JOURNAL OF LUMINESCENCE 5 (1972) 464–471

A PROPOSED MECHANISM FOR THE TRANSIENT SUPPRESSION OF BACTERIAL LUMINESCENCE BY HYDROGEN PEROXIDE

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Received 15 June 1972

It has been shown previously that bacterial luminescence is reversibly suppressed *in vivo* and *in vitro* by both X-rays and hydrogen peroxide. The data presented here show that the mechanism for this phenomenon is based on the transient oxidation of reduced flavin mononucleotide.

1. Introduction

It has been demonstrated that hydrogen peroxide (H_2O_2) is formed in appreciable quantity by the action of ionizing radiations on oxygenated water[1]). Since living cells always contain and are usually bathed in high concentrations of water and adequate concentrations of oxygen, the conditions for production of H_2O_2 exist in most organisms during irradiation. However, successful attempts to demonstrate radiomimetic effects of H_2O_2 on living systems are few[2]).

Steady-state luminescence can be obtained easily from intact luminous bacteria (*in vivo*) or from cell-free extracts (*in vitro*). This biochemical homeostasis is immediately suppressed by X-rays. After irradiation, luminescence recovers promptly[3, 4]). Catalase protects the luminescent system from this effect[5]).

H_2O_2 has been found to mimic this reversible radiation effect *in vivo*[5]). Recently, we have shown that H_2O_2 also mimics radiation effects on luminescence *in vitro*[6]).

The data described in this paper provide the basis for an explanation of this transient suppression of bacterial luminescence produced both by X-rays and by H_2O_2.

2. Background

It has been shown that the reactions which produce bacterial luminescence

are identical *in vivo* and *in vitro*[7]). All of the reactants are known. They are: reduced flavin mononucleotide ($FMNH_2$); molecular oxygen; a long-chain aliphatic aldehyde (RCHO); and the enzyme luciferase (L).

$$NADH + H^+ + FMN \xrightarrow[\text{dehydrogenase}]{\text{NADH-}} FMNH_2 + NAD^+, \quad (1)$$

provides the substrate for luciferase. Luciferase reacts rapidly with $FMNH_2$ to form a luciferase-$FMNH_2$ intermediate. The latter adds molecular oxygen to form another intermediate, and this complex reacts with aldehyde. The aldehyde is oxidized to the corresponding acid, hydroxide is lost, and light is emitted from protonated flavin ($FMNH^+$)[9]).

Several points about reaction (1) are important to this study: *in vivo*, NADH is regenerated continuously by various nutrients[7]); *in vitro*, NADH is added to the extent that it is not rate limiting to the luminescent reactions[4]); bacterial luminescence *in vivo* and *in vitro* is a measure of both the amount of active luciferase and the steady-state concentration of $FMNH_2$[7]); $FMNH_2$ is extremely labile and readily oxidized by many agents including molecular oxygen[10]).

Many enzymes including luciferase are relatively radioresistant *in vivo* because they are literally bathed in molecules which protect them from free radicals and H_2O_2[11]). To mimic this protective effect of the cell's interior, we use bovine serum albumen (BSA) *in vitro*.

3. Methods and materials

3.1. REAGENTS AND EQUIPMENT

Except for NADH-dehydrogenase, all reagents were purchased from Sigma Chemical Co., St. Louis. NADH-dehydrogenase was isolated from *Photobacterium fischeri* and purified 145 fold[12]). Hydrogen peroxide was assayed by the method of Kolthoff[13]).

A Gilford Model 240 spectrophotometer with Model 6040 strip chart recorder was used. Aerobic analyses were done with standard silica cuvettes. Anaerobic analyses were done with a gas-tight, dual side-arm cuvette.

3.2. ASSAY

Table 1 summarizes the composition of solutions used for aerobic and anaerobic assays.

