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Filter-Photometry of Chemiluminescence from Firefly Luciferin Intermediate M_{420} in Deoxygenated Dimethyl Sulfoxide

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The intermediate M_{420} formed in a solution of firefly luciferin in deoxygenated dimethyl-sulfoxide added potassium *t*-butoxide was observed to emit yellow-green and red light by filter-photometry. By H-NMR, M_{420} was found to be deprotonated at the site where luciferin reacts with oxygen.

Key words: firefly luciferin, chemiluminescence, oxyluciferin, dioxetane, superoxide anion

INTRODUCTION

The molecular mechanism and color of firefly chemiluminescence have been interpreted from studies by McCapra and others [1-3] as follows. In the presence of a strong base like potassium *t*-butoxide (*t*-BuOK), the oxygenation of Ln produces a dioxetane which is decomposed immediately to form an electronically-excited oxyluciferin (Oxyln*). The Oxyln* relaxes with light emission to its ground state. A monoanion of Oxyln* in the keto form emits red light at low base concentrations, and its mono- or di-anion in the enolic form emits yellow-green light at high base concentrations [4].

This reaction scheme was established using derivatives of Ln, such as 5,5-dimethylLn [1], because Oxyln has not been detected in the solution of Ln after chemiluminescence, and Ln can be easily oxidized to produce dehydroluciferin (drLn) [5]. In the reaction scheme, the formation process of the Oxyln* through the dioxetane was proposed by McCapra [6] and Koo et al. [7] separately. However, the key intermediate of the dioxetane, which is thought to be unstable, has not yet been detected. Furthermore, in a solution of *t*-BuOK in dimethylsulfoxide (DMSO), the superoxide of O_2^- is produced. The role of O_2^- is not taken into consideration in the scheme [8]. As a step to elucidate these points, we produced intermediates of Ln before light emission by removing oxygen from a Ln chemiluminescence system and then, investigated which intermediate emits light by pouring oxygen into the system. In this paper, we report on the molecular structure of the intermediates produced before chemiluminescence using H-NMR and on the color of the light emitted from the intermediate M_{420} using several types of band pass filters.

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MATERIALS AND METHODS

Analytical grade Ln, *t*-BuOK, and DMSO (the water concentration of 0.2 %) were purchased from Sigma Chemical Co., Merck Co., and Kanto Chemical Co., respectively. The apparatus used for evacuating and pouring oxygen is shown in Fig. 1. Spectra of absorption and H-NMR were measured with cell C and F, respectively. Absorption spectra were recorded with a Hitachi U-3200 spectrophotometer. H-NMR spectra were measured with a JEOL JNM-EX400 FT NMR spectrometer.

The photomultipliers R376 (Hamamatu photonics Co.) used for reference and sample lights were placed on the left-hand side and the down side of the apparatus, respectively. The time courses of the light emission decay measured with both of the

photomultipliers were shown to be almost the same when each of these peak intensities was normalized. An appropriate filter was placed between the apparatus and the downside photomultiplier. Color-glass filters were obtained from Asahi Techno Glass Co. and Toshiba Glass Co.. The spectrum of chemiluminescence of M_{420} was also measured with a Hamamatu PMA-11, which is a multichannel photodiode-array system, but the intensity of this spectrum was too weak to be detected.

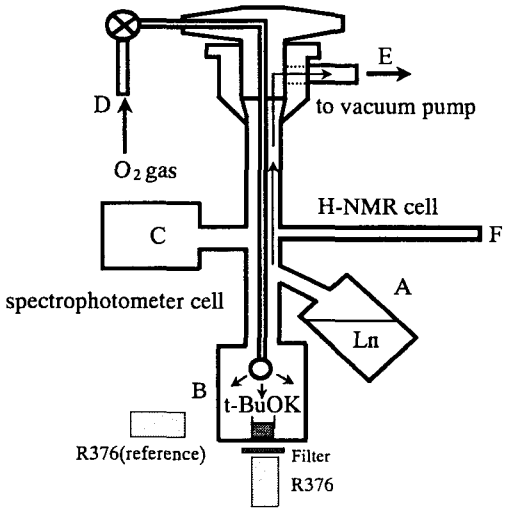

Fig. 1 The equipment for measuring spectra of absorption with cell C, spectra of H-NMR with cell F, and the intensity of the light emission, with the sample photomultiplier R376, transmitted through a filter placed under the apparatus designed for oxygen evacuation at cell A and bubbling at cell B. The reference photomultiplier with no filter, which was used for normalizing, is placed near the left-hand side of cell B.

RESULTS AND DISCUSSION

By absorption spectroscopy, we found two intermediates of Ln in a solution of Ln in deoxygenated DMSO added *t*-BuOK, which is the simplest reaction system of Ln chemiluminescence. At low concentrations of *t*-BuOK, the intermediate with the absorption peak of 440 nm (called M_{440}), and at high concentrations of *t*-BuOK, the other intermediate with the peak of 420 nm (called M_{420}), were observed [9]. These intermediates can be considered to be produced before the formation of the dioxetane proposed, because the oxygenation of Ln can not proceed, and O_2^- can not be formed, in the absence of oxygen.

