MECHANISM OF OXIDATION IN FIREFLY LUMINESCENCE[1]

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Summary

The oxidation of luciferin catalyzed by firefly luciferase results in the emission of light. Molecular oxygen is required and CO_2 is released. Using $^{18}O_2$ and $H^{18}OH$ we have shown that one of the oxygens of the released CO_2 originates from water. Neither of the oxygens of CO_2 is derived from molecular oxygen. A mechanism for the oxidation is proposed.

Introduction

The reactions catalyzed by firefly luciferase are summarized below.

(a) $LH_2 + ATP + E \overset{Mg^{++}}{\rightleftharpoons} E{\cdot}LH_2AMP + PPi$

(b) $E{\cdot}LH_2AMP + O_2 \longrightarrow E + \text{product} + CO_2 + AMP + h\nu$

Reaction (a) is the activation of D-luciferin (LH_2) resulting in formation of enzyme bound luciferyladenylate ($E{\cdot}LH_2AMP$). Reaction (b) requires stoichiometric amounts of molecular oxygen (1) and the quantum yield is about 0.9 (2). Plant *et al.* (3) using ^{14}C-carboxyl labeled luciferin in the presence of excess enzyme, ATP and O_2, demonstrated the quantitative liberation of $^{14}CO_2$ from the luciferin during the reaction. Attempts to isolate the product of reaction (b) have failed probably because the compound is very unstable (3).

Studies of the chemiluminescence of LH_2AMP and various analogs (4,5,6) led to the proposal that the chemiluminescent product was the

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thiazolinone (V, Fig. 1). The mechanisms proposed for the chemiluminescent oxidation as well as the bioluminescent oxidation (3), have an intermediate four membered peroxide ring which decomposes with liberation of CO_2. If these mechanisms are correct then one of the oxygens in the CO_2 must come from O_2. The experiments reported here are designed to test this possibility.

Experimental

Firefly luciferase was isolated and crystallized three times as described previously (7). D-LH_2 was prepared according to White et al. (8) by Dr. Peter Plant.

A solution (3.5 ml) containing ATP (12 μmoles), $MgCl_2$ (30 μmoles), D-LH_2 (33 mμmoles) and 75 μmoles glycylglycine buffer, pH 7.8, was placed along with several glass beads in one sidearm of a reaction vessel. Another solution (3.0 ml) containing luciferase (64 mμmoles) and 62.5 μmoles glycylglycine buffer, pH 7.8, was placed along with several glass beads in the other sidearm of the vessel. The vessel was attached to a high vacuum line and the solutions completely evacuated (i.e., all bubbling ceased). $^{18}O_2$ or $^{16}O_2$ (CO_2 free) was admitted to the vessel. The vessel was removed from the vacuum line and the two solutions mixed. After light emission ceased (15 seconds or less) the vessel was immediately reattached to the vacuum line and the contents frozen in liquid N_2. CO_2 released during the reaction was collected as usual (9). No gases other than CO_2 were detected by mass spectral analysis. Calculation of atom percent excess ^{18}O from mass 46/44 ratio determinations was as described previously (9).

Results and Discussion

The experimental conditions used here are essentially identical to those used by Plant et al., i.e., a large excess of enzyme ensuring a quantitative liberation of CO_2 from luciferin. Since the quantum

yield is approximately 0.9 the released CO_2 must be associated with the light emitting reactions rather than a dark reaction. The pH of the reaction was 7.8 where essentially all of the light emitted is yellow-green.

The data in Table I demonstrate that one oxygen in the CO_2 released during light emission arises from water. The experiments in which light emission was allowed to proceed in an $^{18}O_2$ atmosphere ($H_2{}^{16}O$ medium)

Table I

SOURCE OF OXYGEN IN CARBON DIOXIDE RELEASED DURING LUCIFERASE CATALYSIS

Conditions	Atom % Excess ^{18}O in CO_2 (d)	Oxygens Incorporated
$H_2{}^{18}O$; $^{16}O_2$ (a)	0.634	1.0
$H_2{}^{18}O$; $C^{16}O_2$ (b)		
16 seconds	<0.005	0
30 seconds	0.010	<0.1
80 seconds	0.016	<0.1
$H_2{}^{16}O$; $^{18}O_2$ (c)	<0.005	0

(a) Reaction medium (see Experimental) prepared in $H_2{}^{18}O$ (Miles Laboratories, Elkhart, Indiana) distilled prior to use; enrichment of complete medium during the enzymic reaction was 1.25 atom % excess.

(b) Reaction medium (see Experimental) contained only glycylglycine buffer, pH 7.8, prepared in $H_2{}^{18}O$ (1.35 atom % excess). Times of exposure to $C^{16}O_2$, with vigorous shaking, are indicated. Total exposure times to CO_2 were 15-20 seconds in addition to the shaking times.

