An Improved Assay for Bacterial Luciferase using Ti^{III}

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A novel assay for bacterial luciferase, which gives enhanced and prolonged luminescence, using Ti^{III} is described.

Bacterial luciferase (EC 1.14.14.3) is a commercially available monooxygenase, which catalyses the oxidation by oxygen of $FMNH_2$ to FMN and a long-chain aldehyde to the corresponding acid with the concomitant emission of light,[1] eqn. (1).

$$RCHO + O_2 + FMNH_2 \rightarrow RCO_2H + FMN + H_2O + h\nu \quad (1)$$

The intensity of light depends upon the enzyme, aldehyde, reduced flavin and oxygen concentrations. The mechanism has been extensively studied[2] and a simplified outline is shown in Fig. 1.

Bacterial luciferase is unusual among flavoenzymes in binding FMN weakly[3] ($K = 1 \times 10^{-4}$ mol dm^{-3}) and free $FMNH_2$ must be supplied for luminescence. There is competition between the enzyme-catalysed oxidation of $FMNH_2$ to FMN and the rapid non-enzymatic oxidation of $FMNH_2$ to FMN by oxygen. The assay of luciferase is thus carried out either using pre-prepared $FMNH_2$, or FMN reduced *in situ*.[4] The turnover number for luciferase is low[1] at around 4 min^{-1} and it is a consequence of the rapid oxidation by oxygen of $FMNH_2$, that there is normally no turnover of the enzyme in these assays unless $FMNH_2$ is generated continuously as can

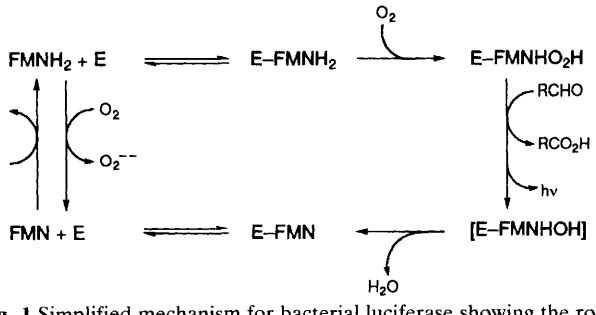

Fig. 1 Simplified mechanism for bacterial luciferase showing the role of $FMNH_2$. (E is bacterial luciferase, RCHO is decanal.)

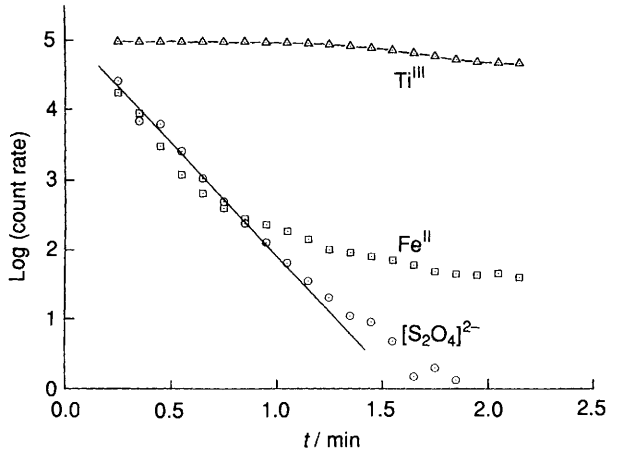

Fig. 2 Change in intensity of light with time for different reducing agents. Line indicates fit to exponential decay. (Conditions in text.)

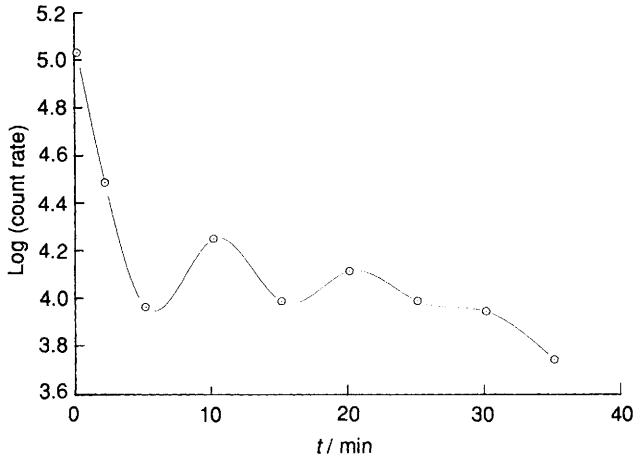

Fig. 3 Change in intensity of light with time for Ti^{III} reduction at low oxygen concentration. [Conditions as in text except lower concentrations of Ti^{III} (6.0×10^{-4} mol dm^{-3}) and FMN (3.2×10^{-8} mol dm^{-3}) used to optimise signal at low oxygen concentrations].

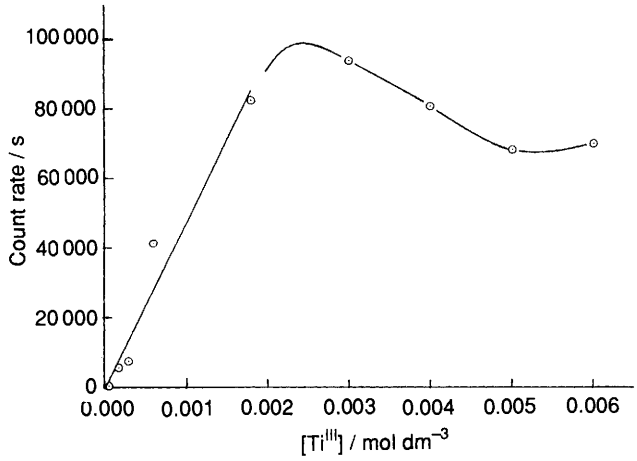

Fig. 4 Effect of Ti^{III} concentration on intensity of light. [Conditions as in text except FMN (3.2×10^{-5} mol dm^{-3}). Readings taken 15 s after mixing].

