The Role of Oxygen in the Photoexcited Luminescence of Bacterial Luciferase*

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SUMMARY

Irradiation of bacterial luciferase results in a long lived luminescence which is qualitatively similar to the chemically induced bioluminescence. It has been found that oxygen is required for the emission of photoexcited bioluminescence, but that it need not be present during irradiation. Oxygen must be added promptly, however, for the intermediate has a relatively short lifetime. In the absence of oxygen still another long lived luminescence has been observed. This is a phosphorescence-like emission which is strongly quenched by oxygen. The observations indicate that this emission involves intermediate states which are distinct and different from those involved in bioluminescence.

It has previously been shown (1, 2) that photoexcitation of bacterial luciferase results in luminescence qualitatively similar to the normal, chemically induced bioluminescence. Fig. 1 depicts the biochemical pathways and intermediates that have been proposed for the reaction (3). A feature which is quite diagnostic of bioluminescence derives from the properties of Intermediate II, which has a lifetime of the order of 5 sec. In particular, its reaction with aldehyde is unique in that the specific lifetime and intensity of the luminescence is dependent on the presence of, and on the kind of, long chain aliphatic aldehyde added to the reaction mixture. The chemically induced luminescence also requires the presence of at least small amounts of molecular oxygen (half-intensity being obtained with 1 × 10^-8 M), which has been deduced to react early in the reaction, as indicated in the figure. The role of oxygen was not investigated earlier for the case of photoexcitation because of technical limitations in the apparatus. The matter is, however, of central importance to the understanding of the mechanism, and this paper now reports the results of this study.

It has been found, first, that oxygen is indeed required for bioluminescence initiated by light, but that it need not be present at the instant of irradiation. The light energy absorbed in the absence of oxygen can be effective for bioluminescent emission if oxygen is added secondarily. However, whereas Intermediate I is known to have an indefinitely long lifetime in the absence of oxygen, the light-induced intermediate has a relatively short lifetime anaerobically. The scheme of Fig. 1, therefore, indicates that light populates Intermediate II in the bioluminescent reaction via a different oxygen-requiring pathway.

A second finding reported here is that when luciferase is irradiated anaerobically or in the presence of minimal amounts of oxygen, a phosphorescence-like emission is observed, which is strongly quenched by oxygen and which is similar in many respects to the delayed light emission in plants discovered by Strehler and Arnold (4). It is also reminiscent of the long lived luminescence observed at liquid nitrogen temperatures by Debye and Edwards (5). The observations reported here suggest that this delayed light involves pathways and intermediates distinct and different from those involved in bioluminescence. The spectra of these two kinds of light-induced luminescence have been determined and are compared.

MATERIALS AND METHODS

The luciferase utilized in these experiments was purified from extracts of Photobacterium fischeri (the same as Achromobacter fischeri, ATCC 7744) by the method of Hastings, Riley, and Massa (6). The enzyme used was of high purity, and had a specific activity in the FMNH2-initiated reaction in the range of 1 to 2 × 10^4 quanta second^-1 mg^-1 with decanal. The enzyme (mol wt, about 60,000) possesses eight free sulfhydryl groups and no disulfide bonds. Maximum enzyme activity is achieved when the enzyme is in the fully reduced condition; mild reductants such as 2-mercaptoethanol and dithiothreitol stabilize activity and were, therefore, utilized in some instances.

Other chemical and reagents were obtained commercially and were of analytical grade. High purity tank nitrogen (The Matheson Company, Inc., East Rutherford, New Jersey) was used without additional purification to deoxygenate solutions.

Light intensity was measured with a photomultiplier operated between 500 and 1500 volts, the output being recorded either on a...
therefore, to transfer the irradiated enzyme to another container.

The bioluminescence obtained by light excitation of the enzyme is weak, even under favorable circumstances, and cannot be increased by using more enzyme because the absorbance at 280 mg soon increases to a point at which much of the enzyme is self screened from effective exciting light. The result is that bioluminescence is, in practice, of about the same order of intensity as the phosphorescence emitted by the walls of the containing vessel (from impurities in the silica). It is necessary, therefore, to transfer the irradiated enzyme to another container in order to measure bioluminescence. Two special pieces of apparatus were constructed for anaerobic irradiation and transfer.

