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Chemiluminescence of flavins in the presence of Fe(II)

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Abstract

The chemiluminescence generated following the reduction of flavin mononucleotide, flavin adenine dinucleotide and riboflavin by Fe(II) in pyrophosphate buffer is reported. These flavins also produce chemiluminescence in the presence of Fe(II) and hydrogen peroxide. The chemiluminescence generated from flavin mononucleotide, hydrogen peroxide and Fe(II) is five to six orders of magnitude lower than bacterial bioluminescence and in the absence of peroxide the chemiluminescence is reduced by a further order of magnitude. The role of hydroxyl radicals in the generation of electronic excited states is discussed and a possible mechanism proposed.

Keywords: Chemiluminescence; Flavins; Fe(II); Peroxide; Bacterial luminescence

1. Introduction

The bioluminescent reaction of the flavoprotein bacterial luciferase (EC 1.14.14.3) has a mechanism which is believed to involve the oxidation of bound reduced flavin mononucleotide ($FMNH_2$) [1]. In bioluminescent assays, flavin mononucleotide (FMN) is normally reduced by H_2 catalysed by Pt, dithionite or nicotinamide adenine dinucleotide (NADH) catalysed by an NAD(P)H:FMN oxidoreductase [2]. Prolonged bioluminescence may be observed using metal ions or complexes [3,4] and this paper reports the chemiluminescence, in the absence of luciferase, that is observed following the reduction of flavins by Fe(II). Results for FMN, flavin adenine dinucleotide disodium salt (FAD) and their precursor riboflavin are described.

Previous reports of chemiluminescence from riboflavin [5] and FMN [6], in aqueous solution enhanced by Cu(II) and aryl-substituted benzopteridines [7] in aqueous dimethylformamide, have all involved hydrogen peroxide. The chemiluminescent reaction has generally been believed to involve the oxidation of flavins by hydrogen peroxide catalysed by copper ions. Studies of chemiluminescence with substituted flavins in modelling bioluminescent reactions have shown that the decomposition of hydroperoxyflavins is involved [8] and implied that the chemiluminescent emitter is a hydroxyflavin radical [9].

We have observed the reduction of flavins when they are mixed with certain metal ions, and the chemiluminescence following the reoxidation of the reduced flavins. In this paper the redox reactions involved are discussed and a mechanism for the chemiluminescence is proposed.

2. Experimental details

2.1. Chemicals

All inorganic chemicals were AnalaR grade and were used as supplied by BDH, UK. Deionised water was purged for 30 min with nitrogen to expel the dissolved oxygen before being used to prepare metal ion solutions. Phosphate and pyrophosphate buffers were 0.1 M and pH 7.0. Fe(II) solutions were made from $FeSO_4 \cdot 7H_2O$. FMN sodium salt (synthetic, 81%), flavin adenine dinucleotide disodium salt (FAD, 95%), riboflavin (98%), bovine serum albumin (BSA, fraction V, 96–99% albumin), n-decanal (99%), *dl*-dithiothreitol (DTT, 99%) and bacterial luciferase from *Vibrio harveyi* (50% protein) were used as supplied by Sigma, UK. Flavin solutions were prepared daily and kept in the dark when not in use. Luciferase solution containing BSA (20 mg l^{-1}), DTT (6.48×10^{-3} M) and luciferase (5×10^{-6} M) in buffer was prepared daily and stored in an ice bath.

2.2. Methods

The absorption spectra of flavins were measured using a Shimadzu 190 UV–Vis spectrophotometer. Luminescence was measured using a Packard 2002 liquid scintillation

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counter in a batch reaction. To a glass vial buffer (3 ml), FMN (10–100 ml, ca. 1.05×10^{-3} M), and in the case of bioluminescence, enzyme solution (10–100 μl, ca. 5×10^{-6} M) were added consecutively. The vial was placed in the scintillation counter to measure the background emission. After that, Fe^{2+} solution (100 μl, ca. 0.1 M) was pipetted into the solution to start the chemiluminescent or bioluminescent reactions. Counting started 15 s after mixing the final component and readings were recorded against time. Usually 0.1 or 0.2 min counts were taken. The first reading is used as initial intensity of chemi- or bio-luminescence. The scintillation counter was calibrated using standards from Biolink Ltd, UK [10].

3. Results and discussion

3.1. Reduction of flavins

The reduction potentials of the free flavins being considered here at pH 7 are all around -0.22 V versus the standard hydrogen electrode for the two electron reduction [11] and several metal ions and complexes have been shown to reduce the flavins [4]. Although Fe(II) in water or phosphate buffer is unable to reduce the flavins at pH 7, in pyrophosphate buffer the reduction potential is sufficiently negative to achieve the reduction.