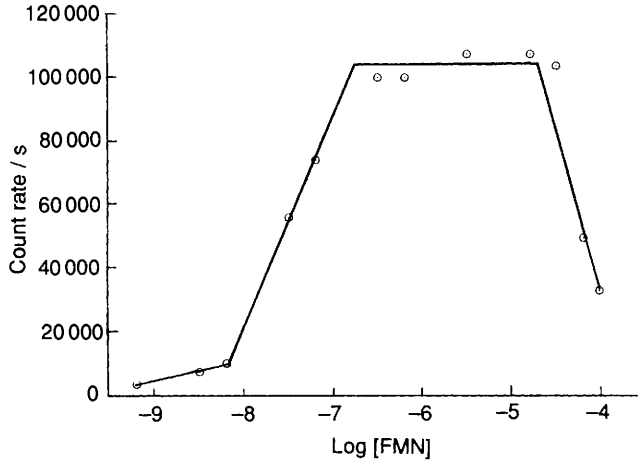

Fig. 5 Effect of FMN concentration on intensity of light. [Conditions as in text except Ti^{III} (1.8×10^{-3} mol dm^{-3}). Readings taken 15 s after mixing].

be achieved by linking to another enzyme-catalysed reaction *via* NAD(P)H.[5]

One-electron reduction of oxygen is less favourable than two-electron reduction[6] and this led us to consider one-electron reducing agents such as transition metal ions and complexes as reducing agents that might reduce FMN more rapidly than oxygen. Those M^{2+} ions known to be inhibitors of bacterial luciferase were excluded.[7]

The rates of many of the kinetic steps in the bioluminescent reaction have been reported[8] and the equilibrium constant for the formation of the $FMNH_2$ enzyme complex is 1×10^5 mol^{-1} dm^3. The reaction of $FMNH_2$ with oxygen shows complex kinetics[9] but $FMNH_2$ typically has a half-life of around 0.1 s.

We have confirmed that reducing agents that reduce oxygen more slowly than FMN will give luminescence directly. In this paper, we report preliminary results on Ti^{III} as a reducing agent and compare this with Fe^{II} and $S_2O_3^{2-}$.

Experiments were carried out using a batch procedure with a Packard 2002 liquid scintillation counter for measurement. Luminescence was measured from a solution (3.1 cm^3) in a vial after mixing with enzyme. In a typical experiment, the mixed solution contained luciferase (1.6×10^{-8} mol dm^{-3}), FMN (3.3×10^{-6} mol dm^{-3}), decanal (3.2×10^{-5} mol dm^{-3}) and Ti^{III} or other reducing agent (3.1×10^{-3} mol dm^{-3}) in pH 7 buffer. The results for Ti^{III}, Fe^{II} and $S_2O_3^{2-}$ as reducing agents are shown in Fig. 2.

With dithionite as reducing agent, the oxygen present in solution was rapidly removed and a single turnover of the enzyme was observed with an exponential decay of the light with a half-life of around 10 s. Similarly, Fe^{2+}, which also reduced oxygen rapidly, showed initially an exponential decay indicating a single turnover and gave similar levels of light emission. In contrast, Ti^{III} in phosphate buffer at pH 7 reduced oxygen more slowly ($t_{1/2}$ = 27 s) than FMN ($t_{1/2}$ = 0.03 s) under typical conditions for bioluminescence and it gave a significant improvement in intensity over both dithionite and NADH assays. In this case the decay of the light was prolonged and no longer exponential, indicating turnover of the enzyme. By reducing the oxygen concentration in the solution, it was possible to prolong further the emission of light since Ti^{III} remained available to reduce further FMN and the reaction was able to cycle. This is illustrated in Fig. 3 in which the solutions have been extensively degassed with nitrogen. There is initially a rapid drop in light as the oxygen concentration is lowered to diffusion levels, followed by a gradual decrease in the signal as the Ti^{III} becomes depleted. Addition of further Ti^{III} increases the signal. The oscillations are characteristic of these reactions and depend on the concentrations of the reagents.

The dependence of initial light intensity upon Ti^{III} concentration is shown in Fig. 4. There is an initial increase in signal as the concentration of Ti^{III} increases corresponding to increased $FMNH_2$ concentration and over this range the

solution was the characteristic yellow of FMN. At 2.0×10^3 mol dm^{-3} the solution was colourless indicative of the absence of unreduced FMN, at 3.0×10^3 mol dm^{-3} the solution was purple indicating excess Ti^{III} and above this a precipitate formed reducing the light measured.

The dependence of initial light intensity upon FMN concentration is shown in Fig. 5. As the concentration of $FMNH_2$ increases there is, initially, the expected increase in light output corresponding to an increased concentration of the E–$FMNH_2$ complex. The competing non-enzymatic oxidation of $FMNH_2$ has been shown to indicate a lag phase, which is reduced in the presence of FMN. Thus, it is possible that the decrease in light at higher FMN concentrations is due to the catalysis of autoxidation of $FMNH_2$ by FMN.

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