In the first apparatus, shown diagrammatically in Fig. 2, two vials were connected together by two lengths of 2-mm capillary tubing which, together with a special glass tap, permitted gas flow from the cylinder either to pass through the observation vial and bubble through the solution in the irradiation vial or, when the tap was reversed, to drive the solution from the irradiation vial into the observation vial. This apparatus was constructed exclusively from glass and Vycor, and all connections were made with ground glass joints.

The irradiation vial was set up inside the flash box of a rectangular chamber was milled in a Lucite block located in a fitting screwing into the lens mounting socket of a Bausch and Lomb "large grating" monochromator. The dimensions of the chamber were 10 x 3 x 5 mm. With this apparatus, the effective transfer time was less than 20 msec. Mechanical synchronization between the flash apparatus and the pneumatic fluid drive was inconvenient, so the time interval between irradiation and fluid flow was measured by triggering an oscilloscope at the time of irradiation and activating the pneumatic drive manually. The time interval was given by the appearance of light at the photomultiplier. It was readily possible to obtain intervals of 100 msec, and it was frequently possible to achieve times of 50 msec or less.

The effective transfer time with this apparatus was approximately 1 sec, which is quite suitable for observing the bioluminescence emission.

For more rapid transfer the apparatus shown schematically in Fig. 4 (see below) was constructed. The enzyme was placed in a Vycor syringe of 2-ml capacity mounted in a Lucite block containing a two-jet mixing chamber. This enabled the contents of the irradiation syringe to be mixed with the contents of a second syringe at any desired time after irradiation. When the experiment did not call for mixing with a second reagent after irradiation, one of the syringes was detached and the hole was plugged. The syringes and mixing chamber were fastened to an aluminum base plate which supported a pneumatic driving piston (model OOME 500 I, Airco Inc., Angola, Indiana). The entire unit slid horizontally into the light box of the flash photolysis apparatus through appropriate holes cut in its sides.

The effluent from the flow apparatus was driven into an observation chamber milled in a Lucite block backed with a mirror, and closed by a Lucite plate facing the end window photomultiplier. This assembly was used in all experiments except when the spectral distribution of the luminescence was being measured. For these experiments a rectangular chamber was milled in a Lucite block located in a fitting screwing into the lens mounting socket of a Bausch and Lomb "large grating" monochromator. The dimensions of the chamber were 10 x 3 x 5 mm. With this apparatus, the effective transfer time was less than 20 msec. Mechanical synchronization between the flash apparatus and the pneumatic fluid drive was inconvenient, so the time interval between irradiation and fluid flow was measured by triggering an oscilloscope at the time of irradiation and activating the pneumatic drive manually. The time interval was given by the appearance of light at the photomultiplier. It was readily possible to obtain intervals of 100 msec, and it was frequently possible to achieve times of 50 msec or less.

Fig. 1. Scheme depicting the hypothesized pathways in bacterial bioluminescence generated chemically by reaction of the enzyme with reduced flavin mononucleotide or by photochemical means. The latter leads to an intermediate (II) which has a lifetime of about 1 sec and which, after reaction with oxygen, may be identified with Intermediate II of the chemical pathway.
The basic circuits of the flash equipment have already been described by Porter (8). Minor changes made were to increase the working voltage to 13.5 K, with the use of 8-μf storage capacitors discharging into two 20-cm hairpin-shaped silica lamps. The loops of the lamps surrounded either the irradiation vial of the gas transfer apparatus or the syringes of liquid flow transfer equipment. The discharge provided an approximately exponential pulse of light with a time constant of about 8 μsec, followed by a longer “tail” lasting for perhaps 200 μsec, and finally by a brilliant, long lasting phosphorescence of the walls of the lamps. To provide a firm working base for the associated equipment together with electrical screening, the lamps were supported in a box made from 1/8-inch thick aluminum sheet with an inset aluminum floor placed to support the flow transfer apparatus at the correct height in relation to the monochromator.

RESULTS

Oxygen Removal—The effect of removing oxygen was first studied with the apparatus of Fig. 2. As shown in Fig. 3, the typical light-induced bioluminescent emission was obtained when air was used as the gas for equilibration and transfer, and the characteristic increases in intensity and quantum yield were also obtained when aldehyde was present.

When, instead, the enzyme solution was deoxygenated prior to irradiation by bubbling for 10 min with purified nitrogen, no bioluminescent emission was observed. Instead, a type of phosphorescence-like emission with a more rapid decay was noted which, unlike the bioluminescence, was unaffected by aldehyde.