The absorption spectrum of the FMN before and after reduction by Fe(II) in pyrophosphate buffer is shown in Fig. 1. The yellow colour of FMN, with a strong band at 445 nm, disappears upon addition of Fe(II) in pyrophosphate buffer, and the colourless reduced $FMNH_2$, is formed. Reduced in situ by Fe(II) in pyrophosphate buffer, $FMNH_2$ has previously been shown to be fully active in the bioluminescent reaction catalysed by bacterial luciferase [3].

3.2. Chemiluminescence of reduced flavins

Chemiluminescent emission in a simple system containing a flavin and Fe(II) in aqueous solutions in the presence of oxygen has been observed. The flavin was first converted by Fe(II) in pyrophosphate buffer to the reduced form, which was readily oxidised back to flavin by the molecular oxygen dissolved in the solution. Chemiluminescence was observed to accompany the reoxidation. The time dependence of this chemiluminescence for reduced FMN, FAD and riboflavin is shown in Fig. 2. The curves (dotted lines) are exponential fits. The half life of the chemiluminescence was about 10 s.

The intensity of chemiluminescence increased both with Fe(II) concentration (Fig. 3) and flavin concentration (Fig. 4). Under anaerobic conditions the flavin remained in the reduced form and no chemiluminescence was observed until the addition of air equilibrated buffer, showing the requirement for oxygen.

The kinetics of the oxidation of $FMNH_2$ following dithionite reduction have been reported [12]. The reaction is auto-

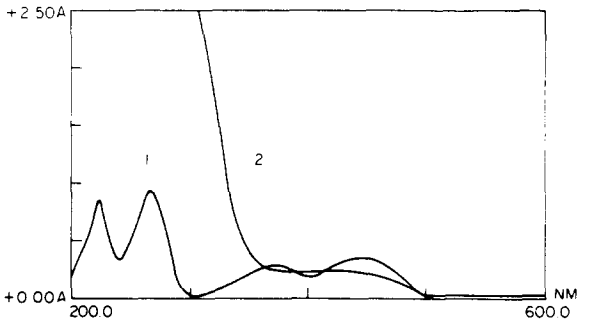

Fig. 1. The spectrum of FMN (1) prior to and (2) after reduction by Fe(II) in pyrophosphate buffer: FMN, 2.9×10^{-5} M; Fe(II), 1.4×10^{-2} M.

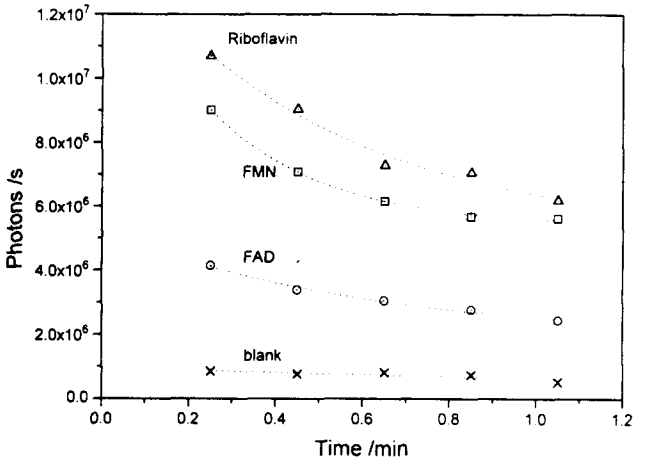

Fig. 2. The change in Fe(II)-induced chemiluminescence of flavins with time; Fe(II), 3.1×10^{-3} M; FMN and riboflavin, 3.1×10^{-6} M; FAD, 7.8×10^{-6} M.

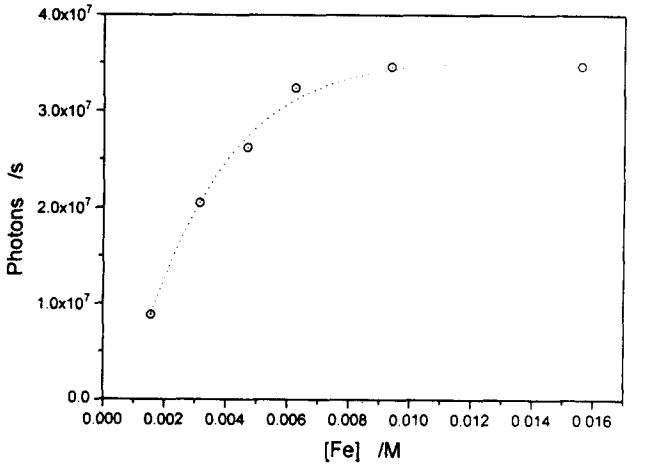

Fig. 3. The initial chemiluminescence of FMN as a function of Fe(II) concentration: FMN, 3.1×10^{-5} M.

