

The Evolution of Bioluminescent Oxygen Consumption as an Ancient Oxygen Detoxification Mechanism

Graham S. Timmins,^{1,2} Simon K. Jackson,¹ Harold M. Swartz²

¹ Department of Medical Microbiology, University of Wales College of Medicine, Cardiff, UK

² Department of Radiology, Dartmouth Medical School, Hanover, NH 03755, USA

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Abstract. Endogenous reductants such as hydrogen sulfide and alkylthiols provided free radical scavenging systems during the early evolution of life. The development of oxygenic photosynthesis spectacularly increased oxygen levels, and ancient life forms were obliged to develop additional antioxidative systems. We develop here the hypothesis of how “prototypical” bioluminescent reactions had a plausible role as an ancient defense against oxygen toxicity through their “futile” consumption of oxygen. As oxygen concentrations increased, sufficient light would have been emitted from such systems for detection by primitive photosensors, and evolutionary pressures could then act upon the light emitting characteristics of such systems independently of their use as futile consumers of oxygen. Finally, an example of survival of this ancient mechanism in present-day bioluminescent bacteria (in the *Euprymna scolopes*–*Vibrio fischeri* mutualism) is discussed. Once increasing ambient oxygen levels reached sufficiently high levels, the use of “futile” oxygen consumption became too bioenergetically costly, so that from this time the evolution of bioluminescence via this role was made impossible, and other mechanisms must be developed to account for the evolution of bioluminescence by a wide range of organisms that patently occurred after this (e.g., by insects).

Key words: Antioxidant — Bioluminescence — Oxygen

Introduction

The hypothesis that bioluminescence may have evolved as an ancient oxygen-detoxifying mechanism that had the sole and express purpose of oxygen consumption, but that was later recruited to provide biological signaling mechanisms, was first suggested some time ago (McElroy and Seliger 1962) and has been the subject of debate since. Despite contributions by many researchers to the field, either supporting (Colepicolo Neto et al. 1992; Barros and Bechara 1998) or rejecting (Seliger 1975; Seliger 1987; Hastings 1983) the concept, no consensus has emerged.

The original hypothesis of McElroy and Seliger (1962) was that “prototypical” bioluminescent reactions developed to detoxify molecular oxygen by its reduction. Later contributions by Seliger suggested, first, that “prototypical” bioluminescent reactions initially developed as mixed function oxidases to metabolize aromatic and alkane molecules (Seliger 1975) and, second, that bacterial bioluminescence evolved to enable lipid oxidation at low oxygen concentrations (Seliger 1987). It has also been hypothesized that the initial function of coelenterazine luciferin was as a radical-scavenging antioxidant (Rees et al. 1998). Each of these hypotheses is perhaps possible, but in light of the simple, attractive nature of McElroy and Seliger’s original hypothesis, coupled with

Correspondence to: Dr. Graham Timmins, Department of Medical Microbiology, University of Wales College of Medicine, Heath Park, Cardiff CF14 4XN, UK; e-mail: timminsgs@cf.ac.uk

our own recent work upon such an oxygen-consuming bioluminescent antioxidative system (Timmins et al. 1999), we have reexamined and refined this hypothesis, and here present our conclusions: in doing so, we critically assess other hypotheses as to the evolution of bioluminescence and, also, analyze the requirement and provision of antioxidant systems through evolutionary time. It is shown that the increase in bioluminescent oxygen consumption required to maintain intracellular anoxia caused by the rise in environmental oxygen concentrations provides a simple mechanism by which organisms possessing these “prototypical” bioluminescent reactions increased their light emission to levels sufficient for its biological detection. Once this occurred, natural selection will have been able to act upon these “prototypical” bioluminescent reactions independently, increasing their quantum efficiency and ensuring their retention irrespective of any antioxidative role. We also discuss how this system has been retained into modern times in the case of bacterial bioluminescence in the symbiosis between *Euprymna scolopes* and *Vibrio fischeri* (Ruby 1996; Weis et al. 1996): here bioluminescent oxygen consumption by the bacteria is used to protect against oxidative stress imposed by the squid, which in doing so ensures the continued bioluminescence of its symbiont. Finally, we show that as ambient oxygen levels reached a certain threshold, it would simply have been too energetically costly for an organism to consume oxygen in a “futile” manner to maintain an antioxidant defense, and so after this threshold oxygen level was exceeded it would not have been possible for bioluminescence to evolve from a preexisting function as a futile oxygen-consuming antioxidative defense mechanism. Thus, the evolution of bioluminescence by organisms after this threshold was reached, such as by insects, must have occurred by other routes.

In the reanalysis of the original hypothesis of McElroy and Seliger (1962) it will become apparent that the only obvious extant possessor of this mechanism is to be found in bacterial bioluminescence: this does not, however, limit the possibility that other such bioluminescent oxygen-consuming chemistries evolved at ancient times but are not now extant.

Evolution of Bioluminescence: A Critical Assessment of Other Hypotheses

Several hypotheses have emerged to try and explain the evolution of bioluminescence: an in-depth analysis of the original hypothesis of McElroy and Seliger (1962) that “prototypical” bioluminescent reactions developed to detoxify molecular oxygen by its reduction forms the basis of this work. The later hypothesis that bioluminescence evolved from reactions whose initial functions were as mixed-function oxidases to metabolize aromatic and al-

kane molecules, thus affording metabolic advantage by providing more readily metabolized substrates (Seliger 1975) is problematic for the following reasons.

- (i) The oxidation of such highly reduced compounds could realistically be performed only by transition-metal-containing monooxygenases (analogous to cytochrome P450). However, extant luciferases (e.g., bacterial, coelenterate, and coleopteran luciferases) do not utilize a catalytic transition metal cofactor and are also dioxygenases. Clearly the enzymes and chemistries required for alkane oxidation and those of extant bioluminescence are very different, and hence it is unlikely that the former evolved into the latter;
- (ii) The products of oxidation of such alkane substrates are compounds such as alcohols, and hence by much more facile oxidation, aldehydes—compounds that are in fact used as luciferins by a range of extant organisms (Wilson and Hastings 1998; Hastings 1983). It would appear more plausible for such a hypothesis to use reactions of such compounds as these aldehydes as the “prototypical” reactions of bioluminescence.
- (iii) As discussed by Seliger (1987), it is difficult to conceive of a way in which the oxidation of such hydrocarbons, with very poor quantum yields of light emission, might have emitted sufficient light for primitive biological photosensing mechanisms to detect and, hence, exert evolutionary pressure upon.