3.2.1. Aerobic analyses

To study peroxide effects on NADH itself, NADH was dissolved in 0.1 M phosphate buffer at pH 7.5. Bovine serum albumin (BSA), when used, was

TABLE 1

Data on the composition of solutions

Reagent	Concentration in stock solution	Volume of stock solution added to cuvette or side-arm		
		Aerobic assay		Anaerobic assay
		NADH only	Reaction (1)	Reaction (1)
Phosphate buffer	0.1 M	3.0	3.0	See NADH below
NADH	10 mg/25 ml buffer	0.1	0.1	3 ml stock
FMN	7.7 mg/100 ml buffer		0.02	1 ml to side-arm No. 1 with 50–200 λ enzyme
BSA	1–10 mg/ml buffer	1 mg/ml buffer	1 mg/ml buffer	1 mg/ml buffer
NADH-dehydrogenase	undetermined		0.05–0.20	0.05–0.20
H_2O_2	A. 1:100 dilution of 30% solution	0.04 (A)	0.04 (A)	0.04 (B) to side-arm No. 2
	B. 1:100 dilution of 3% solution			

added to the buffer before experiments (table 1). Spectrophotometric measurements were made at OD_{340} before and after treatment with H_2O_2.

For studies with the complete dehydrogenase reaction [reaction (1)] NADH, FMN, and NADH-dehydrogenase were suspended in buffer either with or without BSA (table 1). Oxidation of NADH was followed at OD_{340}.

3.2.2. Anaerobic analyses

$FMNH_2$ is rapidly oxidized by molecular oxygen nonenzymatically[10]). Measurements of $FMNH_2$ formation by reaction (1) must be made anaerobically. For these measurements, the gas-tight cuvette was loaded with NADH in buffer. FMN and NADH-dehydrogenase were placed together in one side-arm; H_2O_2 in the other (table 1). This assembly was alternately evacuated at 20 in. of mercury and charged with prepurified N_2 gas at 4 psig for a total of 60 min. To ensure relatively complete anoxia and to rid the system of oxygen liberated by peroxide decomposition, small strips of agar with colonies of viable luminous bacteria were placed inside the cuvette above the solution before degassing. Either NADH or FMN (OD_{445}) can be measured anaerobically.

For both aerobic and anaerobic analyses, measurements were made before H_2O_2 treatment to ensure that no unexpected changes occurred. When freshly isolated NADH-dehydrogenase was used, some measure of its activity was obtained by measuring the rate of NADH oxidation before any peroxide treatment. Of course, dehydrogenase activity depends on its concentration in solution at the final stage of isolation. Thus, depending on this concentration, we added between 0.05–0.20 ml in different experiments (table 1).

After peroxide treatments, all solutions were monitored long enough to ensure observation of all pertinent changes.

4. Results

4.1. NADH

Fig. 1 shows that NADH in phosphate buffer is readily oxidized by 9.3×10^{-3} M H_2O_2 (solid triangles). However, in the presence of BSA, no appreciable oxidation of NADH occurs with similar amounts of peroxide (solid circles). Note that some decomposition of NADH occurs spontaneously (no peroxide, open circles).

Similar protection of NADH by BSA occurs in the presence of FMN and NADH-dehydrogenase. Fig. 1 shows that reaction (1) together with 1 mg ml^{-1} BSA proceeds undisturbed by 9.3×10^{-3} M H_2O_2 (open ∇). Compare this with the same reaction without BSA ($\square$marks).

