with slight changes in the procedure: by the replacement of 1 ml of 80% pyridine with the same volume of methanol, 25% KOH instead of 33% KOH, and measurement of color at 560 nm instead of 480 nm. A standard calibration curve was obtained using α-keto-β-methyl-n-valeric acid, sodium salt (95%, Sigma Chemical Company).

In the study of incorporating ^{18}O into CO_2, 1.4 mg of luciferase dissolved in 4.5 ml of 0.02 M glycylglycine, pH 7.8 (11), containing 0.04 M NaCl, prepared with regular water or $H_2{}^{18}O$, and 2 mg of luciferin dihydrobromide dissolved in 0.12 ml of 60% methanol, were placed separately in a reaction vessel, frozen in dry ice-acetone, and the vessel evacuated. After introduction of $^{16}O_2$ or $^{18}O_2$, the contents were thawed and warmed to 20°C, and the two solutions were

mixed with shaking. The emission of bright light almost ceased in 30 seconds of reaction time, after which the vessel was frozen as before. It was evacuated through a trap in dry ice-acetone and a second trap in liquid nitrogen. The CO_2 collected in the latter trap was analyzed mass spectrometrically for the ratio of m/e 44 and m/e 46 by Morgan-Schaffer Corporation, Montreal.

For the reaction of diglyme, luciferin dissolved in 5 ml of diglyme was mixed with 0.07 ml of 0.3 M sodium acetate, pH 5.6, in pure oxygen. After 15 minutes of reaction, the contents were treated the same as above.

RESULTS AND DISCUSSION

Analyses for the luminescent oxidation products of Cypridina luciferin in several solvent systems are summarized in Table I. In the luciferase catalyzed luminescence, oxyluciferin is the main product, whereas in chemiluminescence the products were always mixture of oxyluciferin, etioluciferin and α-keto acid, with the latter two compounds in approximately equimolar amounts. Moreover, in either diglyme or acetone, which gave exceptionally high quantum yields of chemiluminescence, etioluciferin rather than oxyluciferin was a predominant product. The possible hydrolysis of oxyluciferin to etioluciferin was found to be negligible under the conditions of Table I.

Oxyluciferin and etioluciferin were confirmed by TLC (silicic acid - water saturated n-butanol). The presence of α-keto-β-methyl-n-valeric acid in each reaction mixture was proved as follows. First the keto acid was extracted from acidic aqueous solutions with ether, and was then converted to 2,4-dinitrophenylhydrazone, and the hydrazone confirmed by mass spectrometry or by TLC (silicic acid - water saturated n-butanol) in comparison with an authentic sample. It is noteworthy that even the luciferase-catalyzed reaction gave the keto acid, although the amount was small.

The source of oxygen in the CO_2 product was studied with ^{18}O, as shown in Table II. In the luciferase-catalyzed reaction, oxygen is incorporated into CO_2 mostly from molecular O_2, and only a small part from H_2O. No definite explanation can be given for the unexpected absence of CO_2 in the

TABLE I

PRODUCTS OF THE LUMINESCENT OXIDATION OF CYPRIDINA LUCIFERIN.[1]

Solvents[2] (5 ml)	Reaction time (hours)	Oxy-luciferin (mole %)	Etio-luciferin (mole %)	-Keto acid (mole %)
1.5 mg Luciferase in buffer[3]	0.05	86	10	10
Diglyme + 0.1 ml buffer pH 5.6[4,5]	2	34	51	?[6]
Acetone + 0.1 ml buffer, pH 5.6[4]	2	31	57	?[6]
Pyridine + 50 µl buffer, pH 4.6[4]	4	23	65	67
DMSO + 150 µl buffer, pH 4.6[4]	4	25	69	67
DMSO + Potassium tert-butoxide (10 mg)[7]	0.1	64	35	31
50% Methanol + 50 µl of 28% ammonia	24	55	29	29
H_2O + 5 mg $NaHCO_3$	24	27	49	51

1) In each 5 ml of solvent, 1 to 1.5 mg of luciferin dihydrobromide was reacted at 22°C.
2) In sequence of greater quantum yield. The quantum yields at 22°C for the first five solvents with optimum amounts of luciferin and other additives, typically with 30 ul of buffer and 10 ug of luciferin were: 0.26, 0.023, 0.021, 0.002, 0.0008.
3) 0.05 M Sodium phosphate containing 0.1 M NaCl, pH 6.8. After the reaction, luciferase was inactivated by addition of ethanol and removed by centrifugation.
4) 0.3 M Sodium acetate buffer.
5) Diglyme was first used by Goto et al. (3,9) as an efficient solvent in Cypridina chemiluminescence.
6) Unreliable results because of residual carbonyl due to solvent.
7) Two drops of concentrated HCl were added at the end of the reaction.