A second hypothesis of Seliger (1987) was that bacterial bioluminescence evolved to enable lipid oxidation at low oxygen concentrations, thus affording metabolic advantage. However, bacterial bioluminescence achieves no metabolic advantage other than oxidation of an aldehyde to a carboxylic acid, a function that may readily be performed by other classes of aldehyde oxidases (also termed aldehyde dehydrogenases), enzymes that are widely found in species including bacteria (e.g., Yoshida et al. 1998). There is no known or expected vectorial proton transport coupled to the oxidation of FMNH₂ by bacterial luciferase (in contrast to the respiratory electron transport chain), and hence no utilization of the energy afforded by its oxidation. It would therefore appear that there would be little actual metabolic advantage deriving from this particular hypothesis. In addition, it is known that microaerophilic cytochrome *c* oxidases in the respiratory chains of endosymbiotic nitrogen-fixing bacteria are efficient at low ambient oxygen concentrations (3–30 nM), with a *K_m* for oxygen of, e.g., 7 nM (Preisig et al. 1996), so that bacteria are able to undergo oxidative respiration at low oxygen levels through this pathway.

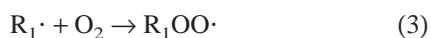
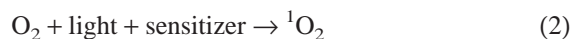
The hypothesis that the initial function of the luciferin coelenterazine was as a radical-scavenging antioxidant (Rees et al. 1998) is certainly not implausible and is, in

concept if not mechanism, similar to the original oxygen-consuming hypothesis of McElroy and Seliger (1962) in that it achieves an antioxidative function. However, as is well known, measurements of radical scavenging antioxidative action from in vitro assays cannot directly be extrapolated to demonstrate that that substance acts in such a manner at physiologically relevant concentrations: for example, the hydrophobic nature of coelenterazine (Teranishi and Shimomura 1997) will greatly favor its uptake into cellular membranes in in vitro systems. Additionally, the presence or absence of other antioxidative systems such as superoxide dismutase or ascorbate in coelenterazine-utilizing organisms has not yet been reported, to the authors' knowledge, and so a final appraisal of this hypothesis awaits the demonstration of a significant antioxidative effect of physiological concentrations of coelenterazine in comparison to any other antioxidants present.

Clearly, there are significant doubts regarding each of these hypotheses, and these have led authors such as Hastings (1983) to cast considerable doubt upon the validity of such hypotheses. Indeed, it is also the opinion of ourselves that most extant forms of bioluminescence will not have evolved through such routes. Instead, for many of these bioluminescent chemistries, it is probably the inherent likelihood of efficient chemiluminescence due to the structure of many of these luciferins that has driven their evolution into bioluminescent systems (McCapra 1990), with either the luciferins themselves or their immediate precursors fulfilling another biological role, until evolution selected for and acted upon their inherently chemiluminescent nature.

The Concept of Antioxidant Defense by "Futile" Oxygen Consumption

Molecular oxygen, O₂, is well known to exert biological toxicity, although the concentration of oxygen required to produce toxicity in a specific organism varies from very low (e.g., obligate anaerobes) to very high. The mechanisms through which oxygen exerts this toxicity include not only the formation of reactive intermediates such as superoxide [Reaction (1)] and, hence, hydrogen peroxide and hydroxyl radical] and singlet oxygen [Reaction (2)], but also the "amplification" of initial free radical damage by substrate peroxidation through Reactions (3) and (4) (Gilbert 1981; Davies 1995; Hatta and Frei 1995; Inkhen et al. 1997; Fridovich 1998).



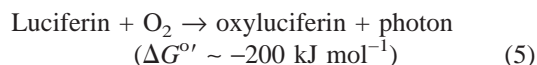
"Conventional" antioxidant systems, i.e., those widely accepted as such, involve the scavenging of these various reactive intermediates by a wide range of molecules to produce less reactive species. Examples of these include low molecular weight antioxidants such as ascorbate and glutathione and the enzymes catalase, superoxide dismutase (SOD), and glutathione peroxidase. However, an alternative direct mechanism to produce an antioxidative effect is to reduce oxygen concentrations by "futile" oxygen consumption, since all of the damaging reactions involving oxygen, detailed above, are dependent upon oxygen concentration. Thus by limiting oxygen concentrations, the deleterious reactions of oxygen can be directly prevented, and it is via this mechanism that an antioxidative role can be most plausibly postulated for bioluminescence. The term "futile" here refers to the fact that although it achieves a biologically useful effect, no free energy from substrate oxidation is harnessed, nor are any of the products "useful" (analogous to thermogenesis). It is interesting to note that such "futile" oxygen consumption (although by different mechanisms) has also been postulated as playing crucial antioxidative roles in the parasite *Ascaris lumbricoides* (Minning et al. 1999) and the mammalian ocular lens (Eaton 1991). Another mechanism of limiting oxygen concentrations is to decrease oxygen solubility, and this is thought to be important in minimizing oxidative damage in some plant tissues (Raven 1991).

Additionally, it has become apparent that many important enzymes present in anaerobes utilize protein-derived free radicals as essential cofactors and that these are highly sensitive to damage by oxygen (e.g., Wagner et al. 1992; Klinman 1995). Thus, in organisms utilizing such enzymes, which are used in diverse metabolic transformations, maintenance of internal anaerobiosis might be utilized to protect specific oxygen-sensitive proteins, rather than a more generalized oxidative damage. The compartmentalization of oxygen-sensitive nitrogenase in microaerophilic heterocysts in cyanobacteria is one such example of how oxygen control can be used to prevent specific damage to proteins (Adams and Duggan 1999).

