The Journal of Experimental Biology 201, 1211–1221 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
JEB1487

THE ORIGINS OF MARINE BIOLUMINESCENCE: TURNING OXYGEN DEFENCE MECHANISMS INTO DEEP-SEA COMMUNICATION TOOLS

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Accepted 13 January; published on WWW 24 March 1998

Summary

Bioluminescence, the emission of ecologically functional light by living organisms, emerged independently on several occasions, yet the evolutionary origins of most bioluminescent systems remain obscure. We propose that the luminescent substrates of the luminous reactions (luciferins) are the evolutionary core of most systems, while luciferases, the enzymes catalysing the photogenic oxidation of the luciferin, serve to optimise the expression of the endogenous chemiluminescent properties of the luciferin. Coelenterazine, a luciferin occurring in many marine bioluminescent groups, has strong antioxidative properties as it is highly reactive with reactive oxygen species such as the superoxide anion or peroxides. We suggest that the primary function of coelenterazine was originally the detoxification of the deleterious oxygen derivatives. The functional shift from its antioxidative to its light-emitting function might have occurred when the strength of selection for antioxidative defence mechanisms decreased. This might have been made possible when marine organisms began colonising deeper layers of the oceans, where exposure to oxidative stress is considerably reduced because of reduced light irradiance and lower oxygen levels. A reduction in metabolic activity with increasing depth would also have decreased the endogenous production of reactive oxygen species. Therefore, in these organisms, mechanisms for harnessing the chemiluminescence of coelenterazine in specialised organs could have developed, while the beneficial antioxidative properties were maintained in other tissues. The full range of graded irradiance in the mesopelagic zone, where the majority of organisms are bioluminescent, would have provided a continuum for the selection and improvement of proto-bioluminescence. Although the requirement for oxygen or reactive oxygen species observed in bioluminescent systems reflects the high energy required to produce visible light, it may suggest that oxygen-detoxifying mechanisms provided excellent foundations for the emergence of many bioluminescent systems.

Key words: bioluminescence, reactive oxygen species, luciferin, luciferase, oxygenase.

Oxygen, oxygenases and the origins of bioluminescence

The utilisation of chemically generated luminescence is one of the most aesthetic forms of animal communication and one that has fascinated observers for centuries. However, relatively few authors have addressed the question of its origin and evolution. One reason that the evolutionary origins of bioluminescence remain obscure may be that most bioluminescent organisms are relatively inaccessible to investigation. Bioluminescence flourishes predominantly in deep ocean waters, and its presence in terrestrial ecosystems remains restricted to a few groups. A survey around Bermuda indicated that 97 % of fishes living between 500 and 1000 m are bioluminescent (Beebe, 1937).

In an early paper, E. N. Harvey (1932) proposed that proto-bioluminescence emerged from proteins linked to the respiratory chain and possessing fluorescent groups. Later, McElroy and Seliger (1962) suggested that luciferases evolved to detoxify molecular oxygen in early anaerobic life forms at the time when photosynthetic processes had begun releasing oxygen into the primitive atmosphere. An alternative to this hypothesis (Seliger, 1975) later proposed that luciferases were more probably mixed-function oxygenases evolved in order to utilise molecular oxygen as an electron acceptor, thereby increasing the metabolic capacity of the organisms and yielding an advantage over their competitors. The same author also developed the idea that bacterial luciferase derived from a flavoprotein oxygenase that could catabolise saturated aldehydes at low oxygen pressures (Seliger, 1987). Finally, Seliger (1993) proposed that luciferases from all present luminous organisms were derived from

oxygenases involved in the metabolism of pigments or toxic substances. Among essays on the evolution of bioluminescence, that of Buck (1978) is seminal in that it clearly defines the essential questions that the evolution of bioluminescence poses.

One of the most striking characteristics of bioluminescence is the very high diversity of mechanisms, structures and functions that bioluminescent organisms have achieved. This is well illustrated by the many different chemistries, reflected by the occurrence of various photogenic substrates (Fig. 1), occurring in bioluminescence. The diversity of chemistries involved in bioluminescent mechanisms renders generalisations hazardous, and attempts to represent bioluminescent reactions schematically have led to confusion. Owing to this high diversity, the safest way to define a bioluminescent reaction would be 'a reaction that produces ecologically functional light occurring in a living organism'. Indeed, light emission is not restricted to bioluminescent organisms, but probably occurs in any tissue of any organism as a by-product of metabolic reactions and oxidations (Murphy and Sies, 1990). The requirement for an enzyme physically interacting with the luciferin is not universal. In the boring clam *Pholas dactylus*, no binding of the luciferin to the luciferase occurs during the light-emitting reaction (Michelson, 1978). Shimomura *et al.* (1993) suggested that some luminescent fungi might lack a proper luciferase enzyme entirely.

