Induction of bacterial luciferase by pure oxygen

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Xenorhabdus luminescens is a terrestrial bacterium that occurs as a symbiont of soil nematodes, and has also been isolated from human wounds. Unlike the several species of marine bioluminescent bacteria, *X. luminescens* has been discovered to grow in an atmosphere of 100% oxygen. Under these conditions the *in vivo* bioluminescence is greater, and this can be attributed in part to an increased synthesis of luciferase. At the same time, cells also produce superoxide dismutase (SOD), whose activity is also increased after growth in 100% oxygen. The patterns of induction suggest that the two enzymes are co-regulated; possible evolutionary relationships are considered.

Introduction

Although many organisms suffer deleterious effects or will not grow in the presence of a high (e.g. 100%) oxygen atmosphere, some will grow (Fridovich, 1981). In several such cases studied (including *Streptococcus faecalis*, *Escherichia coli* B and *Saccharomyces cerevisiae*) (Gregory & Fridovich, 1973; Gregory *et al.*, 1974), high oxygen tension induced the synthesis of superoxide dismutase (SOD), which is postulated to aid the removal of excess reactive oxygen radicals formed in high oxygen conditions. In *Bacteroides fragilis* and *B. gingivalis*, SOD activity increases two-fold (Gregory & Dapper, 1983; Gregory, 1985; Amano *et al.*, 1990), while in *B. thetaiotaomicron* there is a four-fold increase (Pennington & Gregory, 1986).

The action of such radical oxygen species, and the inability of the organism to remove or inactivate them, is presumably responsible for the adverse effects of 100% oxygen in many cases. All bioluminescent bacteria, require molecular O₂ for light emission; however, a low concentration may suffice (Lloyd *et al.*, 1985), and in some species high oxygen tension inhibits light emission promptly *in vivo* (Hastings, 1952). No systematic study of the effects of high oxygen tension on growth and/or luminescence has been reported. As superoxide ion can react *in vitro* with a luciferase–flavin intermediate to give luminescence (Kürfurst *et al.*, 1983), we investigated the effects of 100% oxygen on growth and luminescence.

Five species of luminous bacteria (Hastings *et al.*, 1985; Neilson & Hastings, 1991) were tested for their ability to grow in a medium equilibrated with 100% oxygen. The four marine species, *Vibrio harveyi*, *V. fischeri*, *Photobacterium phosphoreum*, and *P. leiognathi*, failed to grow. However, a strain of *Xenorhabdus luminescens*, a symbiont of soil nematodes (Poinar *et al.*, 1980) that has also been isolated from human wounds (Farmer *et al.*, 1989; Colepico *et al.*, 1989), continued to grow. Moreover, its bioluminescence was increased up to five-fold, due in part to increased cellular luciferase activity. These responses, along with parallel increases in SOD activity, are described here.

The effect of high oxygen concentrations is considered in relation to the physiological response of the organism and the possible role of the luciferase system in the metabolism of oxygen radicals. In this connection, it is interesting that a nonluminous species, *X. poinarii*, did not grow in 100% oxygen.

Methods

Reagents and solutions. Xanthine oxidase, horse heart ferricytochrome c (type VI), bovine blood superoxide dismutase, type I (SOD), flavin mononucleotide (FMN), hypoxanthine, histidine, purine and dithiothreitol (DTT) were from Sigma. Glycerol was from Baker. Superfine Sephadex G-75 and Sepharose 6B were from Pharmacia. Platinized asbestos for the catalytic reduction of FMN with H₂ was from E. H. Sargent and Co., while disodium ethylenediamine tetraacetate (EDTA) was from Fisher Scientific. *n*-Decanal, 3-aminophthalhydrazide (luminol) and 2,2 diphenylpropylamine were from Aldrich. Aldehyde stock solutions were prepared by sonication of 0.1% (v/v) in distilled water and were freshly diluted to 0.001% for use each day. Oxygen and hydrogen gases were from Med-Tech. All other chemicals used were of analytical grade.