The Characteristics of Bioluminescent Reactions Making Them Suitable for "Futile" Oxygen Consumption

Bioluminescent systems utilize molecular oxygen to oxidize a substrate, the luciferin, in a reaction (almost always) catalyzed by an enzyme, the luciferase; therefore they inherently possess the ability to act as oxygen scavenging systems [Reaction (5)]. There is wide variation in structure of both luciferins and luciferases known in differing organisms, but the unifying characteristic of these reactions is, of course, the emission of light of colors from blue to red (Hastings 1983; Wilson and Hastings

1998). Bioluminescent reactions are also highly exergonic, with approximately 200 kJ mol^{-1} being emitted as photons, resulting in equilibrium constants that strongly favor the forward, essentially irreversible bioluminescent oxygen-consuming reaction. This means that, on thermodynamic grounds, O_2 can be scavenged to exceedingly low levels by very modest concentrations of luciferin and luciferase, to provide an effective antioxidant defense. Accordingly, it has been experimentally demonstrated that bioluminescence can proceed at very low levels of oxygen (Hastings 1952). In addition to their favorable thermodynamic parameters, the kinetics of the bioluminescent reaction will vary with the nature of the luciferin and luciferase, allowing a degree of tailoring to the biological requirements, such as the rate of oxygen flux into the organism.



Another characteristic of bioluminescent reactions that makes them suitable as an antioxidative defense is their production of the electronically excited singlet state of the emitter and their high fluorescence quantum yields, which results in very low yields of triplets: this is in sharp contrast to the efficient production of triplet emitters by, e.g., triplet ketones from thermolysis of dioxetanes (Adam and Cilento 1982; Cilento and Adam 1995). This is important, as the products of an antioxidative bioluminescent reaction should not themselves be reactive or damaging, and it is well known that triplet species directly react with and damage many biological molecules and, also, efficiently react with O_2 to form singlet oxygen (Adam et al. 1997; Almeida et al. 1999).

A final feature of bioluminescent reactions favoring their evolution as an antioxidative defense is the wide range of nonrelated structures of both luciferins and luciferases known in prokaryotes and eukaryotes. Indeed sometimes even closely related organisms utilize quite different bioluminescent chemistries. This indicates that bioluminescence has resulted from many independent evolutionary events and, therefore by inference, that it has been relatively common for bioluminescence to evolve from preexisting metabolic pathways. The diversity of control mechanisms known would also suggest that suitable control of bioluminescent systems could also readily be achieved in whatever way required (Hastings 1983; Wilson and Hastings 1998).

The Biological Requirements for Bioluminescent Reactions to Provide a Significant Antioxidative Defense

There is a range of circumstances in which an organism may require “futile” oxygen consumption by bioluminescent reactions to act as a defense, related primarily to the

organism’s environmental exposure to oxygen, but two limiting cases can be defined: continuous and intermittent. Provision of a continuous antioxidant defense would require that

- (i) the organism has appreciable oxygen consumption through bioluminescence compared to any nonbioluminescent metabolism, such as oxidative phosphorylation;
- (ii) the combination of external $[\text{O}_2]$, the rate of oxygen diffusion into the organism, and the rate of bioluminescent oxygen consumption is such that the $[\text{O}_2]$ in the organism is sufficiently lower than in the external environment;
- (iii) the energy requirements for maintenance of this process must be sustainable by the organism; and
- (iv) this mechanism offers some evolutionary advantage compared to “conventional” antioxidant defenses.

The provision of an intermittent antioxidant defense provides more flexibility in these constraints, but it must be the case that (i) the organism is occasionally exposed to episodes of increased external $[\text{O}_2]$, (ii) there is sufficient capacity of the bioluminescent system to consume enough oxygen to protect the organism significantly through such a period of exposure, and (iii) this defense offers advantages compared with other defense systems. In this case, an organism would preemptively accumulate and store sufficient luciferase and luciferin, or perhaps an activated intermediate (analogous to firefly luciferyl-AMP), during its predominantly anaerobic existence to defend against periods of oxygenation. A present-day analogy of preemptive accumulation of antioxidant defenses is the production of SOD even in anaerobic *Escherichia coli* to protect against superoxide mediated toxicity, when it is, in its natural life cycle, exposed to reoxygenation (Kargalioglu and Imlay 1994).

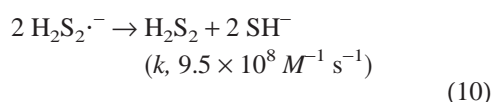
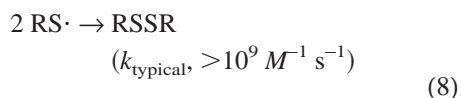
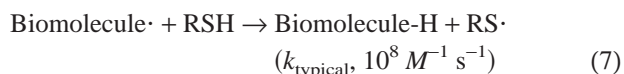
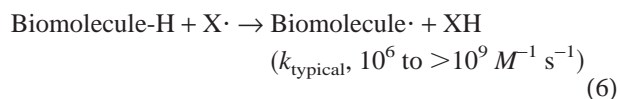
Oxygen, Its Toxicity, and Bioluminescent Detoxification in Ancient Times

There is substantial evidence that cellular life evolved in a low- $[\text{O}_2]$ environment approximately 4 billion years ago (Seliger 1975; Kasting 1993; Krupp et al. 1994). Estimates of the atmospheric partial pressure of oxygen (P_{O_2}) at this time range from a maximum, predicted from water photodissociation, of approx. 0.01 kPa in the upper atmosphere to many orders of magnitude below this value, and it should be noted that there were significant partial pressures of reducing gases such as H_2S (Kasting 1993; Krupp et al. 1994). However, it is certain that life evolved at a P_{O_2} below the prevailing atmospheric level because oxygen production, before the evolution of water-splitting photosynthesis, was primarily atmospheric, whereas oxygen consumption, such as reduction by Fe^{2+} ions (known to occur in aqueous environments in

those times) would occur primarily in the aqueous environment. Thus if life first occurred in a “prebiotic soup” in the water column, the ambient PO_2 would have been very low.

Furthermore, it has been hypothesized that life first occurred, in a chemoautotrophic form, at geothermal vents at the ocean floor, in an environment containing a high concentration of hydrogen sulfide and simple alkylthiols (Huber and Wachterhauser 1997, 1998) and an even lower PO_2 than in the water column. In either case, it is apparent that biomolecules, and the life they engendered, will have evolved in an aqueous environment with PO_2 several orders of magnitude below the atmospheric maximal PO_2 of 0.01 kPa, with estimates of PO_2 such as 10^{-10} kPa leading to aqueous concentrations of ca. 10^{-15} M (Krupp et al. 1994). At these exceedingly low concentrations of O_2 , oxygen-derived reactive intermediates, and peroxidative reactions, would not have exerted any significant toxicity to evolving biomolecules or life forms.