catalytic and the products are FMN and H_2O_2. It has been suggested that there is a direct reaction between $FMNH_2$ and O_2 via an oxygenated intermediate [13].

$$FMNH_2 + O_2 \longrightarrow [FMNH_2\text{–}O_2] \longrightarrow \longrightarrow FMN + H_2O_2 \quad (1)$$

There is also another pathway believed to involve the oxidation of the semiquinone flavin FMNH:

$$FMN + FMNH_2 \longrightarrow 2FMNH \quad (2)$$

$$2FMNH + O_2 \longrightarrow 2FMN + H_2O_2 \quad (3)$$

The electronic excited species may be derived from the oxygenated intermediate as shown in Eq. (1), which is a product of the reaction of $FMNH_2$ and oxygen, but the nature of the final emitting species is not clear.

We have also examined the reoxidation of the $FMNH_2$, following reduction of FMN by dithionite, from which no chemiluminescence was observed. Dithionite has been shown to inhibit the chemiluminescence of riboflavin induced by peroxide [14]. Sulphite, a product of dithionite oxidation, has been shown to form a complex with flavins and this affected the bioluminescence of bacterial luciferase if hydrogen peroxide is present [15–18]. A possible explanation for the inhibition in the dithionite system is that the formation of the FMN–SO_3^{2-} complex may also affect the chemiluminescence of FMN, as upon reoxidation, this complex could lead to a different electronic state from that of FMN in the chemiluminescent system with metal ions.

3.3. Chemiluminescence involving hydrogen peroxide

Hydrogen peroxide reacts with FMN in aqueous solutions to give very weak chemiluminescence. This weak chemiluminescence was slightly enhanced by the addition of superoxide, but was significantly enhanced by the addition of Fe(II) which generates hydroxyl radicals with H_2O_2 (Fenton's reaction) [19].

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO^{\cdot} + HO^- \quad (4)$$

A possible mechanism is that FMN reacts with hydroxyl radicals to form an intermediate similar to that in Eq. (1), which leads to an electronically excited flavin species. This suggestion is supported by the observation that the addition of dimethylsulphoxide, a known hydroxyl radical trap [20],

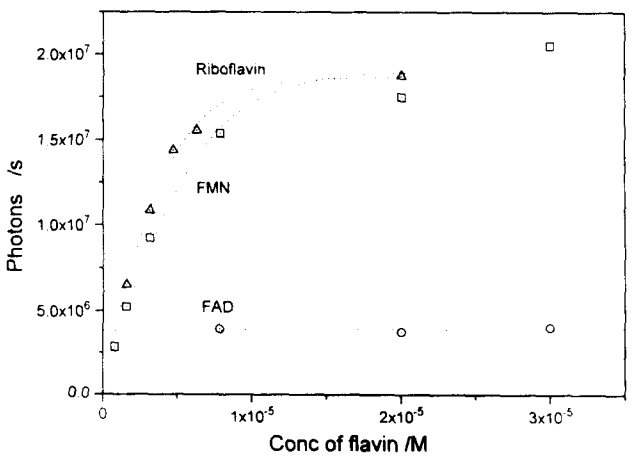

Fig. 4. The initial chemiluminescence with Fe(II) as a function of flavin concentration: Fe(II), 3.1×10^{-3} M.

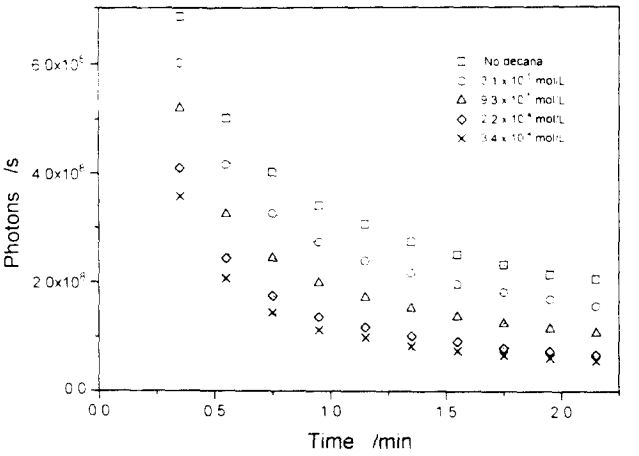

Fig. 5. The effect of decanal on Fe(II)-induced chemiluminescence of FMN: FMN, 3.2×10^{-5} M; H_2O_2, 2.7×10^{-2} M; Fe(II), 3.0×10^{-3} M.