If the enzyme was partially deaerated by bubbling for shorter periods, it was possible to observe bioluminescence together with the rapidly decaying emission, indicating that the quenching of the latter by oxygen requires oxygen concentrations higher than those which will support bioluminescence. This was established more quantitatively in subsequent experiments (Fig. 5).

"Delayed Light"—The rapidly decaying luminescence which occurs following irradiation in the absence of oxygen, which we will refer to as delayed light, is quite incompletely represented in Fig. 3 because of the limitation of time resolution of the apparatus. It is actually considerably brighter and decays very rapidly in its early phases. With the apparatus shown in Fig. 4, the transfer following flash excitation could be accomplished within 50 to 100 msec. The delayed light thereby observed during the 1st sec following irradiation is illustrated in Fig. 5.

![Diagram](http://www.jbc.org/)

Fig. 3. (left). Effects of oxygen and aldehyde upon light-induced light emissions of bacterial luciferase. In the presence of oxygen a typical long lived (k = 0.15 sec\(^{-1}\)) bioluminescence occurs (+), the quantum yield being increased if aldehyde is present (Φ). In the absence of oxygen (×) this bioluminescent emission does not occur; instead, there is a phosphorescence-like emission which is unaffected by aldehyde (Ω). Ordinate, luminescence, in quanta per sec; abscissa, time, in seconds. Irradiation at zero time from a flash tube discharging 600 joules of stored energy. Enzyme, 0.6 mg of luciferase, specific activity, 2 × 10\(^{18}\) quanta sec\(^{-1}\) mg\(^{-2}\), with Antifoam AF (Dow-Corning), 0.01 M phosphate buffer, pH 8.8, in a final volume of 2.5 ml.

Fig. 4 (right). Apparatus for achieving rapid mixing or prompt observation or both, following flash irradiation. Prior to irradiation the enzyme was placed in one of the Vycor syringes which, if desired, could be deoxygenated. When desired, the second syringe was filled with O\(_2\)-containing buffer and aldehyde. Following irradiation the piston was activated by gas pressure from the valve, thereby emptying and mixing the contents of the syringes and transferring them to the remote observation chamber in front of the photomultiplier tube. When rapid transfer of an irradiated solution without mixing was desired, a similar apparatus, but with only a single syringe, was used.
In all cases the decay was not exponential, being more rapid at the outset, suggesting that if observation were begun still earlier (within 1 msec, for example), a significantly greater intensity might have been observed.

Emission Spectra—Determinations of emission spectra were carried out by locating the observation tube or vial (Fig. 2 or Fig. 4) in front of the entrance slit of a large Bausch and Lomb grating monochromator and locating the photomultiplier at the exit slit. The emission spectrum of the light-induced bioluminescence (Fig. 6) corresponds tolerably well, although not exactly, with that of both the chemically induced light emission and the light emission in vivo (6). The agreement constitutes additional support for the assertion that the light-induced bioluminescence emission comes from excited states identical with those generated by the oxidation of FMNH₂.

Because of its shorter lifetime (and also because of the difficulties associated with preparing the deoxygenated enzyme), the color of the delayed light has been more difficult to determine accurately. Several sets of measurements have indicated an emission centered about 430 μm, as illustrated by the spectrum shown in Fig. 6.

Relationship between Delayed Light and Bioluminescence—If oxygen is added to the deaerated enzyme after irradiation, characteristic bioluminescence may still be obtained. This experiment was carried out with the apparatus shown in Fig. 4. Oxygen was removed from the enzyme solution by bubbling with nitrogen in the syringe itself. The second syringe was filled with oxygenated buffer saturated with aldehyde; by actuation of the pneumatic piston, the two could be rapidly mixed at any desired time after irradiation of the anaerobic enzyme.

Since oxygen quenches the photoinduced delayed light but is required for bioluminescence initiated under the same conditions, it was of interest to determine whether the species giving the latter emission is derived from the former. This possibility was excluded by the results shown in Fig. 7. First, this experiment shows that the capacity to obtain typical bioluminescent emission is not dependent upon the presence of O₂ at the time of irradiation.
The experiments showed that samples prepared in this way contained appreciable amounts of oxygen. This was evident from the fact that bioluminescence occurred at a normal intensity. This is indicated by the dashed lines, showing the approximate level of the bioluminescence peak which occurred after the delayed light. On the basis of previous studies (9) it may be deduced that the oxygen concentration in the solution in the the syringe was greater than 0.2% (1.5 mm) O₂. On the basis of past experience with the tonometer method, it is considered unlikely that the O₂ concentration is very much higher than this.

