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THE EFFECT OF LUCIFERASE AND NADH:FMN OXIDOREDUCTASE CONCENTRATIONS ON THE LIGHT KINETICS OF BACTERIAL BIOLUMINESCENCE

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The effects of NADH:FMN oxidoreductase and luciferase concentrations on the light kinetics of the bacterial bioluminescent reaction were investigated. Light emission with low decay rates was obtained by regulating the conversion of NADH to NAD^+ by controlling oxidoreductase activity. Constant light emission can be obtained when the oxidoreductase activity is below 2.5 U/l in the assay system. The luciferase concentration affects the light intensity but it has no effect on the decay rate of light emission. The substrate decanal and the end-products NAD^+ and capric acid had no effect on the light kinetics. The Michaelis constants of bacterial luciferase for $FMNH_2$ and decanal were 3×10^{-6} M and 8×10^{-7} M, respectively, and those of oxidoreductase for FMN and NADH were 6.1×10^{-6}M and 1.6×10^{-5} M, respectively.

Bacterial luciferase (BL) and NADH:FMN oxidoreductase (OR) catalyze the oxidation of NADH, $FMNH_2$ and a long chain aliphatic aldehyde (R-CHO) thereby producing light emission according to the following coupled reactions (1):

$$(1)\ NADH + H^+ + FMN \xrightarrow{OR} NAD + FMNH_2$$

$$(2)\ FMNH_2 + O_2 + R\text{-}CHO \xrightarrow{BL} FMN + R\text{-}COO^- + H_2O + h\nu$$

Specific oxidoreductases exist for both NADH and NADPH and this makes possible the selective measurement of these coenzymes by using the relevant coenzyme dependent enzyme assay (2). Because highly sensitive methods are available for measuring light emission, the OR-BL coupled reaction system is being increasingly used to determine coenzymes and to investigate reactions in which they take part. Concentrations of NADH as low as 10^{-10} M can be easily determined using this bioluminescent technique (3-5).

The light kinetics produced in the bacterial bioluminescence reaction usually results in a relatively rapid increase in light emission followed by a

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somewhat slower decay of light intensity. The reaction rate decay is dependent on the concentrations of substrates for the luciferase enzyme as shown by Meighen and Hastings (6). The light intensity has also been shown to be proportional to the product of the concentrations of BL and OR (7).

Commercially available preparations of bacterial luciferase contain both luciferase and oxidoreductase(s) in an indefinite mixture which produces light emission decay curves of continuously decreasing light intensity. These preparations also contain many impurities such as alcohol dehydrogenase, lactate dehydrogenase, flavins etc., which have made it difficult to examine the effects of BL and OR concentrations on the kinetics of bacterial bioluminescent light emission. As far as we know only Brolin and Hjerten have studied the reaction kinetics using enzymes free of contaminating, interfering compounds (8).

In this study, we evaluate the effects of the concentrations of OR and BL on the light kinetics of the bacterial bioluminescent reaction. The aim was to produce a stable level of light emission for at least a minute or more and thus decrease the rate of light decay. The use of very highly purified luciferase and oxidoreductase preparations made it possible to obtain controlled kinetics more suited to analytical applications.

MATERIALS AND METHODS

FMN, decanal, NADH and FMN-agarose were purchased from the Sigma Chemical Company, Saint Louis, Missouri 63178, USA. All other reagents were of analytical grade. Luciferase and oxidoreductase were isolated from the luminous bacterium Vibrio harveyi (ATCC 14126). The cells were grown up to late exponential phase, harvested and stored frozen at -20°C until used (9). Luciferase was purified according to the method of Baldwin et al. (10) but with an additional purification step in which FMN-agarose was used as an affinity chromatography medium. Oxidoreductase was purified according to the method of Jablonski and DeLuca (2).