This is not to say that no free radical-mediated damage occurred at this time, as background ionizing radiation levels must have been higher than today (Seliger 1975). Additionally, the significant fluxes of UV light would have generated $HO\cdot$ and, hence, H_2O_2 at the water surface. However, it is likely that an inherent repair system for radical damage of primitive organisms was provided by the “antioxidative” functions of molecules already present in their environments. Particularly suitable antioxidants would be low molecular weight thiols [Reactions (6–10) shown with “typical” or specific rate constants], present as alkylthiols and/or H_2S [in which case disproportionation; Reaction (10) is also possible] that would have provided sufficient, endogenous radical repair capacity, before more specific defenses had time to develop (Asmus 1990; Mills et al. 1987). Biological damage by a species $X\cdot$, such as $HO\cdot$ could have been readily repaired thus:



Estimated of P_{H_2S} before 2.35 billion years ago are approximately 10^{-7} kPa, leading to concentrations in the

oceans of approximately 10^{-10} M, six orders of magnitude higher than that of oxygen (Krupp et al. 1994), thus repair of radicals by H_2S [Reaction (7)] would have greatly outcompeted addition of oxygen [Reaction (3); $k_{\text{typical}} \sim 10^9 \text{ M}^{-1} \text{ s}^{-1}$].

Once a cellular membrane had developed, early organisms evolving in the low extracellular PO_2 of this environment, would have had a similarly low intracellular PO_2 in equilibrium with their environment, and the diffusion of simple thiols into the cell, and of their oxidized products (such as disulfides) out of the cell, would have maintained their rudimentary free radical damage repair system. Indeed, thiols such as glutathione are still extremely commonly found antioxidants in biology, with the membrane-impermeable nature of glutathione, unless mediated by a transport mechanism, enabling cells to retain this species for recycling (as almost all are no longer supplied with membrane-diffusible simple thiols to use in a consumptive manner). This low- PO_2 environment is thought to have continued throughout the biosphere for about 1.5 to 2 billion years, providing ample time for most fundamental biochemical processes (perhaps even including primitive oxidative phosphorylation) to have evolved (Castresana and Saraste 1995). The known importance of (fermentive) sugar redox disproportionation reactions in energy supply for ancient biosynthetic metabolism also supports the low availability of oxygen during the development of cellular metabolism (Weber 1997).

This state of affairs continued until the evolution of oxygen-producing photosynthetic organisms, 2.5 to 3 billion years ago, that began to increase the environmental PO_2 (Kasting 1993; Krupp et al. 1994). The precise estimation of these low oxygen levels in this time period, and hence the rate of its increase, is not possible, although it took from 1 to 2 billion years after evolution of oxygenic photosynthesis until atmospheric oxygen concentrations were sufficient to provide an ozone layer for UV protection allowing colonization of the terrestrial environment ($PO_2 \sim 2.5$ kPa). This is likely a result of the buffering effect of terrestrial and aqueous reductants (sulfides, Fe^{2+} , etc.), resulting in a prolonged gradual rise in external PO_2 until this buffer was depleted. Figure 1 shows some of the constraints that can be made upon oxygen levels at these times in two separate compartments: the atmosphere and ocean surface at or near equilibrium with the atmosphere and the deep ocean, >200 m. Since the extant organisms had not previously needed specific antioxidant mechanisms to deal with such elevated oxygen levels, they might then have been required to develop them, with significant evolutionary advantage being afforded those that did. Additionally, their simple thiol/disulfide-based antioxidative system would now, in the presence of appreciable levels of O_2 , have of itself produced superoxide, Reaction (11) (Winterbourn 1993; Kopenol 1993; Wardman 1998). Organisms that did not

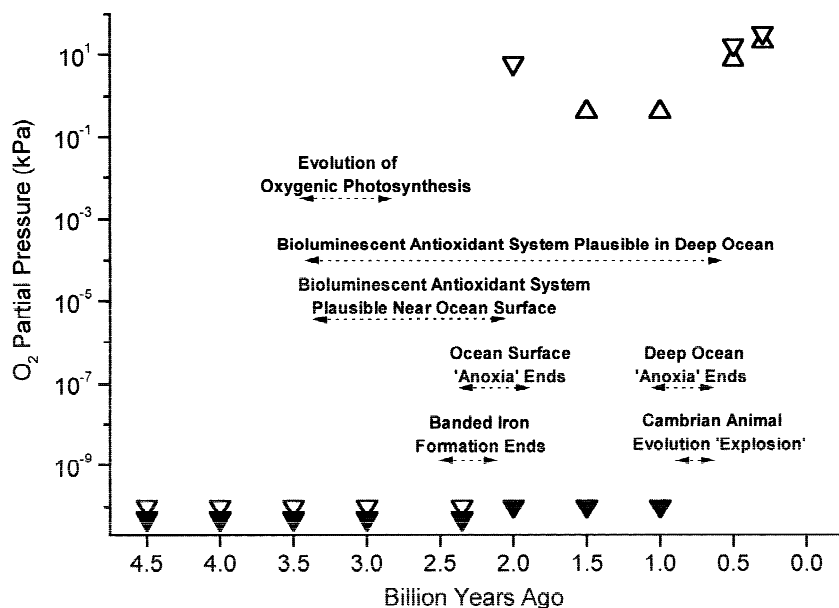
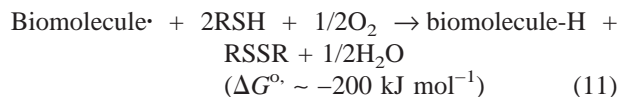


Fig. 1. Constraints through geological time upon the P_{O_2} in two separate compartments: the atmosphere and ocean surface at or near equilibrium with the atmosphere (∇ , upper constraint; \triangle , lower constraint) and the deep ocean, >200 m (∇ , upper constraint). Also shown horizontally are periods of time in which a bioluminescent antioxidant system could reasonably have operated, together with other important events in the history of oxygen levels on Earth.

evolve additional antioxidant defenses would have been faced with a decreasing number of anaerobic environments in which to survive, and the two essential “choices” to avoid this fate and survive in the increasingly aerobic environment would have been either to accept rising extracellular and intracellular P_{O_2} and develop “conventional” antioxidant defenses against, e.g., superoxide and H_2O_2 (i.e., SOD, catalase, glutathione peroxidase, etc.), or to attempt to control the intracellular P_{O_2} .