The H-NMR spectrum of M_{440} showed the signals of the three protons on the benzothiazole ring of Ln and the proton signals of the CH, and CH_2 on the thiazoline ring of Ln. Hence, M_{440} was identified as the intermediate deprotonated at the OH and COOH groups of Ln. In the spectrum of M_{420}, the resonance signal of the CH was not observed. Accordingly, M_{420} was speculated as the intermediate additionally deprotonated at the CH. By oxygen-bubbling into the intermediate M_{420}, we observed light emission and found a new product with the absorption peak of 535 nm (called P_{535}) [9]. Therefore, M_{420} is considered to react with oxygen at the CH site on the thiazoline ring of Ln.

Figure 2 shows the dependence of light intensity of M_{420} transmitted through a filter on oxygen-bubbling duration. A M_{420} solution was prepared from a solution of 0.25 mM of Ln in deoxygenated DMSO added 5 mM of *t*-BuOK. Oxygen gas was poured into the M_{420} solution at a bubbling-rate of 200 ml/min. The intensity of the light transmitted through the filter was calibrated with the normalized peak-intensity of the reference light. The reference light shown in the figure indicates different decays in the two regions of the bubbling duration. In the region from the starting time to ~ 10 seconds a strong intensity and fast decay curve (named the fast-phase) was observed. The peak intensity in the fast-phase was decreased by 10 % with the Y49-filter, which cut off the light with wavelengths of shorter than 490 nm, by 75 % with the O54-filter and by 95 % with the R62-filter, respectively. As a result, the emission in the fast-phase was found to be a green-colored light with a maximum wavelength of emission of about 520 nm. This is, however, about 40 nm shorter than the emission peak of the bioluminescence reported [2]. The wavelength of 520 nm was estimated as the wavelength cut off by 50 % of the peak intensity of the reference light in the fast-phase, by supposing that the intensity of the spectrum has symmetrical strength with the peak of the spectrum. In the region from the end time of the fast-phase to 1.5 minutes, a slow decay curve was observed. During any bubbling duration time in this region, the decrease rates of intensity of the light transmitted through the filters were the same as those of the light in the fast-phase when the light intensity transmitted through the R62-filter was subtracted from

the intensities observed with the other filters. According to this, the slow-phase is considered to be due to the increase of the intensity of the red light with wavelengths between 620 nm and 660 nm.

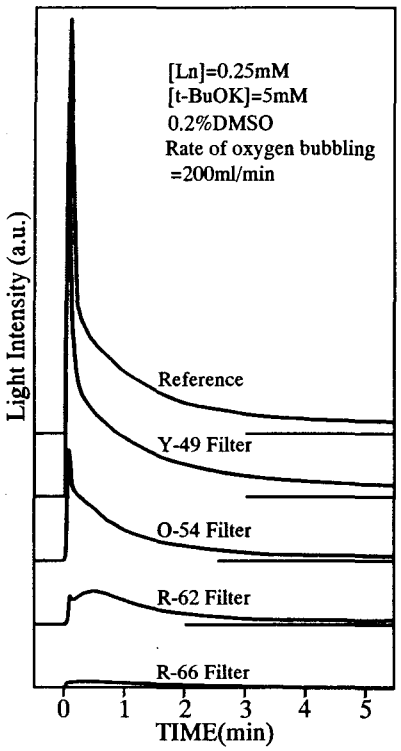

Fig. 2 The dependence of the intensities of light transmitted through Y-49, O-54, R-62 and R-66 filters on oxygen bubbling duration. The intensity of the reference light was equal with the normalized intensity of light measured with no filter. Yellow-green light was observed in the duration of 0 - 10 secs and red light, which was very weak, in that of 10 secs - 1.5 mins.

We measured the absorption spectra of the products at specific times throughout the oxygen-bubbling duration; the time before oxygen-bubbling (0 minutes; T_0), the end time of the slow-phase (1.5 minutes; $T_{1.5}$), and the time after a glow was emitted (5 minutes; T_5). The sample at $T_{1.5}$ was prepared by immediately stopping the oxygen-bubbling after observing the slow-phase, and the one at T_5 was prepared by repouring oxygen into the sample after measuring the spectrum of the sample at $T_{1.5}$. The spectrum of the product at $T_{1.5}$ shows the absorption maximum of 420 nm, almost the same as that of M_{420} observed at T_0, and another weak peak at 535 nm. The spectrum at T_5 clearly shows the formation of the single product P_{535}. The work to elucidate the molecular structure of the products formed after chemiluminescence and the correlation of Oxyln with these products are in progress.

From these experiments, it was found that the intermediate M_{420} is considered to react with oxygen at the deprotonated CH site on the thiazoline ring of Ln, and that M_{420} emits yellow-green light followed by a very weak red light on oxygen-bubbling.

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