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Yellow-Green and Red Firefly Bioluminescence from 5,5-Dimethyloxyluciferin

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Bioluminescence, the conversion of chemical energy into light by a living organism, is beautifully illustrated by the flashing light produced by the firefly. Mainly through research[1−4] on the common North American firefly *Photinus pyralis*, there is a very good understanding of the chemical transformations leading to light emission (Scheme 1). Beetle luciferases (including those of the firefly) use the same LH_2[5] substrate (Chart 1) to naturally display light ranging in color from green ($\lambda_{max} \sim 530$ nm) to red ($\lambda_{max} \sim 635$ nm).[6−8] Moreover, luciferase mutants have been produced that display emission maxima from ∼550 to ∼620 nm.[2,9,10] White et al.[4] proposed that red light emission from Luc at pH ∼6 results from the keto form of the emitter oxyluciferin (Chart 1). At pH ∼8, the familiar yellow-green emission ($\lambda_{max} \sim 560$ nm) is produced from the enol of excited-state oxyluciferin formed by a presumed Luc-assisted tautomerization.[4] Among the beetle luciferases, the variation of bioluminescence color with pH observed in the firefly does not occur in the families of click beetles or railroad worms.[2,6]

Elucidation of the relationship between the wide range of bioluminescence colors and luciferase structures is an intriguing problem.[2,11] Color modulation of the tautomeric emitters, which represent the approximate extremes in wavelength, may occur through changes in the polarity of the emitter binding site.[12] Alternatively, McCapra proposed[11] that color variation is associated with conformations of the keto form of excited-state oxyluciferin related by rotation about the C2−C2′ bond. At the extremes, red emission is attributed to a minimum energy conformation of a TICT excited state with $\Phi \sim 90°$ (Chart 1) and green to a higher energy conformer with $\Phi < 90°$. Further, luciferase−emitter interactions are proposed to maintain specific conformers of the excited state thus influencing color.[11] Since the minimum energy conformer is the red emitter, perturbations that disrupt protein−emitter constraints through changes in protein tertiary structure, e.g., acid or heat denaturation, would be expected to give red light.

In view of the high chemical reactivity of 5-methyloxyluciferin and the parent oxyluciferin, which has not yet been prepared chemically pure,[4d] we undertook a study of the mechanism of bioluminescence color determination focusing on the relatively stable potential light emitter 5,5-dimethyloxyluciferin (Chart 1). This compound, unlike oxyluciferin and its monomethyl analogue, is constrained to exist in the keto form and fluoresces in the red ($\lambda_{max} \sim 635$ nm) as the phenolate ion. We first prepared D-$DiMeLH_2$ (Chart 1), the substrate analogue that would be expected to produce the bioluminescence product 5,5-dimethyloxyluciferin. Unfortunately, D-$DiMeLH_2$, like the racemic compound reported by White,[4e] did not emit light with Ppy (or Luc) and ATP-Mg. We then synthesized D-$DiMeLH_2$-AMP, effectively bypassing the required

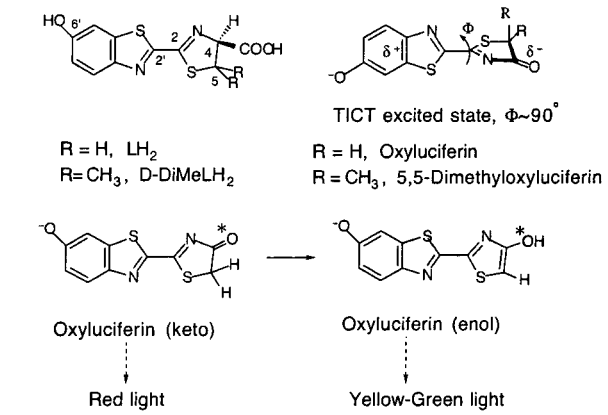
Scheme 1

$$\text{Luc} + LH_2 + \text{ATP-Mg} \rightleftarrows \text{Luc}\cdot LH_2\text{-AMP} + PP_i \quad (1)$$

$$\text{Luc}\cdot LH_2\text{-AMP} + O_2 \rightarrow \text{Luc}\cdot\text{oxyluciferin}^* + \text{AMP} + CO_2 \quad (2)$$

$$\text{Luc}\cdot\text{oxyluciferin}^* \rightarrow \text{Luc}\cdot\text{oxyluciferin} + h\upsilon \quad (3)$$

Chart 1



enzymic adenylation of D-$DiMeLH_2$ (eq 1). With Ppy, this synthetic adenylate surprisingly produced yellow-green light ($\lambda_{max} = 560$ nm) with a slightly broader emission spectrum than observed with LH_2 plus ATP-Mg or synthetic LH_2-AMP (Figure 1). Light emission from D-$DiMeLH_2$-AMP (pH optimum 8.6) is slow and sustained (rise time = 21 s and decay = 12 min), unlike that of LH_2-AMP, which is very rapid (rise time = 0.4 s and decay = 2 s).

In marked contrast to our results with Ppy, red ($\lambda_{max} = 624$ nm) bioluminescence (Figure 1) was observed from D-$DiMeLH_2$-AMP in a reaction catalyzed by the green click beetle isozyme PplGR,[13] a luciferase that produces green emission (546 nm) from LH_2 and ATP-Mg, as well as from LH_2-AMP. The pH optimum and kinetics of light emission with PplGR and D-$DiMeLH_2$-AMP were similar to those of Luc with this adenylate. Unfortunately, we could not make bioluminescence measurements at acidic pH because light emission from $DiMeLH_2$-AMP and the luciferases was too weak below pH ∼7.5. Control experiments for both enzymic reactions eliminated the possibilities that light emission from D-$DiMeLH_2$-AMP was caused by nonspecific protein interactions, LH_2 contamination, buffer components, or synthetic contaminants. Additional controls eliminated protein denaturation as the cause of the red emission from PplGR.