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Protein was estimated using Bio-Rad Coomassie-Blue-binding assay (Bradford, 1976) with ovalbumin (Sigma) as the standard.

Cultures and media. We used a primary form of the luminous terrestrial bacterium *X. luminescens* (Akhurst & Boemare, 1988, Boemare & Akhurst, 1988) isolated from a human wound at the Center for Disease Control, Atlanta, Georgia (# 3265-86; Farmer *et al.*, 1989). Cells were plated on solid low salt medium [25% water, 3.0 ml glycerol, 5.0 g bactotryptone (Difco) and 3.0 g yeast extract (Difco) per litre (Nealson, 1978)] at 25 °C. A single bright colony was selected for study.

Liquid cultures were grown in one litre flasks in a temperature-controlled New Brunswick G-10 Shaker at 150 r.p.m. Samples (3.0 ml) were removed from the flasks at the times indicated and used for measurements of cell density (OD₆₆₀ in a Kontron spectrophotometer), *in vivo* luminescence, and extractable luciferase and SOD activities.

Light emission. Bioluminescence was measured using 3.0 ml samples in a scintillation vial placed in a photometer (Mitchell & Hastings, 1971), calibrated in quanta per second using the standard of Hastings & Weber (1963) corrected by a factor of 3 (Dunlap & Hastings, 1981).

Luciferase and antiluciferase: luciferase specific activity. Luciferase activity was measured by rapidly injecting 1.0 ml of 50 µM-FMNH₂ (catalytically reduced) with 100 µl cell extract containing luciferase and 0.001% *n*-decanal in 1.0 ml of the same buffer (Waters & Hastings, 1977). For the isolation of luciferase, cells were harvested by centrifugation and frozen. Upon thawing, cells were lysed osmotically with ultrasonic disruption in 30 mM-phosphate buffer, pH 7.0, 1.0 mM-DTT and 10 mM-EDTA (1 g cells per 5 ml buffer). The purification of luciferase was carried out using 2.2-diphenylpropylamine-Sepharose for affinity chromatography (Holzman & Baldwin, 1981, 1982, Colepiccolo *et al.*, 1989).

Rabbit antiluciferase was isolated 30 d after an initial 1 mg sensitizing injection of purified protein emulsified in Freund's complete adjuvant, followed 14 d later by a 3 mg booster injection.

The specific activity of the luciferase molecule was determined by an immunological titration, in which we measured the amount of luciferase activity inactivated by a given amount of antiluciferase (Waters & Hastings, 1977; Anderson *et al.*, 1980). Inactivation was complete within one hour. If a luciferase molecule formed in cells grown in 100% O₂ had twice the specific activity, the amount of luciferase activity inactivated by a fixed amount of antibody would be doubled, since its titre per molecule with antiluciferase should remain the same. On the other hand, if the effect of 100% oxygen involves an increase in the amount of enzyme, then a given amount of antiluciferase will inactivate a fixed amount of luciferase activity.

SOD activity. The SOD activity was measured by an indirect method using the inhibition of the chemiluminescent reaction produced in the oxygen hypoxanthine/xanthine oxidase/luminol system, in which the SOD competes with luminol for superoxide anions generated by the xanthine oxidase (Hodgson & Fridovich, 1973). The assay was done in 0.1 M-potassium phosphate buffer, pH 7.8, containing 10⁻⁴ M-EDTA, 1 µM-luminol, 0.5 µg ml⁻¹ xanthine oxidase and various amounts of SOD or luciferase. The reaction was initiated with 1.0 ml 0.2 mM-hypoxanthine injected with a hypodermic syringe into the vial containing the reaction mixture at 25 °C. Measurements of light intensity were performed as described above. The quantity of enzyme causing 50% inhibition was defined as one unit (McCord & Fridovich, 1969). An alternative method for measuring SOD activity was based on the inhibition of cytochrome *c* reduction, using the hypoxanthine/xanthine oxidase system as the source of O₂·⁻ (McCord & Fridovich, 1969).

