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FREE RADICAL PARTICIPATION IN BACTERIAL BIOLUMINESCENCE

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The metastable intermediate II produced on reaction of bacterial luciferase with reduced flavin mononucleotide and O_2, reacts with any of several stable free radicals to produce bioluminescence. The bioluminescence spectrum is very similar to that from the well-studied intermediate II and aldehyde reaction, and the number of photons per luciferase molecule reacted is at least 40% of the aldehyde reaction.

Key words: Free Radical, Bacterial Bioluminescence

INTRODUCTION

The traditional *in vitro* reaction sequence for bacterial bioluminescence[1] is the reaction of reduced flavin mononucleotide ($FMNH_2$) with the bacterial enzyme luciferase and molecular oxygen to yield an isolable intermediate II which then reacts with long chain aldehyde to yield light. Long chain aldehyde has heretofore been regarded as being absolutely required for this reaction[2]. Intermediate II has been characterized spectroscopically[3,4] and shown to be stabilised by long chain alcohol[5].

We now demonstrate that long chain aldehyde is not absolutely required for the *in vitro* bacterial bioluminescence reaction. Using luciferase purified from *Photobacterium phosphoreum* (strain A13) and *Vibrio harveyi* (aldehyde deficient mutant),[6] intermediate II reacts with the stable free radicals 1,1-diphenyl-1-picrylhydrazyl (DPPH), galvinoxyl and nitric oxide to produce light.

EXPERIMENTAL

The bacterial luciferases were purified as described elsewhere[7]. Bioluminescence measurements at room temperature were made using a photodiode photometer calibrated using the luminol standard. Low temperature bioluminescence and fluorescence measurements were made using a computer controlled fluorimeter whose calibration has been previously described[8]. Intermediate II was purified by adding $FMNH_2$ to *V. harveyi* luciferase in pH 7 buffer containing dodecanol at 0°C and putting the mixture

on an HPLC column of TSK-5PW (LKB Bromma, Sweden), eluting excess FMN with 0.05 M phosphate buffer, and eluting intermediate II with 0.35 M phosphate buffer, both buffers contained 100 μM dodecanol. The eluted intermediate II was concentrated by centrifugation and its concentration determined spectrophotometrically[4].

RESULTS

The time courses of the radical induced bioluminescence reaction of intermediate II under various conditions are shown in Fig. 1. A mixture of luciferase (3–50 μM) and dodecanol or tetradecanol (generally 100 μM), is reacted with $FMNH_2$ produced by photoreduction, producing a rapidly rising and decaying emission spike, the "aldehydeless" reaction. The nominal concentration of intermediate II is calculated from the concentration of the kinetically limiting component, luciferase, unless otherwise noted. After the initial spike had largely died away the free radical was added, as a 1% v/v of a methanolic solution in the cases of DPPH and galvinoxyl or gas bubbling in the case of NO. Panel A shows the reaction of intermediate II from *V. harveyi* luciferase (4 μM) with 80 μM DPPH at 23°C. A rapid rise in luminescence is observed followed by a slow first order 7×10^{-3} s^{-1} decay. A similar result is observed when the reaction is carried out at 0°C in the absence of long chain alcohol. Panel B shows the reaction of intermediate II from *P. phosphoreum* luciferase. $FMNH_2$ was limiting in this case, II = 25 μM with 150 μM DPPH at 0°C, in the presence and absence of lumazine protein (Lump) from *P. phosphoreum*. The reactions are

