THE ENZYME CATALYZED OXIDATION OF *CYPRIDINA* LUCIFERIN

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Received March 26, 1968

Evidence has been found which supports the pathway presented in Fig. 1, for the catalytic oxidation of *Cypridina* luciferin (L-H) by *Cypridina* luciferin oxygenase (CLO). This mechanism is in accord with the conclusions of McCapra and Chang (1967) for the chemiluminescent oxidation of a Cypridina luciferin analogue.

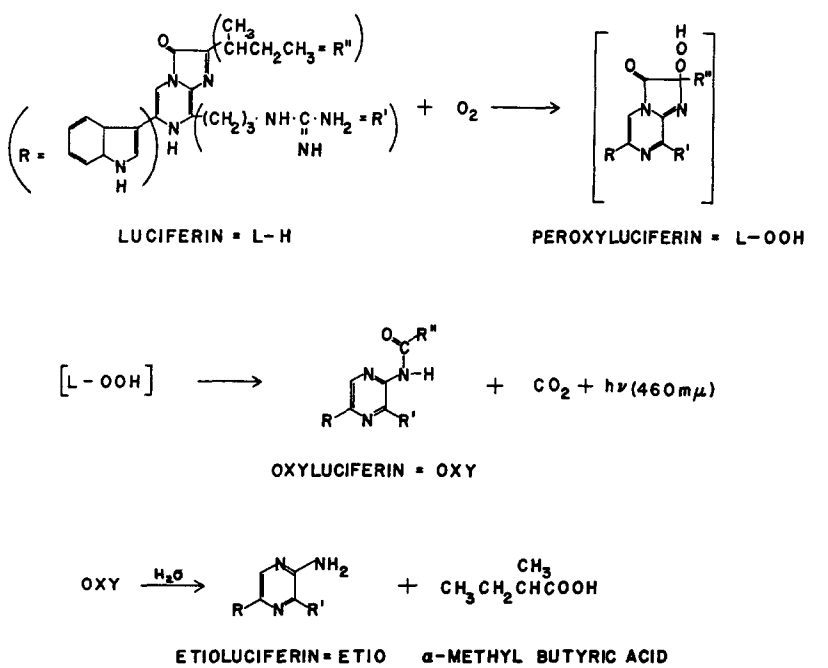

Fig. 1. Pathway for the degradation of *Cypridina* luciferin.

CLO in a crude aqueous extract, prepared from 10% by weight of dried organisms, was precipitated at 40 to 70% saturation with ammonium sulfate, then dialyzed, adsorbed on DEAE cellulose,

eluted by a sodium chloride gradient, and purified further by a Bio-Gel P-200 column and a second DEAE column. The pooled most active fractions, representing 20% of the total activity of the crude extract, had a specific activity 137 times greater than the crude extract. An ultracentrifuge run of this material revealed a single, slightly unsymmetrical peak with S = 3.72. Shimomura et al. (1961) reported S values of 3.93 and 4.30, while Tsuji et al. (1961) reported S = 4.58. Disc gel electrophoresis showed two dark and two faint bands.

The stoichiometry of luciferin oxidized to O_2 consumed was determined by means of the Clark-Type Oxygen Electrode under a variety of conditions (TABLE I). Determinations were also made with Warburg manometers; representative data are given (TABLE II).

TABLE I

OXYGEN ELECTRODE DATA

Temp °C	M Na-phos., pH 6.7	M NaCl	Act. Units* of CLO x 10^{-6}	mμmole L-H used	mμmole O_2 consumed	L-H/O_2
30	0.1	0.2	0.455	55.	57.	0.97
30	0.1	0.2	0.228	110.	110.	1.00
23	0.1	0.2	0.130	270.	294.	0.92
1	0.001	0.002	0.130	100.	88.	1.10
1	0.01	0.02	0.130	100.	88.	1.10
1	0.1	0.2	0.130	100.	120.	0.84

*The specific activity of the CLO preparation (pooled most active fractions off the second DEAE column) used in the experiments presented in this paper, was 1.13 x 10^6 activity units (arbitrary) per mg. protein. 1 Act. Unit = 5.86 x 10^{-3} mμmoles of O_2 consumed per minute = 5.94 x 10^9 photons emitted per second.

TABLE II

WARBURG DATA

Flask	μmole L-H	μmole O_2 Before acid	Temp. 19.7°C Ratio (μmole L-H)/ (μmole O_2)	μmole CO_2 After acid	μmole CO_2 corrected	Ratio (μmole L-H)/ (μmole CO_2)
9	3.02	2.97	1.02	----	----	----
10	3.02	1.61*	1.88	1.44	2.58**	1.17
11	----	----	----	0.49	----	----

Only flask 9 had 10% KOH in the center well. Each flask contained 1.25×10^6 activity units of CLO in 0.05 M Na-phosphate, pH 6.8, in 0.01 M NaCl. The side-arms of flasks 9 and 10 had 1.71 mg. L-H·2HBr in 30% aqueous methanol; the side-arm of flask 11 contained HCl equivalent to the acidity of the luciferin. "Before acid" indicates the results at the end of the period of oxygen consumption. H_2SO_4 was then added to the side-arms of flasks 10 and 11. "After acid" indicates the results of adding the H_2SO_4 to the spent reaction mixture, bringing the pH to 1.5.