Oxygen concentration. A growing culture was divided into two at an OD₆₆₀ of about 0.20. One flask was kept at atmospheric (21%) oxygen, while in the other a 100% oxygen atmosphere was maintained in the gas

phase of the culture flask. Both cultures were shaken vigorously in order to equilibrate with the gas phases, which were maintained by circulating the gas. In some of the experiments, O₂ was bubbled directly into the culture; similar results were obtained. Samples were removed under sterile conditions for measurements at the times indicated.

Results

Although the growth rate of *X. luminescens* was slower in 100% oxygen (Fig. 1), the bioluminescence of the culture was considerably greater (Fig. 2a). Based on the light emission per cell, which eliminates the need to correct for differences in growth rate, light emission was up to five times higher in 100% oxygen. Note that a log scale is used.

This difference could be partly or completely due to an effect of oxygen on the luciferase reaction itself, where oxygen is involved as a reactant. This seemed unlikely, since studies with other bacterial luciferases (Hastings, 1955) have indicated that the reaction *in vitro* is saturated at very low concentrations (<1% oxygen). That luciferase activity actually increased was confirmed by extractions and *in vitro* measurements at ambient oxygen; the luciferase activity was greater by at least two-fold in cells grown in 100% oxygen (Fig. 2b).

We first wanted to establish whether this increase in luciferase activity was due to differences in the amount of active protein (luciferase) synthesized, or simply to some change in or effect on the enzyme molecule causing an increase in its specific activity. Immunological titration showed that the same amount of luciferase activity was inactivated by a given amount of antiluciferase, irrespective of whether the luciferase was extracted from cells grown in 21% or in 100% oxygen (Fig. 3). Thus, the increased luciferase activity in cells grown in 100% oxygen is due to an increase in the cellular luciferase content.

If oxygen radicals induce luciferase synthesis, then a decrease in their cellular level should decrease luminescence and luciferase activity. Several scavengers of oxygen radicals, including histidine, carnosine, vitamin E and carotenoids decreased luminescence and luciferase activity when added to the medium during growth. The results with 2 and 20 mM-histidine are shown in Fig. 4. Although histidine resulted in a substantial decrease in the bacterial growth rate (data not shown), the data are plotted on a per cell basis, eliminating the need for a correction.

These observations raised the question as to whether luciferase reacts with oxygen radicals, as suggested in prior studies, in which a luciferase-flavin mononucleotide radical in the *V. harveyi* system reacted *in vitro* with superoxide anion to give bioluminescence (Kurfüst *et al.*, 1983). Tests for SOD activity with purified luciferase

Fig. 1. Time course of growth of *X. luminescens* in 21% (○) and 100% oxygen (●).

Fig. 2. Effect of oxygen tension on bioluminescence (a) and extractable luciferase activity (b) in *X. luminescens*.

preparations gave positive results, and in the luminol test for SOD activity, the superoxide-anion-dependent chemiluminescence of luminol was quenched by the addition of luciferase (Fig. 5). Does this luciferase have SOD activity?

As mentioned in the Introduction, high oxygen tension is known to induce SOD synthesis in several species. A similar induction of SOD activity was observed in *X. luminescens* (Fig. 6). Quantitatively, it was increased by no more than a factor of 2, considerably less than the induction of luciferase, suggesting that the two activities are different.

Fig. 3. Increased luciferase activity is due to increased amounts of luciferase protein. Different amounts of luciferase extracted from cells grown in 100% oxygen (■) and 21% oxygen (●) were added to tubes containing 20 μ l *X. luminescens* antiluciferase, and the final volume of each sample was made up to 0.4 ml with 10 mM-phosphate buffer. After 1 h at room temperature, each sample was assayed for remaining luciferase activity, which was plotted against the corresponding added activity. No further decrease in luciferase activity occurred after 1 h. Controls (open symbols) contain various amounts of *X. luminescens* luciferases from cells grown in either 21% (○) or 100% oxygen (□) assayed without added antiluciferase serum. Similar values were obtained with preimmune serum. This experiment thus shows that a total luciferase activity of 10⁸ quanta s⁻¹ is neutralized by 20 μ l *X. luminescens* luciferase antiserum with luciferases extracted from *X. luminescens* cells grown in either 21% or 100% oxygen.