Luciferase activity was measured using bioluminescence in a reaction in which FMN was reduced by dithionite in the presence of hydrogen peroxide and catalase, the products being $FMNH_2$ and oxygen (5, 11). Decanal was used as the aldehyde. In addition to the luciferase sample, the 0.6 ml reaction mixture consisted of 5.3 μM FMN, 3.4 mM dithionite, 2.7 mM decanal and 130 U of catalase. The buffer used for the assay was 0.1 M potassium phosphate pH 7.0 containing 0.08 % bovine serum albumin. The reaction was initiated by injecting 0.05 ml of 1.0 M H_2O_2 and the peak height of the resulting flash of light was recorded. The luciferase activity was expressed as millivolts (mV). 7 mV of activity corresponded approximately to 0.13 ng of luciferase. The activity of the oxidoreductase was measured spectrophotometrically by following the absorbance decrease of NADH at 340 nm in a reaction mixture containing NADH and FMN in phosphate buffer at pH 7.0 (2, 5).

The coupled bioluminescent assay contained FMN (2×10^{-6} M), decanal (2×10^{-5} M) and varying amounts of NADH, BL and OR in a total volume of 0.5 ml.

The luminescence was recorded in a 1250 Luminometer coupled to a chart recorder (LKB-Wallac, Turku, Finland).

RESULTS

1. Interdependence of luciferase and oxidoreductase activities

Although the light produced in the bacterial bioluminescent system arises from the BL reaction, the interdependence of the two enzymes involved apparently regulates the intensity of light emitted over a period of time. The light emission curves produced by varying the concentration of either OR or BL when they are both present in the same reaction mixture are shown in Figure 1. In the presence of a constant amount of BL the flash-like signal of light emission increases with an increasing amount of OR (Fig. 1A). However, the period of constant light emission decreases and the rate of light decay increases. If the amount of OR is kept low and constant (Fig. 1B) and the BL concentration is increased, the rate of light decay is unaffected.

Figure 2 shows the effect of BL and OR activities on the light signal decay rate. This result confirms the fact that OR (Fig. 2A) but not BL (Fig. 2B) influences the decay rate. The overall peak light intensity is a function

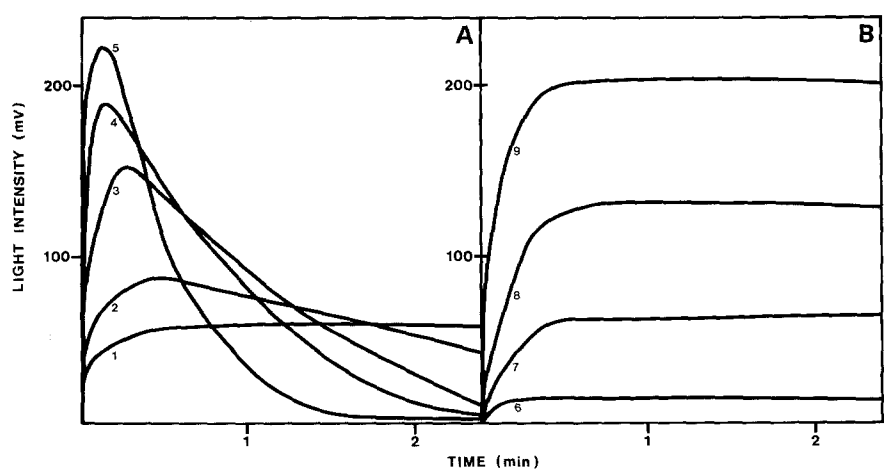

Fig. 1. The dependence of light kinetics on NADH:FMN oxidoreductase (A) and bacterial luciferase (B) activities. In curves 1-5 the BL content was kept constant (300 V/0.5 ml) and the OR activity in an assay volume of 0.5 ml was as follows: 1 = 1.0 mU, 2 = 1.6 mU, 3 = 3.8 mU, 4 = 6.5 mU, 5 = 20.2 mU. In curves 6-9 the OR activity was kept constant (1.0 mU) and the BL content in the assay volume of 0.5 ml was as follows: 6 = 70 V, 7 = 200 V, 8 = 400 V, and 9 = 550 V. The composition of the reaction mixture is detailed in Materials and Methods. The concentrations of NADH, FMN and decanal were 10^{-8} M, 2 x 10^{-6} M, and 2 x 10^{-5} M respectively.