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diglyme solvent, although it could be due to insufficient water in the reaction mixture to hydrolyze an intermediate.

The foregoing data suggest that at least 3 different pathways are involved in the luminescent oxidation of luciferin, as illustrated in Fig. 1.

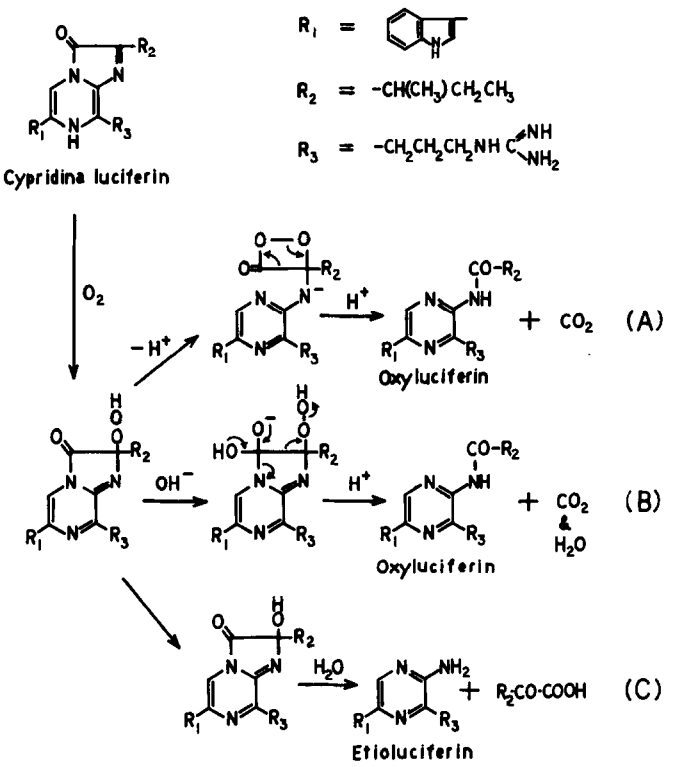

Fig. 1. Pathway of the oxidative degradation of Cypridina luciferin.

Pathway A was first suggested by McCapra and Chang (6) for the chemiluminescence of a synthetic analog, pathway B is the same mechanism that DeLuca and Dempsey (11) proposed for the bioluminescence of the firefly, and pathway C is proposed herewith to explain the formation of etioluciferin and the α-keto acid. In pathway C, an intermediate tertiary alcohol is formed probably by a base-catalyzed oxygen elimination or by a reaction of hydroperoxide with unreacted luciferin.

From the data of Tables I and II, in the bioluminescent reaction, the ratio of luciferin oxidized by pathways A, B and C is approximately 7:1:1, whereas in the chemiluminescent reaction in diglyme or in acetone, more than half of the luciferin is oxidized by pathway C, whereas a smaller fraction goes by pathways A or B, or by both, without distinguishing between these two.

TABLE II

INCORPORATION OF OXYGEN INTO CO_2 DURING THE LUMINESCENT OXIDATION OF *CYPRIDINA* LUCIFERIN.[1]

Conditions	^{18}O in CO_2 (Atom % excess)	Oxygens[2] Incorporated
$H_2{}^{16}O + {}^{18}O_2$ (89 atom % excess)	35.8	0.80 from O_2
$H_2{}^{18}O$ (3.76 atom % + $^{16}O_2$ excess)	0.29	0.15 from H_2O
$H_2{}^{18}O$ (3.76 atom % + $C^{16}O_2$[3] excess)	0.035	0.02 from H_2O
Diglyme + Acetate buffer + O_2	No CO_2 detected[4]	

1) Luciferase-catalyzed, except the last condition. See "Materials and Methods" for details.
2) Half of the oxygens in the total CO_2 is taken as 1.0.
3) A control run, in which luciferin was omitted and CO_2 produced by heat-decomposition of $NaHCO_3$ was admitted immediately before introduction of O_2.
4) The same negative result when 1 drop of concentrated H_2SO_4 was added after the reaction.

Because the maximum quantum yield of bioluminescence is approximately 0.3 (12), pathway A must be a light-emitting reaction in this system. No conclusive evidence is available, however, showing whether pathway B and C are completely dark or weakly luminescent.

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