It is at this time compared to any other that the development of bioluminescence for oxygen detoxification would have been most favorable. As noted previously, it appears that the development of bioluminescence is a very common event in evolutionary terms, certainly with many more separate evolutionary events than present in other “conventional” antioxidant systems such as SOD and catalase. This is probably linked to the fact that bioluminescence might readily evolve from either preexisting (and common) ligase or oxidase enzyme activities, such as CoA synthetases in beetles (Wood 1995). In addition, since SOD produces damaging hydrogen peroxide, the simultaneous presence (evolution) of SOD and catalase is required to exert a maximal antioxidative effect. Therefore, it is highly likely that a bioluminescent “futile” oxygen-consuming antioxidant system would have appeared before any others, affording a significant evolutionary advantage to those organisms in its possession.

Not only would such a mechanism be more likely to evolve first, but also it would have directly counteracted

the environmental pressure—raised the intracellular oxygen levels—as these could easily be scavenged by bioluminescent reactions to the low levels that these organisms had previously evolved in and adapted to. Additionally, other antioxidative enzyme activities may not have provided a suitable defense, e.g., in the unicellular organism (*E. coli*) it appears that catalase provides only a “quorum” defense mechanism (i.e., protects a bacterial colony), and does not defend isolated bacteria (Ma and Eaton 1992); or if specific damage to oxygen-sensitive proteins is required to be protected against, this will be achieved only by oxygen limitation.

A Quantitative Analysis of Bioluminescent Oxygen Consumption

As discussed previously, the highly exergonic nature of bioluminescent reactions and their low K_m for oxygen mean that oxygen can be scavenged to exceedingly low levels (equilibrium constant, $K_{eq} > 10^{36}$) by modest concentrations of luciferin. To determine, however, if such a form of defense is plausible, one can estimate the rate of oxygen consumption required of a spherical, model cell to maintain itself in an anoxic state in any given environmental P_{O_2} from the following equation (Subczynski et al. 1992):

$$\Delta C_{ext} = V/4\pi RD$$

where ΔC_{ext} is the difference in oxygen concentration from an infinitely distant point to the cell membrane, V is the rate of oxygen consumption per cell per second, R is the cell radius (assumed here to be 5 μm), D is the diffusion coefficient of oxygen in water ($3 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$), and the solubility of oxygen in water at 293 K is

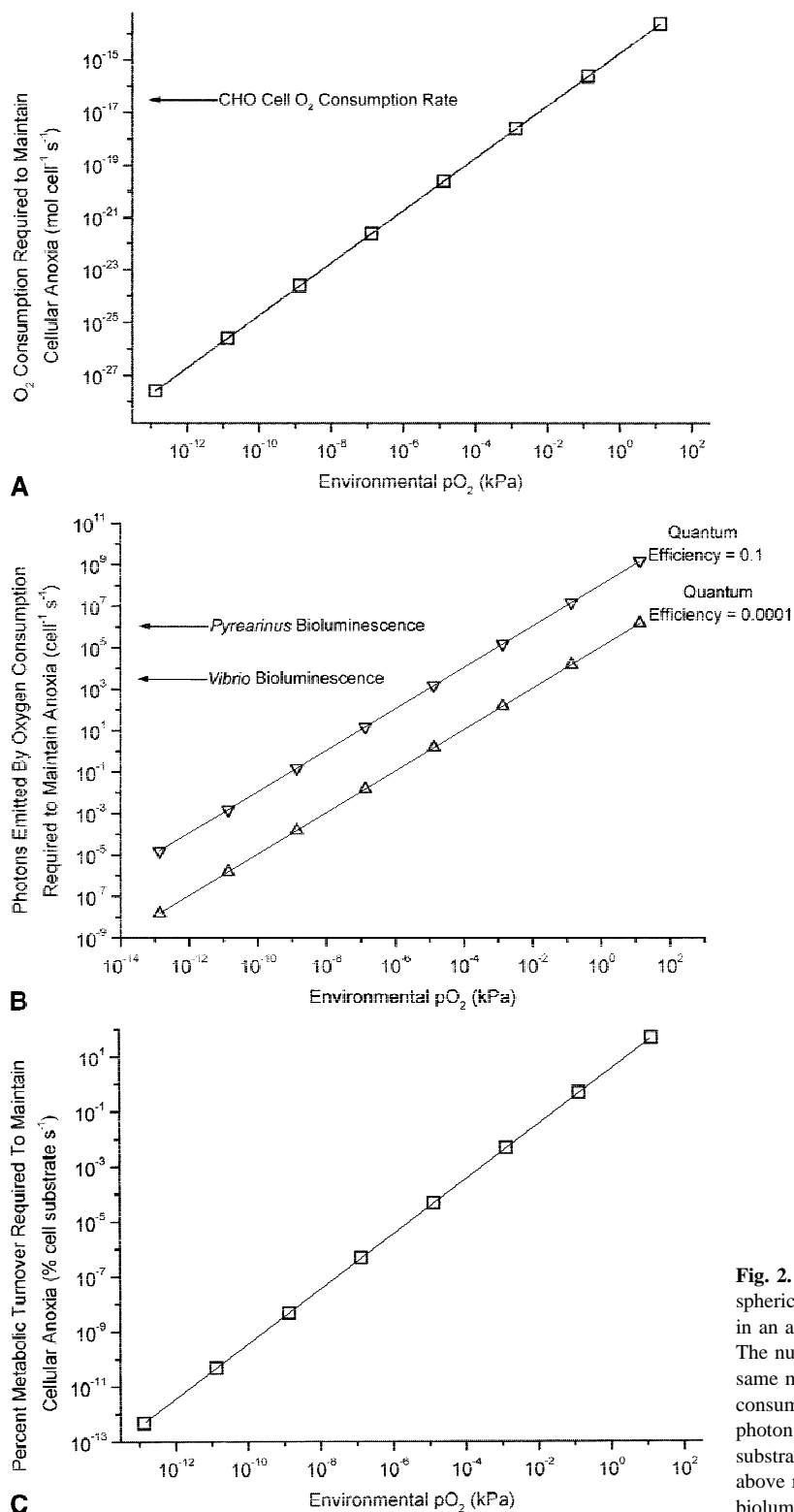


Fig. 2. **a** The rate of oxygen consumption required of a spherical model cell (see text for details) to maintain itself in an anoxic state as a function of environmental PO_2 . **b** The number of photons emitted per cell per second in the same model cell resulting from bioluminescent oxygen consumption (assuming a quantum efficiency of 0.1 photon per O_2 consumed). **c** Percentage of the total cellular substrate required to be metabolized per second by the above model cell to maintain anoxia through bioluminescence (assumptions in text).