With air-equilibrated luciferase (21% O₂), the delayed light was quenched by a factor of about 100, whereas with about 2% (15 mm) O₂ the quenching was between 2- and 3-fold (Fig. 5). This suggests that quenching is directly proportional to oxygen concentration over this range.

**DISCUSSION**

It is desirable to relate, insofar as is possible, the results of the experiments described above both to the scheme for the chemically initiated bioluminescence (Fig. 1) and to protein fluorescence and phosphorescence (Fig. 8).

The light-induced luminescent emission from anaerobic solutions (delayed light) corresponds spectrally to protein phosphorescence and is quenched by molecular oxygen (although the concentrations required for quenching are high as compared with those necessary to give bioluminescence). Note that the decay constant for the delayed light is independent of oxygen concentration (Fig. 5), so that quenching by oxygen cannot be due to reaction with the species which determines the observed lifetime.

Two ways to accommodate these observations are shown in Schemes 1 and 2, where P is used to represent an excitable species, which may well be an aromatic amino acid residue in the protein, and P₁* and P₂* are used to designate excited intermediates, the exact natures of which need not be specified for these schemes. It should be pointed out, however, that P₁* is not to be identified with S₁ in Fig. 8, although additional rapid steps involving S₁ or other excited states may be inserted before or after the formation of P₁*.

In Scheme 1, k₁ and k₂ are both much greater than k₄. The characteristic lifetime of the delayed light is given by k₄.

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**FIG. 7.** This figure summarizes experiments in which flash irradiation was carried out anaerobically and oxygen was added subsequently at the times indicated on the abscissa. The mixing was achieved with the apparatus shown in Fig. 4, and the maximum intensities following oxygen addition are plotted for each experiment. Note that the lifetime of this light-induced “anaerobic” intermediate (k \( \approx 0.9 \text{ sec}^{-1} \)) is considerably longer than the delayed phosphorescence-like emission which occurs in the absence of oxygen (k \( \approx 20 \text{ sec}^{-1} \)) but shorter than bioluminescence emission which occurs under similar conditions when oxygen is added (k \( \approx 0.2 \text{ sec}^{-1} \)).

Second, it shows that the intermediate formed upon irradiation (which is capable of bioluminescence when oxygen is added) has a lifetime much longer (about 20 times longer) than that of the delayed light, so that the intermediate giving rise to the delayed light cannot be an immediate precursor of the one which gives rise to bioluminescence.

**Luminescence from Other Proteins and Quenching by Oxygen—**

The observation of delayed light prompted us to question whether or not this kind of delayed light emission was a feature especially associated with luciferase. We therefore examined a number of other proteins for emission of this type. These included Armour crystalline bovine serum albumin, crystalline ribonuclease (Worthington), diaphorase (Calbiochem), and globin. In all of these cases it was readily possible to detect a similar luminescence, and equally possible to show that it was strongly quenched by oxygen. This is illustrated for bovine serum albumin and RNase in Fig. 5. In none of these instances, however, was the intensity of the luminescence so great as that observed with luciferase. Among those examined the brightest was bovine serum albumin, which gave an emission equal to about 7% of that of luciferase when compared on the basis of equal absorbance at 280 nm.

The experiments illustrated in Fig. 5 also serve to indicate the relative oxygen levels which produce the effects being studied. The deaeration in this case was carried out not by direct flushing with gas, as in the experiments of Fig. 3, but by the use of a tonometer in which a larger quantity of the protein was deoxygenated by repeated evacuation and equilibration with N₂. The samples were then transferred individually to a Vycor syringe, and the experiment was carried out in the apparatus of Fig. 4, with only a single syringe, without mixing.

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**FIG. 8.** Conventional energy diagram depicting excited singlet (S₁) and triplet (T₁) states of protein, illustrating the proposal that delayed light of protein in liquid at room temperature derives from the triplet, populated by a long lived radical intermediate, the lifetime of which determines the duration of delayed light, as proposed in Scheme 2 in the text.
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so oxygen for its development. Economy of hypothesis demands that an attempt be made to relate the molecular species involved one possibility is that the formation of the long lived (5 sec) species associated with bioluminescence is competitive with Pz* with Scheme 2, and identifying PI* with the radical storage state and the lifetime of the luminescence would remain unaffected, since in the two processes. Since the rate of decay of delayed light is media on the route to bioluminescence. The relative energies 350 nm, it would appear that Si cannot be an obligatory inter-

mediate on the route to bioluminescence. The relative energies of protein fluorescence is observed at around 450 nm (2), and since protein fluorescence is observed at around 200 to 200 sec), a further light output following a different rate law continued for several hours. This they attributed to a charge separation mechanism with the formation of radicals of the organic compound and trapping of electrons in the glass in which their experiments were conducted.