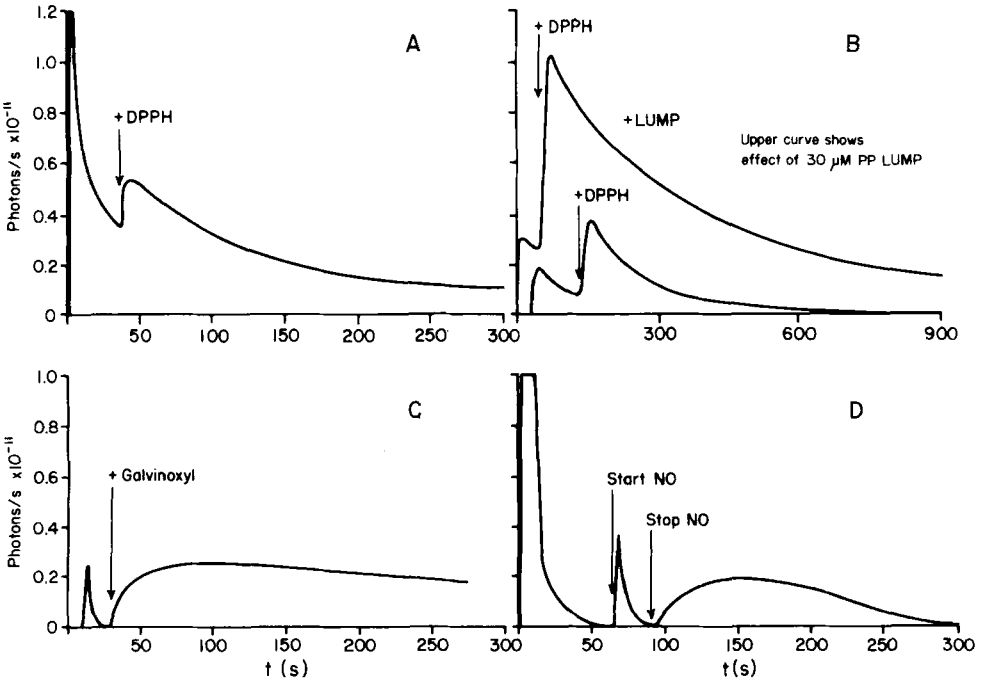

FIGURE 1 Luminescence time courses for the reactions of intermediate II with various radicals.

generally similar to that from *V. harveyi* with more rapid decays, 24×10^{-3} s^{-1} with Lump and 7×10^{-3} s^{-1} without. The 3-fold stimulation produced by Lump is typical to that observed for the normal long chain aldehyde with *P. phosphoreum* luciferase[9]. Panel C shows the reaction of intermediate II from *V. harveyi luciferase* (3 μM), with 12 μM galvinoxyl at 23°C. A rapid rise followed by a relatively slow first order (3.7×10^{-3} s^{-1}, decay is observed. Panel D shows the reaction of intermediate II from *P. phosphoreum* luciferase (3 μM) with NO. Nitric oxide bubbling produced a rapidly rising and decaying emission spike whereupon the bubbling was stopped. The gas induces bioluminescence from intermediate II but is also a fluorescence quencher and eventually kills the emission. A short time after the NO bubbling ceases the bioluminescence reappears as the NO is chemically depleted or exchanged with air. The time profile of the emission decay is complex and non first order.

Figure 2 shows the bioluminescence spectrum for the intermediate II + DPPH reaction. The reaction conditions are identical to those of Fig. 1, Panel B, lower curve. This spectrum is corrected for bioluminescence and instrumental response[8] and is very similar to that obtained from the normal intermediate II + aldehyde reaction.

Bioluminescence quantum yields, calculated as moles photons per mole of intermediate II initially present, are shown in Table I. The yields for *P. phosphoreum* luciferase at room temperature were obtained using the same protocol as in Fig. 1 experiments and integrating the time profile of the bioluminescence. The yields for