Since both Ppy and PplGR can produce light from D-$DiMeLH_2$-AMP, but not D-$DiMeLH_2$, the steric effects of the 5,5-dimethyl substituents evidently severely impede the adenylation partial reactions (eq 1). The methyl groups probably disrupt the proper orientation that enables the carboxylate ion of D-$DiMeLH_2$ to

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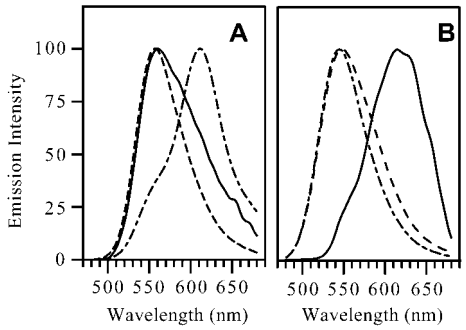

Figure 1. Normalized bioluminescence emission spectra of Ppy (Panel A) and PplGR (Panel B) catalyzed reactions with LH_2 plus ATP-Mg at pH 8.6 (- - -) and pH 6.0 (— - —) and D-DiMeLH$_2$-AMP at pH 8.6 (—). Conditions used to obtain the spectra are in the Supporting Information.

approach the α-phosphate of ATP. The slow light emission kinetics of the luciferases with D-DiMeLH$_2$-AMP indicate that steric effects also hinder the oxidative steps (eq 2), possibly by impairing C-4 proton abstraction.

A RP-HPLC-based analysis of the Ppy- and PplGR-catalyzed reactions with D-DiMeLH$_2$-AMP revealed that the only reaction product was 5,5-dimethyloxyluciferin formed in nearly quantitative yield. We have therefore demonstrated that the keto form of 5,5-dimethyloxyluciferin emits both yellow-green and red bioluminescence. The corrected relative total light yields from D-DiMeLH$_2$-AMP were ∼16% and ∼11% of those obtained from LH_2-AMP with Ppy and PplGR, respectively. These values show that we have documented a significant bioluminescence process.

While it seems likely that we have produced convincing experimental evidence supporting the mechanism[11] that conformations of the keto form of excited-state oxyluciferin alone can determine bioluminescence color, it also is conceivable that 5,5-dimethyloxyluciferin emits green light with Ppy because the 6′-phenolic substituent is un-ionized in the excited state. This possibility must be considered because McCapra has observed[11a] green fluorescence from 5,5-dimethyloxyluciferin in SDS-micelles that presumably inhibit phenol deprotonation in the excited state. The bulky substituents of 5,5-dimethyloxyluciferin could force the excited-state emitter into an anionic protein environment that impedes ionization of the phenolic group, as is thought to be the case in SDS-micelles.[11a] We do not feel that direct experimental evidence to determine the ionization state of the luciferase-bound emitter is readily obtainable. However, we believe that the results[14] of steady-state kinetics studies at pH 8.6 with Ppy are better accounted for by the phenolate ion of excited state 5,5-dimethyloxyluciferin being the yellow-green light emitter. D-DiMeLH$_2$ is a very good competitive inhibitor with respect to LH_2 with a K_I value ∼10-fold lower than the K_m value of Ppy for LH_2. Similarly, the K_m value for D-DiMeLH$_2$-AMP is ∼30-fold lower than that of LH_2-AMP. Thus, the 5,5-dimethyl analogues are accommodated very well by the respective Ppy sites for LH_2 and LH_2-AMP. Moreover, since 5,5-dimethyloxyluciferin is a reversible competitive inhibitor with respect to LH_2-AMP, the adenylate binding site is very likely also the emitter site. Perhaps, the guanidinium ion of stringently conserved Ppy active site residue Arg218 stabilizes the phenolate ion of D-DiMeLH$_2$ and 5,5-dimethyloxyluciferin as mutagenesis studies have led us to propose[15] is the case for LH_2 and oxyluciferin.

The results of this study may be taken as the first experimental support for McCapra's mechanism[11] of firefly bioluminescence color or any other proposal that requires only a single keto form of oxyluciferin. While our results also demonstrate that the keto−enol tautomerization mechanism of White[4] is not required to explain red and green firefly bioluminescence, they do not preclude the occurrence of this phenomenon. Currently, we are extending the present study with additional mechanistic and mutagenesis experiments designed to explain the unusual occurrence of red bioluminescence from PplGR, a luciferase that emits green light at acidic pH and with 6′-aminoluciferin, a substrate analogue that produces red light emission with Luc.[16] We anticipate that the ongoing studies will enable us to propose a new mechanism of bioluminescence color determination in the firefly.

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Supporting Information Available: Experimental details including synthesis and characterization of D-DiMeLH$_2$ and D-DiMeLH$_2$-AMP, RP-HPLC analyses of reactions of Ppy and PplGR with D-DiMeLH$_2$-AMP, bioluminescence emission spectra, determination of relative light yields, kinetic measurements, and subcloning of PplGR (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(5) Abbreviations used: DiMeLH$_2$, 5,5-dimethylluciferin; LH_2, D-firefly luciferin; Luc, *Photinus pyralis* luciferase (E.C. 1.13.12.7); PplGR, recombinant green-emitting *Pyrophorus plagiophthalamus* luciferase containing the additional N-terminal peptide GPLGS-; Ppy, recombinant *Photinus pyralis* luciferase containing the additional N-terminal peptide GPLGS-; SDS, sodium dodecyl sulfate; TICT, twisted intramolecular charge transfer.

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