(c) Reaction medium (see Experimental) prepared in $H_2{}^{16}O$; $^{18}O_2$ (91.0 atom % excess; Miles Laboratories, Elkhart, Indiana) admitted after complete evacuation of the medium.

(d) Mass 46/44 measured with a Hitachi-Perkin-Elmer RMU-6D mass spectrometer; CO_2 admitted to the instrument via an all glass inlet system. Results are the mean of duplicate or triplicate experiments differing by less than 5%.

showed that no ^{18}O was incorporated into CO_2.

At pH 7 and below it is known that CO_2 is rapidly hydrated and dehydrated (10-13). It was important to the validity of the results presented here to determine the maximum amount of nonenzymic exchange of CO_2 oxygens with water oxygens in glycylglycine at pH 7.8. Control experiments (Table I) in which $C^{16}O_2$ was vigorously shaken with glycylglycine buffer prepared in $H_2{}^{18}O$ demonstrated that measurable but insignificant ^{18}O incorporation occurred during exposures lasting 2-5 times longer than the enzymic experiments (less than one minute total exposure time). Additional control experiments in which solid $NaHCO_3$ and deaerated glycylglycine buffer, pH 7.8, were mixed showed that no significant dehydration would be expected of CO_2 that may have been hydrated during the enzymic reactions. Furthermore, the partial pressure of CO_2 during the enzymic reactions was extremely low (micron range) and would not favor solution in the medium of appreciable quantities of CO_2.

These experiments confirm the observation (3) of CO_2 liberation during light emission and permit the formulation of a modified mechanism Fig. 1. Starting with luciferyladenylate (I) the first step is removal of a proton from the number 4 carbon atom of luciferin. The fact that the rate of the overall light emission is slower if deuterium is substituted for hydrogen at the number 4 carbon supports this conclusion (White and DeLuca, unpublished). The carbanion (II) then adds oxygen at carbon number 4. The peroxide (III) does not cyclize but OH^- is added at the carbonyl carbon. If the reaction medium is $H_2{}^{18}O$ this is the step where ^{18}OH is incorporated. Reaction 4 is considered to be a simultaneous dehydration, decarboxylation, and is the light emitting step.

If the reaction was carried out in the presence of $^{18}O_2$, it can be seen one of the oxygen atoms would appear in the keto group of compound V, while the other oxygen would be released to the medium water, no ^{18}O would be incorporated into the CO_2.

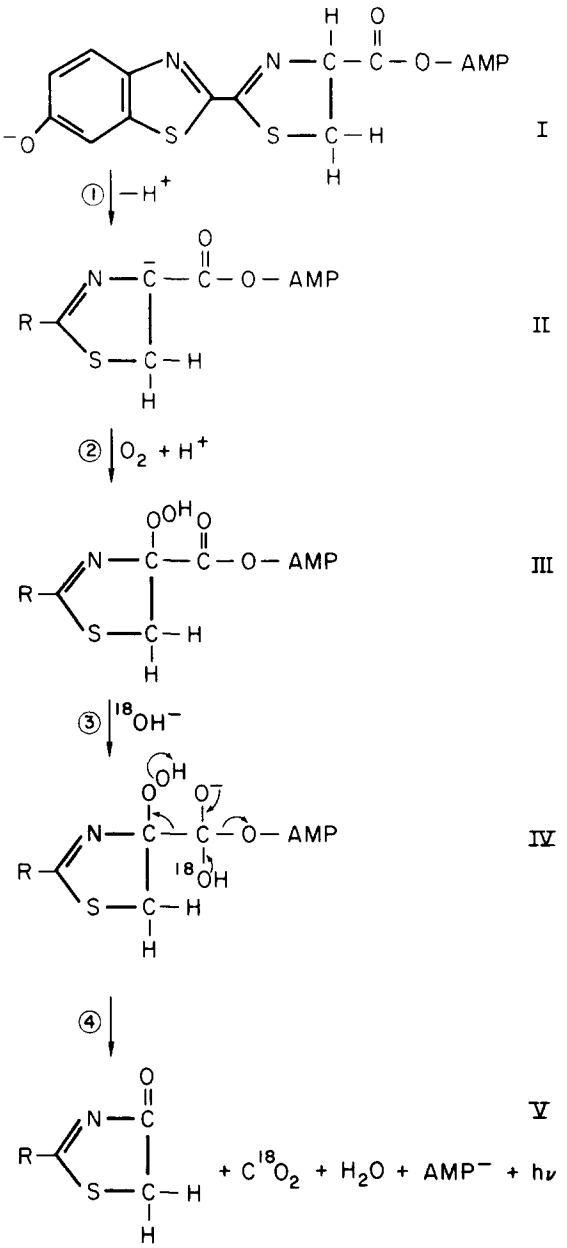

Fig. 1 Mechanism of oxidative and light emitting steps catalyzed by firefly luciferase.

A mechanism similar to this may apply to other bioluminescent and chemiluminescent systems.

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