assumed. The final degree of anoxia (i.e., the remaining trace oxygen concentration achieved) is dependent upon the scavenging system; for bioluminescent reactions the trace levels resultant from a $K_{eq} > 10^{36}$ will be vanishingly small. The results of such a simulation are shown in Fig. 2a (noting the dual log plot), together with the oxy-

gen consumption of a typical mammalian present-day Chinese hamster ovary (CHO) cell for comparison [$6 \times 10^{-17} \text{ mol } O_2 \text{ s}^{-1} \text{ cell}^{-1}$ (Subczynski et al. 1992)]. It can be seen that a model cell with a bioluminescent oxygen consumption 1000-fold less than that of respiratory consumption by a CHO cell can still maintain internal an-

oxia over a wide range of geologically relevant PO_2 levels. Similarly, assuming quantum efficiencies of between 0.1 (similar to that of present-day bioluminescent bacteria) and 0.0001 photon per O_2 consumed, the number of photons emitted per cell per second resulting from bioluminescent oxygen consumption can be estimated (Fig. 2b). Also shown in Fig. 2b is the photon emission rate of other cell types such as the photocytes of *Pyrearinus termitilluminans* [$\sim 10^6$ photons s^{-1} cell $^{-1}$ (Timmins et al. 1999)] and bioluminescent *Vibrio* sp. bacteria [10^3 to 10^4 photons cell $^{-1}$ s $^{-1}$ (Dunlap 1984; Makemson 1986), and it can be seen that the levels of bioluminescence required to protect against geologically relevant PO_2 values are very modest indeed and, certainly, do not entail bioluminescence light emission greater than that currently observed.

Finally, to determine whether such a method of antioxidant defense is energetically viable, one can estimate the turnover of cellular substrates required to achieve the necessary rate of oxygen consumption. Assuming that the total concentration of such substrates in the model cell above is 0.1 M, and the stoichiometry is such that 1 mol of substrate is consumed for each mole of O_2 , the percentage of total cellular substrate required to be metabolized per second to maintain anoxia through bioluminescence is shown in Fig. 2c. Again, it can be seen that, below a PO_2 of say 10^{-4} kPa, the metabolic requirement to maintain this defense is really rather low. In fact, such a mechanism could well be *more* efficient than maintaining more complicated antioxidant defenses. Although this is difficult to estimate quantitatively, it is apparent from the fact that antioxidant enzymes such as SOD, catalase, etc., are almost always up-regulated in times of increased oxidative stress in organisms that utilize them, that there is a significant metabolic cost of maintenance of such defenses; otherwise they would be permanently expressed at these higher levels. Additionally, these estimates do not assume that the luciferin is recycled, although there is the possibility of some recycling of firefly, bacterial, and coelenterate luciferins (McCapra 1990) that would minimize the metabolic cost of such a mechanism.

Two (at first sight) apparent contradictions in this hypothesis are that the oxygen production that brought about the increase in atmospheric PO_2 was due to cellular photosynthesis: Why did this oxygen, formed in cell membranes, not poison the first photosynthetic bacteria? and Why, if these cells had to develop enzymatic antioxidant defenses (SOD/catalase, etc., as they now possess) to cope with raised intracellular oxygen levels, did this not occur elsewhere, making futile oxygen consumption irrelevant as an antioxidant?

These questions can be dismissed relatively easily, as early photosynthetic organisms would have been able to rely upon passive diffusion of oxygen out of the cell and into the sink provided by the external anoxic environ-

ment. For example, a model cell representative of an isolated cyanobacterium (spherical, with a radius of 1 μ m) undergoing photosynthetic oxygen production of 1×10^{-19} mol O_2 s $^{-1}$ cell $^{-1}$ will result in a rather low intracellular oxygen concentration, $\sim 2 \times 10^{-9}$ M (PO_2 , 2×10^{-4} kPa). In addition, the outward diffusion of poisonous oxygen could have afforded competitive advantage against other organisms. Finally, the great metabolic advantage afforded by oxygenic photosynthesis would have provided additional metabolic energy for the provision of antioxidant defenses such as SOD, catalase, etc., once required.

Finally, in organisms dependent upon the activity of oxygen-sensitive free radical-cofactor enzymes, maintenance of internal anaerobiosis might well be considerably more energetically favorable than the requirement for proteolysis and resynthesis of such enzymes damaged by the presence of oxygen.

The Development of Bioluminescence as a Signaling Mechanism Driven by the Increasing Environmental Oxygen Partial Pressure

As Seliger (1975) has pointed out, one of the major difficulties in explaining the evolution of bioluminescence as a signaling mechanism has been explaining how progressive evolution acted upon prototypical bioluminescent reactions of low efficiency and light emission, to select for and amplify this bioluminescence until it was of a sufficient intensity to be perceived by light-sensing organisms. However, let us assume that the *original* purpose of these early reactions was to reduce and detoxify molecular oxygen, with the light emission being merely a consequence of the large free energy change required to make such a system essentially irreversible and capable of scavenging oxygen to very low levels. It can then be seen from Fig. 2b that as the environmental PO_2 increased from say 10^{-13} to 10^{-6} kPa, then the number of photons emitted by the model organism (above, assuming a quantum efficiency of 0.1 mol photon per mol O_2) increases from approximately 10^{-5} to 100 photons s $^{-1}$ cell $^{-1}$. These levels could have been detected by organisms possessing primitive photosensors. Even assuming a much lower quantum efficiency of 0.0001 mol photon per mol O_2 , for "prototypical" bioluminescence, such a phenomenon would still have operated over similar ranges of PO_2 if the light-emitting organisms were present at higher cell densities, such as a small bacterial colony of a thousand organisms. Once this threshold had been reached, evolutionary selection would have been able to act upon the light-emitting, rather than the oxygen-consuming, properties of these systems, thus enabling the retention and further evolution of bioluminescence, independent of any antioxidative role.