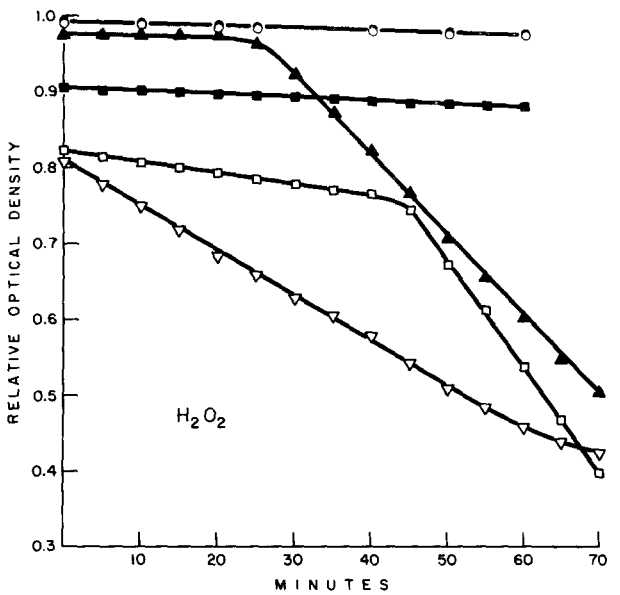

Fig. 1. The effects of 10^{-4} M H_2O_2 on NADH in phosphate buffer, pH 7.5 with the following: ○ NADH only, no H_2O_2 added, ● NADH + BSA (1 mg ml^{-1}), H_2O_2 added at 22 min; ▲ NADH only, H_2O_2 added at 22 min; ■ NADH + FMN + BSA (1 mg ml^{-1}) in phosphate buffer, H_2O_2 added at 43 min; □ NADH + FMN + 0.5 ml NADH-dehydrogenase preparation, H_2O_2 added at 43 min; △ NADH + FMN + 0.2 ml NADH-dehydrogenase preparation + BSA, H_2O_2 added at 43 min.

4.2. $FMNH_2$

Our method measures the rate of disappearance of FMN in the presence of N_2 gas as $FMNH_2$ is formed by NADH-dehydrogenase with NADH as substrate. Fig. 2 shows that 9.3×10^{-3} M H_2O_2 instantaneously oxidizes $FMNH_2$ and that this oxidation is transient. The addition of BSA up to 10 mg ml^{-1} buffer causes no significant change of this result.

5. Discussion

It has been established that bacterial luminescence is reversibly suppressed *in vivo* and *in vitro* by both X-rays and hydrogen peroxide, a product of radiation reactions in aqueous media. The transient nature of this suppression suggests that X-rays and H_2O_2 attack an intermediate which is under continuous production by some biochemical pathway.

The results presented here show for the first time that $FMNH_2$ under continuous production by the NADH-dehydrogenase reaction is transiently oxidized by H_2O_2 even in the presence of relatively high concentrations of

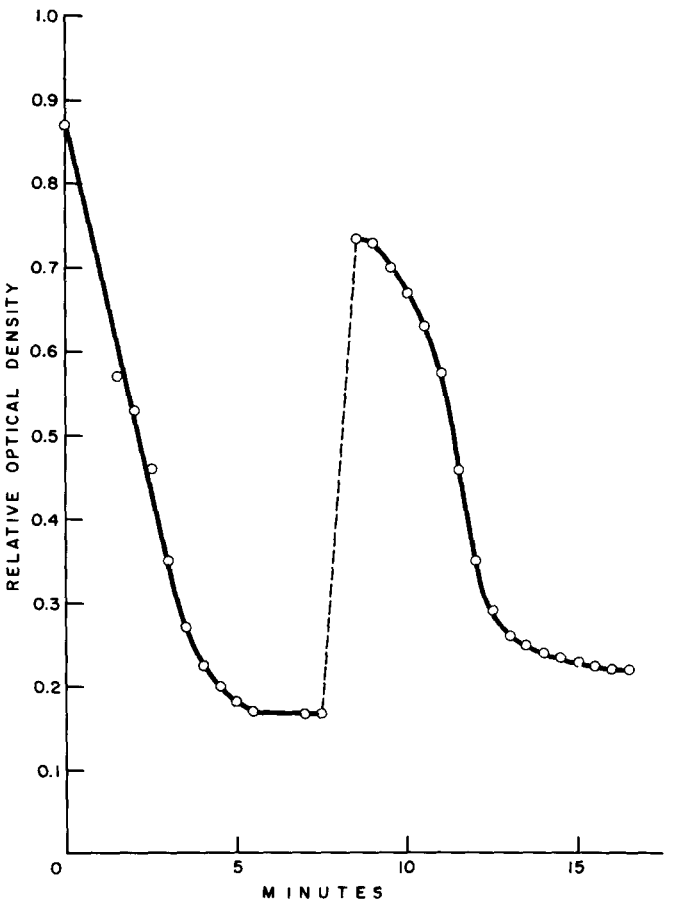

Fig. 2. The transient oxidation of reduced flavin mononucleotide by H_2O_2. The evacuated, gas-tight cuvette contained NADH + FMN + 50λ NADH-dehydrogenase preparation in phosphate buffer, pH 7.5. H_2O_2 (10^{-4} M) was added at 7.5 min. The dashed portion of the curve represents the time necessary to remove the cuvette from the spectrophotometer, add H_2O_2 from one side-arm, and replace the cuvette in the sample compartment.

exogenous protein. These observations provide the basis for a mechanism which would explain the transient suppression of bacterial luminescence by H_2O_2.