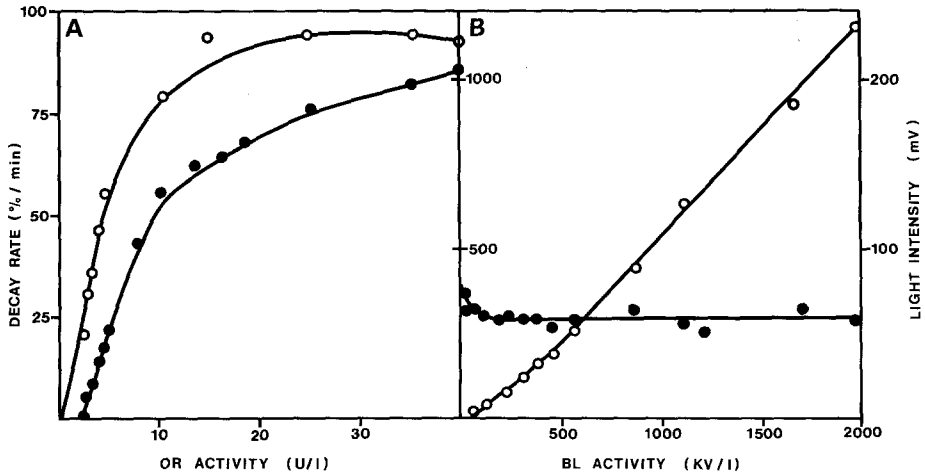

Fig. 2. Light decay rate (•) and light intensity (o) as a function of NADH: FMN oxidoreductase (A) and bacterial luciferase (B) activities. In 2A the BL activity was 600 kV/L in all assays, and in 2B the OR activity was kept constant at 5.6 U/l. The initial concentrations of NADH, FMN and decanal were 10^{-8} M, 2 x 10^{-6} M and 2 x 10^{-5} M, respectively.

of the two reactions. The constant level of light output at high OR concentrations (>15 U/l) can be explained by the rate at which NADH is consumed in the assay system.

Fig. 3 shows the effect of NADH concentration on the light decay rate and light intensity when in the presence of high (3A) and low (3B) OR concentrations. In the presence of low oxidoreductase activity (B) the light decay

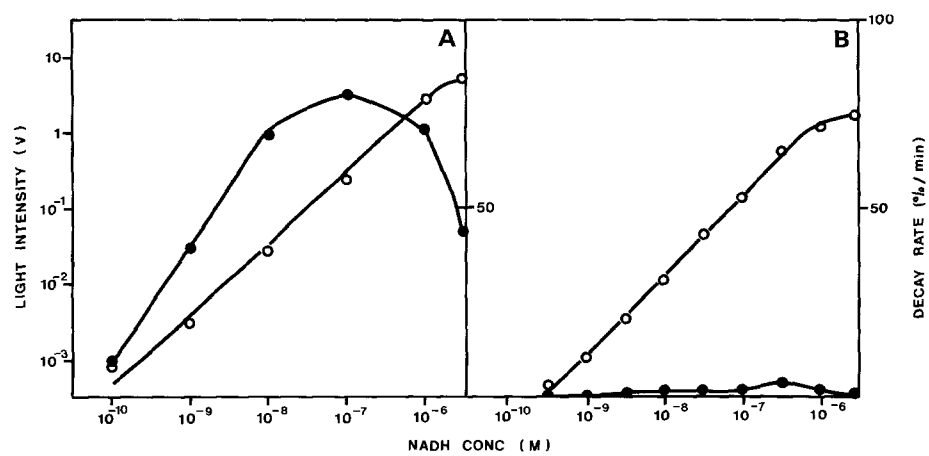

Fig. 3. Effect of NADH concentration on the light decay rate (•) and light intensity (o) of luminescence in the coupled bioluminescent assay at high (A) and low (B) oxidoreductase levels.
3A: The enzyme activities were 60 kV/L for BL and 20 U/L for OR.
3B: The corresponding enzyme activities were 200 kV/L and 1.5 U/L.
The light decay rate was calculated as the percentage decrease in the light intensity per minute after the peak light intensity had been achieved.

rate was low and constant over the whole range of the standard curve for NADH. However, in the presence of high oxidoreductase activity (A) the light decay rate was high and varied with NADH concentration. The decay rate increased with an increase in NADH concentration up to 10^{-6} M but thereafter it declined in response to an increase in NADH concentration.