Fig. 4. Effect of histidine on the expression of the bioluminescent system. At the time indicated by the arrows, the culture was split into different flasks and sterile histidine was added (control, solid line). Histidine inhibits bioluminescence and luciferase synthesis. The effects of 2 mM-histidine (○) and 20 mM-histidine (□) on the luminescence *in vivo* (a) and *in vitro* (b) are shown.

Fig. 5. Quenching of superoxide anion-dependent light emission of luminol. Time course of superoxide-dependent luminol luminescence (solid line). Luminescence was inhibited (broken line) by partially purified luciferase preparation from *X. luminescens* injected at the time indicated (arrow).

Fig. 6. Effect of oxygen tension on extractable SOD activity. Cells were inoculated and grown with shaking for 5 h, at which time the culture was split, with one flask being grown in an atmosphere of 100% O_2 (●) and the other as a control (○). Samples were removed at the times indicated and extractable SOD activities measured. See methods for units of SOD activity.

Fig. 7. Proteolytic inactivation of luciferase. Effect on luciferase and SOD activities of incubation of the partially purified luciferase fraction (Fig. 5) with trypsin (22 °C, 0.1 M-phosphate buffer, pH 7.2).

Experiments in which the luciferase activity was proteolytically destroyed also indicate that the two activities are distinct. Bacterial luciferase is known to be inactivated by proteases (Njus *et al.*, 1974). This effect involves cleavage of bonds in the α subunit only, without a concomitant change in the molecular mass of the protein (Baldwin *et al.*, 1978). In our preparations, trypsin rapidly inactivates luciferase while leaving the SOD activity intact (Fig. 7), indicating that the two enzymes are different molecules. This conclusion is further confirmed by the fact that the antiluciferase (Fig. 3) does not inhibit SOD activity.

Discussion

As described above, extractable SOD activity is greater in cells grown in 100% oxygen, by a factor of about two. An interesting but unexplained fact is that what appears to be induction of SOD occurs even in cells growing in atmospheric oxygen. This could be a type of autoinduction, in which the enzyme content and activity per cell decrease during the early phase of growth and then rise dramatically above a certain cell density. The pattern is similar to that for luciferase in certain species and strains, being illustrated for *X. luminescens* in Fig. 2. In *V. harveyi* and *V. fischeri*, the entire operon is regulated by genes that control the production of a small inducer molecule (autoinducer) and a binding protein (Eberhard *et al.*, 1981; Meighen, 1988; Cao & Meighen, 1989). The present results suggest that the gene(s) coding for the luciferase-associated SOD may be co-regulated with those of the luciferase system, since they exhibit similar patterns of induction and similar responses to high oxygen tension. The *LUX* gene in *X. luminescens* has recently been cloned (Johnston *et al.*, 1990; Xi *et al.*, 1991); studies of its regulatory elements in relation to oxygen will be of interest.

In their provocative hypothesis concerning the evolutionary origin of bioluminescence, McElroy & Seliger (1962) argued that the many different luciferases (Hastings, 1983) arose in evolution at the time oxygen first occurred on earth, and that they served as specific oxidases able to remove oxygen rapidly. The system could thus be viewed as detoxifying maintaining low oxygen concentrations necessary for the anaerobic organisms. They speculated that in such a highly exergonic 'luciferase' reaction, one of the product molecules could be formed in a electronically excited state, and thus emit light. Although that specific hypothesis has been withdrawn (Seliger, 1975), the involvement and importance of luciferases in oxygen metabolism (Hastings, 1982), as well as that of oxygen in luciferase induction (Nealson & Hastings, 1977), are both firmly established.

However, the present observations suggest that the McElroy & Seliger hypothesis should be revived and reconsidered, at least for some organisms. The increase in the synthesis of luciferase and its consequent activity in cells grown in 100% oxygen implicates the light-emitting system in the metabolism of oxygen radicals. Since the two enzymes may be co-regulated, the possibility that bacterial luciferase and SOD have evolutionary relationships should be investigated.

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