* uncorrected for CO_2 liberation.

** CO_2 liberated before acidification was 1.63 μmole.

The production of CO_2 was not detected in preliminary experiments by Johnson et al. (1962) and their analysis of manometric data, obtained without KOH in the vessel inset, indicated a stoichiometry of L-H/O_2 = 2, as in the present data with the KOH omitted (TABLE II).

After oxidation was complete in the Warburg flasks, the solutions were dehydrated and the residues extracted with methanol. Chromatography on silica gel with butanol:glacial acetic acid: H_2O (4:1:1) as a solvent, gave only one fluorescent spot, in each case, which had an R_f corresponding to oxyluciferin, formerly called oxyluciferin A, Kishi et al. (1966).

A direct test for CO_2 was run by placing a saturated solution of $Ba(OH)_2$ in the center well of the Warburg flask, flushing with O_2 gas let in through the side-arm vent, stoppering

the flask and then mixing the substrate (side-arm) with the enzyme (cup). A blank with an amount of HCl equivalent to luciferin·2HBr was also run. $BaCO_3$ was formed in sufficient excess in the substrate plus enzyme flask compared to the blank that there was no doubt of a positive test for CO_2 production.

The oxygen electrode and Warburg experiments led to the conclusions that:

1) $L\text{-}H/O_2 = 1$
2) CO_2 was formed together with oxyluciferin
3) $L\text{-}H/CO_2 = 1$

Present evidence indicates that α-methyl butyric acid rather than α-keto-β-methyl valeric acid as reported by Kishi et al. (1965), is formed when oxyluciferin is hydrolyzed. Chromatography on silica gel of the ether extracted organic acid, formed on acid hydrolysis of the product of the catalytic oxidation of luciferin is presented in Fig. 2.

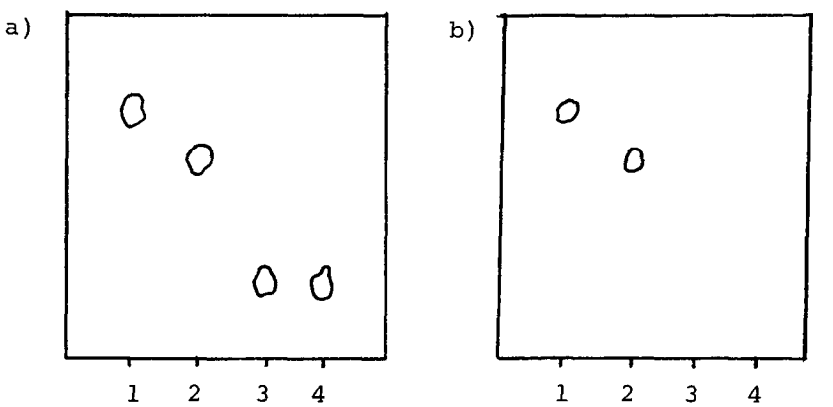

Fig. 2. Chromatography of organic acid formed on hydrolysis of oxyluciferin.

1) α-keto isocaproic acid
2) α-keto isovaleric acid
3) isobutyric acid
4) 640 mμm L-H·2HCl + 7.65 x 10^6 ACT. UNITS CLO in .01 M KPO_4 pH 6.8 + .02 M NaCl (ether extract of ACIDIFIED SOLUTION).
a) Spray used was .04% Bromo cresol green aq. soln., pH adjusted to 7 (Organic acid indicator).
b) 2,4 dinitrophenylhydrazine, .1% soln. in 2 N HCl (α-keto acid indicator).

Solvent for a and b was butanol + 1% NH_3. After running the chromatograms, the ammonium salt of each acid was converted to the organic acid + $NH_3\uparrow$ by heating for 5 minutes at 100°C.