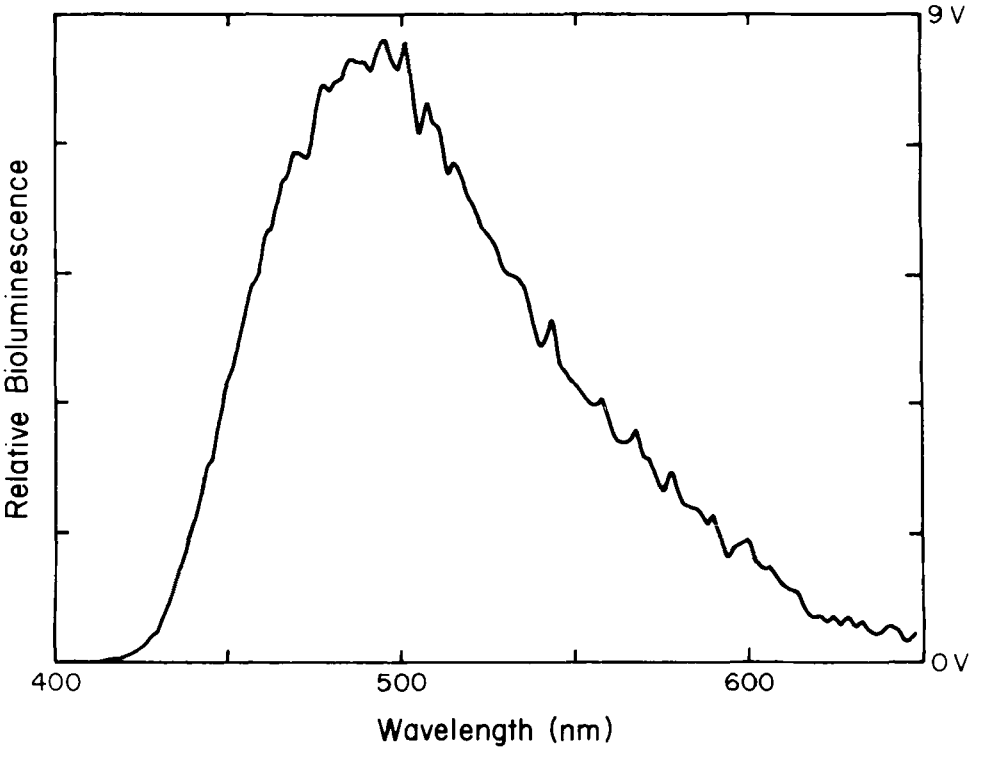

FIGURE 2 Corrected bioluminescence spectrum for II derived from P. phosphreum luciferase reacting with DPPH. Minor correction for DPPH absorption has not been made.

TABLE I
Quantum yields percent

Temp.	DPPH	1-tetradecanal
23°	0.07	0.17
0°	4.5	10.0

both the DPPH and tetradecanal reactions are very low suggesting the yield of intermediate II at room temperature is low. Accordingly quantum yield determinations were made at 0°C using intermediate II purified by the method described in the experimental section. The quantum yield of the purified intermediate II + DPPH reaction is 4.5% to be compared with tetradecanal quantum yield of 10% with FMNH limiting.

Both the free radical label 4-hydroxyl-TEMPO and the xanthine/xanthine oxidase enzyme system were ineffective in producing light from intermediate II.

DISCUSSION

The simplest rationalisation of the present results is that intermediate II is in equilibrium to a small extent (10^{-6} would account the current data) with a radical intermediate III. Intermediate III and the added stable free radicals then give rise to light by a chemically induced electron exchange mechanism[10,11]. Intermediate III is postulated to be a luciferase-flavin cationic radical with hydroxy substitution at the 4α-position[12] which has been proposed in the bioluminescence literature. A reaction scheme appropriate for a reducing radical sources RS$^{\cdot}$ such as DPPH is shown in the following scheme:

$$\text{Intermediate II} \rightarrow \text{Intermediate radical III} + RS^{\cdot} \rightarrow h\nu + P + RS^{+}$$

P is the product of the light reaction. Inspection of the luminescence rise times in Fig. 1 shows a very rapid rise only with DPPH. Thus it is likely that a reducing radical source is preferred for this reaction.

The present data show that DPPH, galvinoxyl and NO are effective in producing light from intermediate II made from *P. phosphoreum* or *V. harveyi* luciferases, the emission band profile is very similar to that of the normal reaction, and that the quantum yield is a significant of that of the normal aldehyde reaction. The present results imply a radical mechanism is not unlikely for bacterial bioluminescence.

Acknowledgement

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