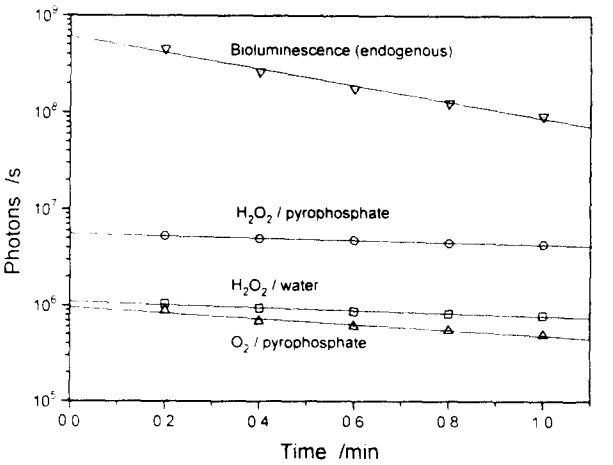

Fig. 6. The comparison of Fe(II)-induced endogenous bioluminescence with chemiluminescence of FMN with and without hydrogen peroxide: FMN, 3.2×10^{-5} M; H_2O_2, 8.9×10^{-2} M; Fe(II), 3.0×10^{-3} M; luciferase, 4.6×10^{-7} M.

inhibits the chemiluminescence from both $FMNH_2$ and FMN–H_2O_2.

The effect of decanal, which enhances the bioluminescence in the presence of luciferase, was to increase the rate of decay of the chemiluminescence without significantly changing the initial level of chemiluminescence (Fig. 5). The scavenging effect of decanal on the hydroxyl radicals is probably the cause of this decay.

In the presence of hydrogen peroxide in pyrophosphate buffer, the Fe(II) is able to reduce the FMN, and at the same time reacts with H_2O_2. This leads to a significant increase in the light output indicating that, in the presence of hydroxyl radicals, the chemiluminescence is from both the reoxidation of $FMNH_2$ and the reaction of FMN with $OH^{\cdot}$. The relative intensities are shown and compared with endogenous bioluminescence in Fig. 6. Endogenous bioluminescence refers to bacterial bioluminescence in the absence of aldehyde and is about three orders of magnitude lower in intensity than normal bioluminescence. Thus the chemiluminescence is five to six orders of magnitude lower than bioluminescence.

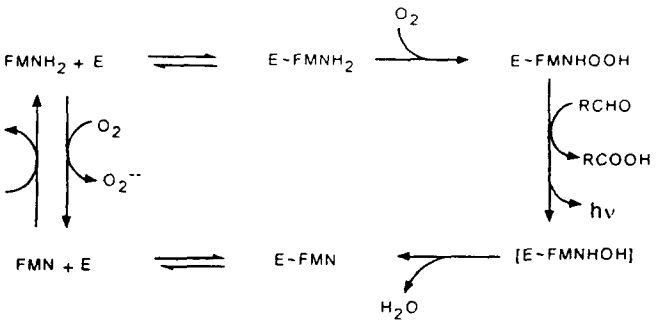

Fig. 7. Simplified mechanism for bacterial luciferase. E is bacterial luciferase, RCHO is decanal.

The proposed mechanism [1] for bioluminescence of bacterial luciferase (Fig. 7) involves an enzyme–FMN intermediate [E–FMNHOH] which generates an electronically excited light-emitting species. Very little is known about the chemiluminescence of FMN alone, mainly due to the lability of oxygenated FMN in aqueous solutions. By using a more stable model compound, *N*(5)-alkyl derivatised FMN, chemiluminescence in the presence of aldehyde has been demonstrated suggesting an intermediate involving –OOH attached at the 4a position of the flavin [21]. A similar intermediate has been reported to be generated at an electrode [9].

It is possible that an intermediate, leading to the formation of an electronic excited emitting species, could be produced directly from flavin and hydroxyl radicals generated in Fenton's reaction:

$$FMN + OH^{\cdot} \longrightarrow FMN\text{–}OH \qquad (5)$$

A similar flavin intermediate may also be formed in the reoxidation of flavins as chemiluminescence is the product of the reaction:

$$FMNH_2 + O_2 \longrightarrow FMN\text{–}OH + OH^{-} \qquad (6)$$

The quenching of luminescence with dimethylsulphoxide is consistent with $HO^{\cdot}$ radicals being of importance in chemiluminescence.

It has not yet been possible to obtain emission spectra of these chemiluminescent reactions due to the low intensity of the emission.

4. Conclusion

It has been shown that chemiluminescence can be observed from both the aerial oxidation of reduced flavins and the oxidation of flavins by hydroxyl radicals and it is proposed that both chemiluminescent reactions may involve the same emitter following the formation of a hydroxyflavin species.

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