The results presented in Figs. 1, 2 and 3 show that the key factor affecting the light kinetics of the bioluminescent reaction is not the amount of NADH present in the assay system but the conversion rate of NADH which is itself affected by OR concentration.

2. Correlation between the light decay rate and the luciferase activity resulting from NADH consumption by oxidoreductase activity

Table I lists the Michaelis-Menten constants of purified OR and BL for their relevant substrates. The measurements were carried out at 20°C, pH 7.0. In the coupled bioluminescent assay the upper limit of the linear range for NADH concentration is 10^{-6} M and thus below the K_m constant (1.6×10^{-5} M), as is also the case with the concentration of the FMN (2×10^{-6} M). The decanal concentration (2×10^{-5} M), however, lies above the K_m value (8×10^{-7} M). The production of $FMNH_2$ should therefore be rate limiting for the bioluminescence reaction because of the low OR activity and its low substrate concentrations (NADH and FMN).

In the oxidoreductase reaction, if the rate of change in the concentration of either the NADH substrate or one of the $FMNH_2$ or NAD^+ products is calculated in the presence of varying amounts of OR for a one minute reaction time and then plotted against the measured decay rates, the result obtained is as shown in Figure 4. This figure shows that the rate of light decay

Table I The Michaelis-Menten constants of bacterial luciferase and NADH:FMN:oxidoreductase for some of their substrates. The assay systems used to measure the separate activities is presented in Materials and Methods.

Enzyme	Substrates	K_m (M)
Luciferase	$FMNH_2$	3×10^{-6}
	decanal	8×10^{-7}
Oxidoreductase	FMN	6.1×10^{-6}
	NADH	1.6×10^{-5}

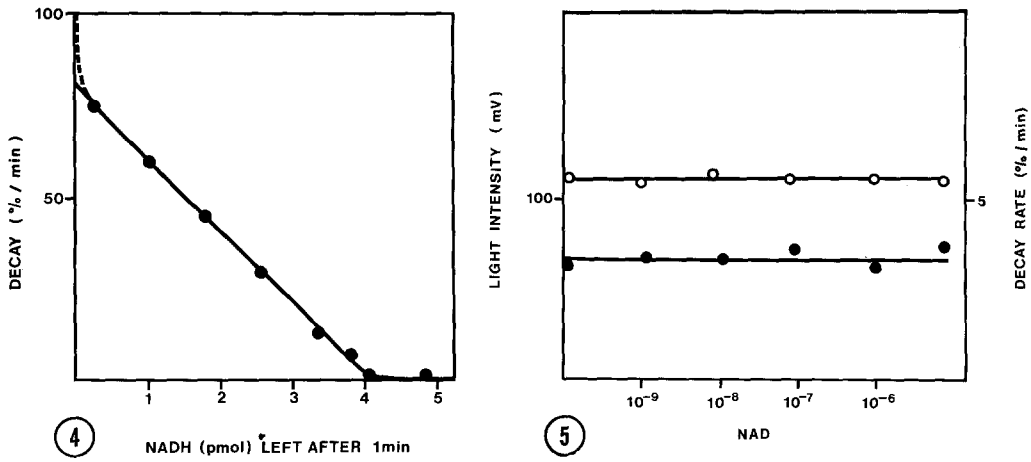

Fig. 4. Correlation between light decay and the calculated NADH content in the assay sample after one minute reaction time. The amount of NADH left after one minute reaction was calculated at different OR concentrations (0.3 - 20.2 mU) using the Michaelis-Menten equation and the K_m value for NADH presented in Table I. The decay rates are expressed as the decrease in light emission after 1 minute reaction (%/min).