The working hypothesis for this study is that steady-state luminescence is the manifestation of biochemical homeostasis that exists between the rate of production of $FMNH_2$ by the NADH-dehydrogenase reaction and the rate of oxidation of $FMNH_2$ by the luminescence pathway, electron transport, and autoxidation by dissolved oxygen. When this competition is increased by X-rays or H_2O_2, luminescence is suppressed in proportion to

the amount of increased competition. Recovery of luminescence, then follows the withdrawal or depletion of this exogenous competition.

The model in fig. 3 suggests the central importance of $FMNH_2$. In our experiments, NADH is added in large quantity while in bacterial cells it is turned over normally using a variety of substrates (XH_2). Oxidation of NADH by H_2O_2 in our experiments would not be recoupable since there is no mechanism for rehydrogenation of NAD^+. Actually, as our results indicate, (section 4.1), NADH in the presence of even low concentrations of

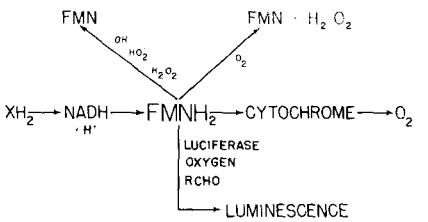

Fig. 3. The relation of various biochemical intermediates to the production of and competition for $FMNH_2$. HO· and HO_2· represent radiation-induced free radicals.

exogenous protein (BSA) is not oxidized significantly by H_2O_2. Further, the rate of oxidation of NADH by NADH-dehydrogenase in the presence of BSA is not altered by H_2O_2. Thus, any NADH-H_2O_2 reactions cannot account for the reversible peroxide suppression of bacterial luminescence.

On the other hand, the primary substrate for bacterial luciferase, $FMNH_2$, is rapidly but only momentarily oxidized by H_2O_2 even in the presence of exogenous protein (10 mg ml^{-1} BSA). This treatment does not affect NADH-dehydrogenase activity to any measurable extent (section 4.1).

Maximum suppression of luminescence in living cells by H_2O_2 occurs within 0.5 min[2]). Luminescence *in vitro* is maximally suppressed by H_2O_2 more slowly but within 1 min in most cases[3]). Our measurements of $FMNH_2$ do not allow precise estimates of the time required for maximum oxidation by H_2O_2, but indicate that this time is compatible with the proposed mechanism (see fig. 2).

Time from H_2O_2 addition to one-half maximum recovery of luminescence *in vivo* varies with the concentration of peroxide, but for 3×10^{-4} M H_2O_2 equals 5 min[2]). For recovery *in vitro*, this time after treatment with 3×10^{-4} M H_2O_2 is 1 min[3]). Our measurements of the recovery half-time for the NADH-dehydrogenase production of $FMNH_2$ is 3 min. Thus, in spite of several variables, the recovery of initial concentrations of $FMNH_2$ as measured spectrophotometrically is compatible with the observations of recovery of luminescence after H_2O_2 addition to luminescent systems *in vivo* and *in vitro* and thus compatible with the proposed mechanism.

We conclude that radiomimetic effects of H_2O_2 do occur provided measurements are made on appropriate systems continuously or with a minimum of interruption since some of these effects are reversible within minutes. We also conclude that the reversible suppression of bacterial luminescence is due to the momentary oxidation of $FMNH_2$ by H_2O_2. Further, peroxide causes no significant change of NADH or NADH-dehydrogenase activity provided protein is present.

Previous data[4]) show that the X-ray-induced suppression of bacterial luminescence is prevented by catalase. This observation very strongly implicates H_2O_2 as an important part of the mechanism which is responsible for this radiation effect. We are exploring this hypothesis.

Acknowledgement

The critical review of Harry Pettengill and Dr. Robert Kaman is acknowledged and appreciated.

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