In our experiments, carried out in the liquid state at room temperature, the lifetime of T1 (Fig. 8) would be expected to be short because of collisional deactivation, and phosphorescence would be correspondingly brief. The mechanism proposed by Debye and Edwards might, however, operate to maintain a population of T1 and give a small, but relatively long lived, emission with a spectral distribution the same as that of protein phosphorescence. In such a scheme, quenching by oxygen would occur by reaction with T1 in competition with the radiative process, but the lifetime of the luminescence would remain unaffected, since this is determined by the rate of formation of T1 from the long lived radical intermediate. This proposal amounts to accepting Scheme 2, and identifying P1* with the radical storage state and P2* with T1.

The excitation spectrum for light-induced bioluminescence follows the absorption spectrum of the enzyme solutions up to 450 nm (2), and since protein fluorescence is observed at around 350 nm, it would appear that S1 cannot be an obligatory inter-

mediate on the route to bioluminescence. The relative energies of protein phosphorescence and bioluminescence do not, however, exclude T1 as an intermediate; if it is included in the bioluminescence scheme, excitation at about 400 nm would presumably induce the forbidden So → T1 transition (Fig. 9), although the ratio of oscillator strengths as between S0 → S1 and S0 → T1 seems, at 106, to be somewhat low.

The light-initiated bioluminescence occurs at wave lengths longer than that of the delayed light and requires molecular oxygen for its development. Economy of hypothesis demands that an attempt be made to relate the molecular species involved in the two processes. Since the rate of decay of delayed light is of the same order as the rate of appearance of bioluminescence, one possibility is that the formation of the long lived (5 sec) species associated with bioluminescence is competitive with one or the other of the processes in Schemes 1 and 2. It is also necessary to accommodate the finding that when enzyme is irradiated anaerobically, the capacity to give bioluminescence on secondary addition of oxygen is lost at a rate intermediate between that of the decay of delayed light and the decay of bioluminescence. The second finding excludes the possibility that the processes described by $k_1$ in both schemes represent the formation of the bioluminescent intermediate. In the first case, $k_1 > k_2$, and the bioluminescent intermediate would have to be formed rapidly or not at all, while in the second case its formation would be competitive with delayed light emission and should have the same time dependence as $k_1$. To account for bioluminescence it is therefore necessary to propose a reaction of oxygen with an intermediate different from those in Schemes 1 and 2. A suitable scheme is shown in Fig. 9, in which the formation of the intermediate with a lifetime of 1 sec is proposed. This is stabilized by reaction with oxygen to form a long lived (5 sec) intermediate which may be identical with Intermediate II of Fig. 1. From this the emitting species (L*) is populated.

This scheme thus formulates the bioluminescent reaction as different in kind from delayed light emission and involving other intermediates. Such a proposal, although perhaps untidy, is consistent with the wide distribution of delayed light, which has been obtained from all the proteins so far examined, and with the restricted occurrence of aldehyde-dependent bioluminescence, so far confined to the bacterial enzyme alone.

Weak, long lived luminescence has been observed by Stauff (10) following irradiation of many compounds with visible light. This luminescence was obtained with aqueous solutions of several proteins as well as with simple organic compounds. As the data were not reported in terms of quanta and since emission spectra were not determined, accurate comparison with our results is difficult. Repetition of some of his experiments indicates that the light-induced emission we have called bioluminescence is at least 10^9 times more intense, on a quantum per protein molecule basis, than the luminescence he described. Moreover, the mechanism of excitation in his experiments by visible light in the region of 450 to 700 nm (where these solutions exhibit minimal absorption) is even more difficult to explain, and may call for a 2-photon process. For these reasons it seems preferable not to attempt to reconcile the two sets of findings at this time.

FIG. 9. Energy diagram depicting intermediate states proposed for the light-induced bioluminescence, showing that it may be populated either via $S_1$ or $T_1$. The energy levels of the intermediates states between $T_1$ and $L^*$ (the emitting species in bioluminescence) are arbitrary; the relative levels of these two are deduced from the spectral distribution of phosphorescence and bioluminescence.
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