Fig. 5. The reaction (o) and light decay (•) rate kinetics of NADH:FMN oxidoreductase (3 U/L) and bacterial luciferase (200 kV/L) with various concentrations of NAD at pH 7.0 in 0.1 M phosphate buffer. The FMN concentration was 2 x 10^{-5} M, and that of NADH 10^{-7} M.

of the coupled bioluminescent reaction in which NADH, FMN, oxygen and aldehyde are substrates, will decrease because of the low consumption of NADH, which in turn reduces the availability of $FMNH_2$ at low OR activity.

NAD^+ produced during the first of the coupled enzyme reactions has no effect on the decay rate of light emitted as a result of the second reaction. This is shown in Fig. 5. This rules out NAD^+ as a light decay rate regulator. In addition, capric acid (an end product of the bioluminescent reaction) at concentrations of between 10^{-8} and 10^{-4} M did not influence the decay rate of the coupled reaction.

DISCUSSION

Constant light emission is preferable when using bioluminescence as an analytical technique because this allows better measurement of the reactants in the assay system. Such a system has been worked out for analyses using firefly luciferase bioluminescence (12). However, no systematic analysis of the kinetics of delayed light emission has been carried out for the bacterial bioluminescence system in which the conversion of NADH or NADPH carried out by the relevant oxidoreductase reaction initiates light production in the bacterial luciferase reaction.

The results presented in this study clearly indicate that light emission with low decay rates can be obtained by regulating the conversion of NADH to

NAD^+ (or FMN to $FMNH_2$) by controlling oxidoreductase activity. Luciferase activity has no influence on the light kinetics, it only affects the peak light emission. These conclusions are based on the data presented in Figures 1 and 2. Peak light emission can thus be regulated by both oxidoreductase and luciferase activity, while the decay rate of luminescence depends only on the concentration of oxidoreductase in the reaction system. This is attributed to the rate of NADH conversion taking place in the OR reaction. At high oxido-reduction activity, repeated additions of NADH give reproducible signals of light emission having rapid decay rates. This makes it possible to perform several successive NADH assays in the same reaction mixture and to calibrate the system by a second addition of a known amount of NADH (5). These results show that both the luciferase and oxidoreductase retain their original activities and thus the fast decay rate cannot be attributed to irreversible denaturation of either of these enzymes.

The results presented in Fig. 3 confirm these conclusions. In the presence of high oxidoreductase concentrations the decay rate of luminescence increases as a function NADH concentration up to 10^{-7} M. When the NADH concentration increases further, the light decay rate begins to fall because a build up of this substrate caused by its low rate of consumption in the reaction. When the NADH concentration is far below the oxidoreductase K_m value the oxidoreductase activity is low, and as a result of this the decay rate decreases because the consumption of NADH decreases. However, at very low oxidoreductase activities (Fig. 3B), NADH concentration has no effect on the kinetics of light emission because the oxidoreductase activity is so low that only a very small amount of NADH is consumed.

We can conclude that all the factors affecting the oxidoreductase activity also have an effect on the decay rate of luminescence in the coupled bioluminescent assay system. The oxidoreductase concentration must be below 2.5 U/l to allow constant light emission. In this study we have only presented the results of the NADH:FMN oxidoreductase-luciferase system, but similar results are obtained using NADPH:FMN oxidoreductase, except that the concentration of NADPH:FMN:oxidoreductase required is much lower to produce constant light kinetics due to its lower K_m value for NADPH, which was found to be 5×10^{-7} M.

NAD and decanal have no influence on the light decay rate over the concentration ranges 5×10^{-10} - 10^{-5} M and 10^{-8} - 10^{-4} M respectively. However, the alkyl chain length of the aldehyde has an effect on the decay rates of luminescence as already shown by Hastings et al. (13). It was shown by

Hastings and Gibson (14) that $FMNH_2$, oxygen and luciferase concentrations have no effect on the decay rate of the luciferase reaction. Our experiments confirm these results.

According to the results presented here a "steady state" can be achieved in the bacterial bioluminescence reaction in which none of the reactants is significantly consumed. This is made possible by limiting the rate of NADH consumption by lowering the amount of OR in the assay system.

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