The Decline of Bioluminescent Antioxidative Systems

Eventually, perhaps as a result of a “threshold” oxygen concentration being reached in the atmosphere and ocean surfaces at some time approximately 2.5 to 1 billion years ago (Kasting 1993; Krupp et al. 1994), it would have become too inefficient to consume oxygen in a “futile” manner as an antioxidative system. It is indeed possible that this provided the selective force for the development of primitive, thermodynamically inefficient but efficiently oxygen-scavenging, oxidase enzymes for oxidative phosphorylation pathways, as the provision of even a limited amount of metabolically useful energy from oxygen consumption would have provided a significant advantage. Alternatively, evolution and selection of “conventional” antioxidant systems, such as SOD and catalase, could have been responsible for the eclipse of bioluminescently antioxidant organisms, as their catalytic action would be more efficient than the stoichiometric consumption of luciferin. Certainly, for unicellular organisms the ability rapidly to convert membrane-impermeable $O_2^{\cdot-}$ into the membrane-permeable and more stable H_2O_2 via SOD would allow for its removal by diffusion out of the cell. Additionally, this outward diffusion of H_2O_2 would allow poisoning of other competing organisms in its environment. Such a concept is supported by the finding that in *E. coli*, catalase provides quorum, rather than individual, protection against H_2O_2 -producing competitors (Ma and Eaton 1992).

However, there is strong evidence that anoxia of the deep oceans (>200 m) ended much later than that at the surface, approximately 1 to 0.5 billion years ago (Canfield 1998). Therefore, bioluminescent antioxidant systems might have been effective in deep oceans until up to approximately 0.5 billion years ago, and hence significant selection for development/retention of bioluminescence as an antioxidative system would have occurred for organisms in this environment compared to those inhabiting surface waters. This might be partly responsible for the high proportion of present-day bioluminescent organisms in this ecosystem (in addition to the low levels of penetrating sunlight). Once these waters finally became oxic, in addition to their antioxidative system no longer being as efficient as those from those of the ocean surface (previously forced to develop efficient antioxidant defenses such as SOD and catalase), the latter would be fully adapted to the use of oxygen as a terminal receptor of electrons from the respiratory electron transport chain, affording them considerable metabolic advantage.

Finally, the “Cambrian explosion” of animal evolution, and hence large predators known to possess eyes, is thought to have resulted from the ocean floors becoming oxic (Canfield 1998; Ohno 1996), and these predators could also have contributed to the decline of some permanently bioluminescent species through their easy visual detection. The development of ascorbate synthesis is

also linked to this time period (Ohno 1996) and ascorbate is thought to be the “ultimate” sink for radical damage in those organisms that possess it (Wardman 1998).

The Retention and Development of Bioluminescence as a “Modern” Antioxidant Through Symbiotic Relations

Although it might at first appear difficult to conceive of present-day circumstances in which bioluminescence plays a continuous antioxidative role, there is evidence that this may occur in the bioluminescent symbiotic bacterium *Vibrio fischeri* in its association with specific host light organs of the squid *Euprymna scolopes* (Ruby 1996; Weis et al. 1996; Wilson and Hastings 1998). In this case, nonluminous mutant bacteria are defective in colonization of the host, indicating that strain selection by the host for bioluminescence occurs, although its mechanism is as yet unclear (Visick et al. 2000). Since it is known that those areas of host squid light organ that are adjacent to the extracellular *V. fischeri* contain high concentrations of myeloperoxidase (an enzyme used in the oxidative destruction of bacteria by neutrophils producing damaging species such as hypochlorite), the local environment of *V. fischeri* in the light organ is likely to be subjected to high levels of oxidative stress (Weis et al. 1996). Through bioluminescent oxygen consumption, *V. fischeri* would be able to reduce the ambient PO_2 to very low levels, resulting in a minimization of the oxidative stress caused by the host-derived oxidative factors, and simultaneously, the host would achieve selection for bioluminescent strains. Furthermore, recent evidence suggests that bioluminescent *Vibrio* species including *V. fischeri*, growing in anaerobic environments, can use alternate host-derived electron acceptors such as trimethylamine *N*-oxide for respiration and growth, providing a mechanism for continued cell growth despite bioluminescence-mediated reduction of the PO_2 to very low levels (Proctor and Gunsalus 2000).

Although a lowering of the PO_2 by bioluminescence has been suggested (Ruby 1996), the mechanism was hypothesized as acting to reduce the PO_2 to below the K_m of the host NADPH oxidase for oxygen, thereby decreasing the production of H_2O_2 (and hence HOCl): however, the most plausible mechanism, as described here, is a reduction in HOCl-initiated peroxidative damage by maintaining bacterial anoxia. There is much evidence supporting this hypothesis including the following.

- (i) The control of bacterial cell number to prevent overgrowth is achieved by a once-daily expulsion of approximately 90% of light organ contents (Nyholm and McFall-Ngai 1998), therefore it is unlikely that the myeloperoxidase is involved in control of cell number, considering that more regu-

lar expulsion of light organ contents could achieve greater control.