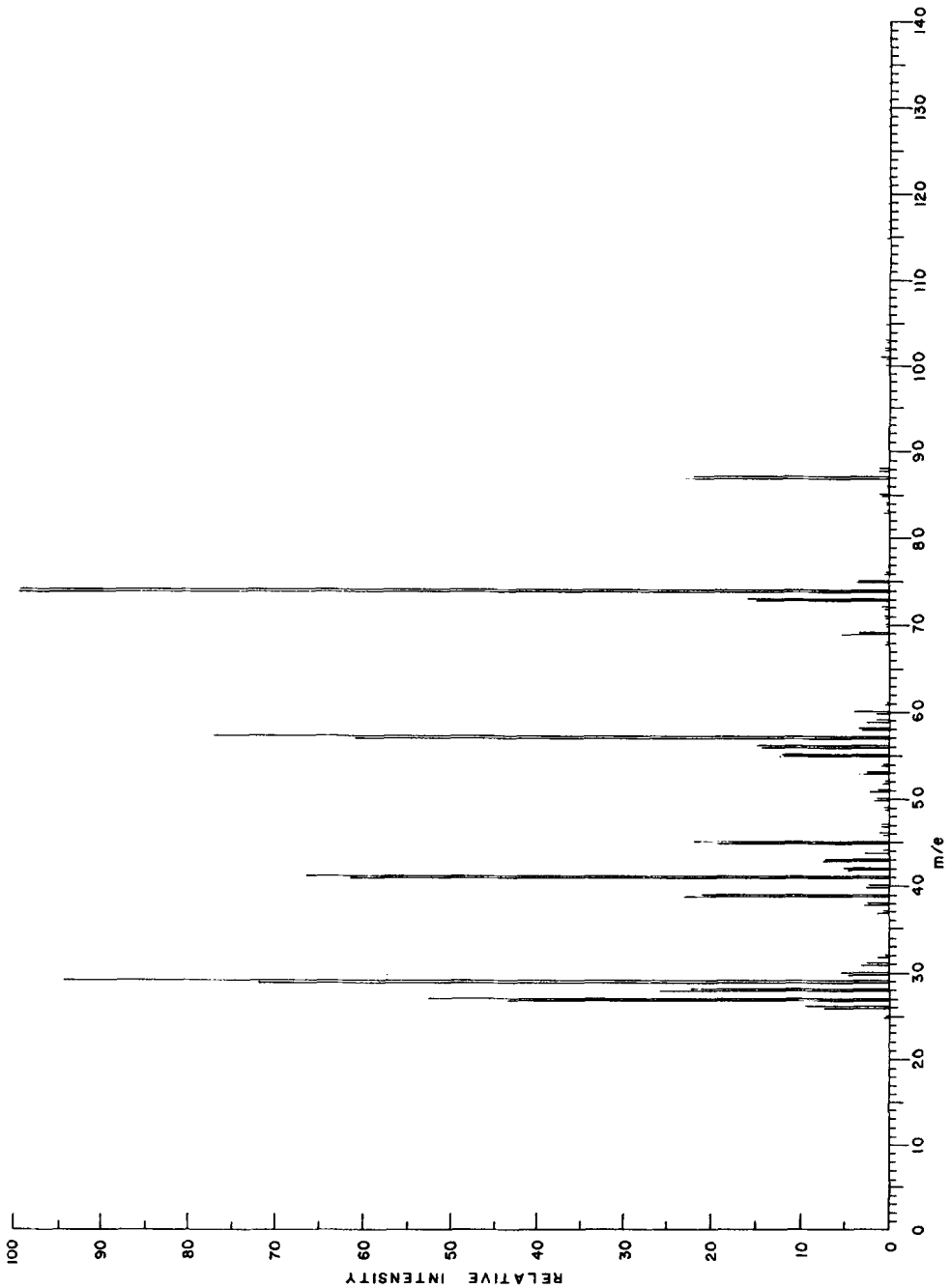

Fig. 3. Mass spectra of organic acid isolated from the hydrolysate of oxyluciferin (lines at left of each m/e number; Morgan-Schaffer Corp.) and of α-methyl butyric acid (lines at right; American Petroleum Institute, 1951).

Mass spectral analysis of the acid gave a fragmentation pattern which is similar to the published spectrum for α-methyl butyric acid, Fig. 3.

The structure of oxyluciferin given in Fig. 1 is supported by its synthesis from the anhydride of α-methyl butyric acid and etioluciferin in pyridine. The product was isolated on a CM-cellulose column followed by TLC on silica gel with butanol: glacial acetic acid:H_2O (4:1:1) as solvent. The spot with an R_f similar to that of oxyluciferin was removed and treated with methanol. TLC on silica gel of this extract gave R_f's similar to natural oxyluciferin in butanol saturated with H_2O, and in butanol + 2% NH_3. The U.V. spectrum of this material was exactly similar to that of natural oxyluciferin.

We therefore conclude that Cypridina luciferin oxygenase catalyzes the reaction,

$$\text{Luciferin} + O_2 \longrightarrow \text{Oxyluciferin} + CO_2 + h\nu$$

$$\lambda_{max} = 460\ m\mu$$

Acknowledgements

The author is grateful to Dr. Frank Johnson and Dr. Osamu Shimomura for their support and advice throughout this work.

This research was supported by Grant GM 962 from the U.S. Public Health Service, NSF Grant GB-6836 and ONR contract 4246(00).

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