- (ii) It is likely that the high level of myeloperoxidase would result in some local oxidative stress to the host—indeed the light organ lens itself contains an aldehyde dehydrogenase-like crystallin (Weis et al. 1993)—and so such a damaging system is unlikely to be involved in bacterial cell number regulation when the above, simple expulsion mechanism exists.
- (iii) The regulation of bioluminescent genes of *V. fischeri* is unlike that in some other bioluminescent bacteria, in that bioluminescence continues even at low oxygen concentrations (Nealson and Hastings 1977a).
- (iv) Myeloperoxidase-derived oxidants such as HOCl cannot be enzymatically converted into harmless products (unlike superoxide or hydrogen peroxide), hence oxidation of some bacterial components is inevitable once these are formed. Through inhibiting the peroxidative damage caused by the presence of oxygen, bioluminescent oxygen consumption may represent the most logical defense to this oxidative stress.
- (v) A catalase-deficient *V. fischeri* mutant was unable to colonize squid light organ competitively in comparison to the wild type (Visick and Ruby 1998), with this enzyme's periplasmic nature indicating that *V. fischeri* in its host is likely exposed to externally applied oxidative stress. This also argues circumstantially against the role of lowered PO_2 in preventing H_2O_2 production—if this were the case, catalase would not be expected to be crucial.
- (vi) The percentage of bioluminescent to total oxygen consumption is thought to be rather high where known, between 1.7 and 17% in ex vivo *Photobacter leiognathi*, twice that in vivo, and up to 12% in ex vivo *Vibrio harveyi* (Dunlap 1984; Makemson 1986). Reasonable models suggest that regions of anoxia can exist within the *E. scolopes* light organ (calculations not shown). However, in the light of the number of assumptions that have to be made (oxygen consumption rate, external PO_2 , oxygen diffusion and solubility coefficients), a definitive conclusion awaits an in vivo measurement of PO_2 in the light organ-containing wild-type and nonbioluminescent mutants.
- (vii) Perhaps most importantly, it has been demonstrated that the microbicidal action of hypochlorite is strongly dependent upon the presence of oxygen, e.g., in the case of *E. coli* treated with 38 to 76 μM HOCl, anaerobiosis resulted in an approximately 50- to 100-fold increase in cell survival compared to identical aerobic treatment (Dukan et al. 1999). Since such a large effect occurs in this in vitro

system, where any inhibition of host NADPH oxidase by lowered PO_2 cannot have any effect (see viii), it would appear that this direct action of anaerobiosis is most important in the protection of bioluminescent bacteria against host-derived oxidants, rather than any such inhibition of H_2O_2 production.

- (viii) The apparent K_m of NADPH oxidase (rat) for oxygen ranges from 9.6 to 3.7 μM , values substantially higher than that of mitochondrial *a/a*₃ oxidase, $\leq 1 \mu M$ (Edwards et al. 1983). Thus, normal respiration by the bacteria would be able to reduce oxygen levels to substantially lower than the K_m of NADPH oxidase and thereby inhibit production of superoxide (and hence H_2O_2 and HOCl). It is therefore apparent that the real reason for the high level of bioluminescent oxygen consumption is to “scavenge” oxygen to near-anoxic levels in the bacteria to inhibit the HOCl-initiated oxidation.
- (ix) “Wild-type” bioluminescent *V. fischeri* are able to outcompete nonbioluminescent mutants when coinoculated as a 1:1 mixture in juvenile *E. scolopes* (Visick et al. 2000). If the most important action of bioluminescent oxygen consumption were to decrease the squid tissue PO_2 to below the K_m of NADPH oxidase for oxygen (decreasing the production of H_2O_2), then nonbioluminescent mutants would be able passively to use the defenses afforded by the wild type, and so the observed competitive pressure might not be exerted upon the mutant. If, however, the most important bioluminescent oxygen consumption were the maintenance of anoxia at the surface of and within the bacteria (to minimize HOCl-derived damage), this would allow for the observed competitive pressure upon the nonbioluminescent mutant.

Furthermore, in support of the overall hypothesis of bioluminescent antioxidative defenses, the development of associations between such bacteria and larger host animals could well have provided a mechanism for the retention of bioluminescent oxygen consumption as an antioxidative defense during the final phases of deep ocean oxygenation 1 to 0.5 billion years ago. As these larger host organisms developed, they would, due to their increased size and their aerobic metabolism (through increasing the distance of diffusion of oxygen from the environment and consumption of oxygen, respectively), have provided environments within themselves at a substantially lower PO_2 than the external environment. Small (unicellular) organisms with bioluminescent antioxidative systems may well have been able to colonize these environments, with the host animals deriving advantage (and hence undergoing evolutionary selection for) through the acquisition of bioluminescence. During

the ongoing evolution of animal species, this “reservoir” of bacteria using bioluminescent oxygen consumption as an antioxidative defense would have continued to be present, evolving new associations with new hosts. Obviously, another factor favoring the development of these associations is the fact that this would have prevented the easy predation of the bioluminescent bacteria—only predators able to attack its much larger host would have posed a threat.

As oxygen levels in the environment increased so as to make it difficult for such bacteria to survive the transition between the light organs of one host and another (absolutely required for this life cycle), due to the oxygen-rich environment [e.g., the atmospheric PO_2 was approximately double the present levels 300 million years ago (Graham et al. 1995)], successful bacteria may have been required either to sporulate to achieve infection or to acquire other suitable defense systems (SOD, catalase, etc.) analogous to the anaerobic expression of SOD by *E. coli* to survive its aerobic trek between anaerobic intestinal environments (Kargalioglu and Imlay 1994). This helps explain why modern-day symbiotic bioluminescent bacteria possess such defenses for their passage through oxic environments between hosts, in addition to their bioluminescent system needed specifically to withstand host myeloperoxidase-derived oxidants during symbiosis.

Another finding has been that fish fecal pellets can exhibit bioluminescence from marine enterobacteria such as *Beneckea* (Nealson and Hastings 1977b) that is of sufficient intensity to be visible by fish, hence stimulating ingestion and promoting bacterial propagation. A plausible hypothesis for the development of this behavior related to the overall concept of bioluminescent oxygen consumption is that the bioluminescence was initially involved in maintaining anoxia within the fecal pellet during the time the bacteria were outside the anaerobic environment of the gut and in the aerobic ocean, an episodic exposure to an increased PO_2 required for propagation.

In the free environment today, it is probably likely only that bioluminescent oxygen consumption can provide an intermittent defense. Such a defense could prove useful to organisms present in hypoxic sediments that were intermittently disturbed (by tides or animal movement) and exposed to a raised PO_2 before resettling. However, surprising discoveries may still wait, particularly in relatively hypoxic environments such as the deep ocean.

Conclusions

We have shown here how “futile” oxygen consumption by bioluminescent reactions could have acted as an antioxidant system in primitive times. The selection for

bioluminescence of sufficient intensity for biological photosensing would have been a natural result of the rise in the environmental PO_2 . Additionally, in the case of bacterial bioluminescence, the possibility of retention of such systems through the development of symbiotic relationships is demonstrated. We hope that this paper provides fuel for thought in the design of experiments either to prove or to disprove these theories and, also, stimulates explorations of how bioluminescence might have evolved in other species, such as insects, where the oxygen-consuming antioxidative hypothesis discussed here would not appear to hold true.

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