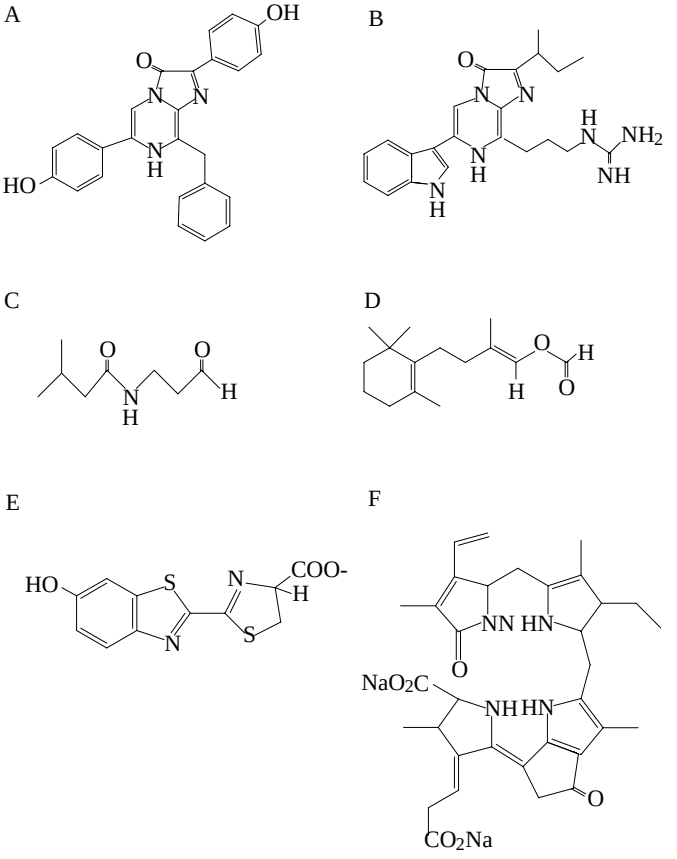

Fig. 1. Structure of several luciferins found in bioluminescent organisms. (A) Coelenterazine, (B) *Vargula* luciferin, (C) *Diplocardia* luciferin, (D) *Latia* luciferin, (E) Coleoptera luciferin and (F) dinoflagellate luciferin.

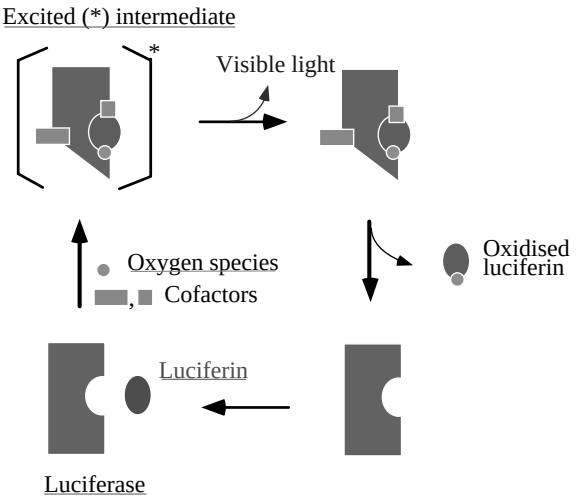

Fig. 2. Schematic illustration of a bioluminescent reaction. Steps and components common to all known systems are underlined.

Furthermore, in addition to the endogenous luciferase, metals are able to catalyse the photogenic oxidation of luciferins in scale worms (*Chaetopterus variopedatus*) (Shimomura and Johnson, 1968*a*). Finally, the true luciferin can be difficult to determine in multi-component systems such as those in bacteria. With these qualifiers in mind, a luminescent reaction could be defined as follows (Fig. 2): a substrate, the luciferin, or a complex formed by the luciferin and cofactors, is oxidised by either oxygen or one of its derivatives (hydrogen peroxide, superoxide anion). The reaction, catalysed or not by a luciferase, yields an excited intermediate which emits light upon returning to the ground state. Cofactors taking part in the reaction either act on the luciferase (e.g. Ca^{2+} inducing conformational changes and the activation of aequorin) or on the luciferin.

This high diversity suggests multiple independent origins of bioluminescence over the course of evolution (Buck, 1978; Hastings, 1983). That bioluminescent chemistries emerged several times independently in various phyla is perhaps not surprising. Although sometimes regarded as a rare phenomenon, chemiluminescence is widespread in biology. Indeed, all cells are ultraweak chemiluminescence emitters as the oxidation of many organic molecules (e.g. proteins, lipids, DNA) leads to the production of light (Murphy and Sies, 1990). The light emitted from these metabolic reactions was not of sufficient intensity to be of selective advantage in itself, but probably provided the basis from which useful bioluminescent mechanisms could evolve. Yield, kinetics, spectrum and localisation would have been the selective parameters on which subsequent evolution was based.

Hypotheses on the origins of bioluminescence have tended to focus on luciferases as mixed-function oxygenases that happened to produce light upon reaction with a substrate, thus conferring metabolic or protective advantages on proto-bioluminescent organisms. The view that luciferases were oxygenases and were the key to the development of bioluminescence (Seliger, 1987, 1993) has prevailed for years.

Although this possibility exists, it is not necessarily the main evolutionary pathway for the emergence of luciferases. Current knowledge suggests that luciferases are phylogenetically different from oxygenases. A search of the SwissProt database yielded 682 partial or complete oxygenase sequences representing 0.4 % of the total of 175 546 registered sequences. Homology searches using the FastA algorithm were then carried out with the following luciferase sequences: *Gonyaulax polyhedra*, *Clytia gregaria*, *Aequoria victoria*, *Renilla reniformis*, *Vargula hilgendorfii*, *Luciola cruciata* and *Photinus pyralis*. No significant homologies were found with any other oxygenase sequences. When 10 representative oxygenase sequences (cytochrome P450 oxygenase, pentachlorophenol 4-mono-oxygenase, 4-hydroxyphenylpyruvate dioxygenase, steroid 21-mono-oxygenase, indoleamine 2,3-dioxygenase, flavin-containing mono-oxygenase, ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit, biphenyl dioxygenase, dopamine beta-mono-oxygenase, tryptophan 2-mono-oxygenase) were chosen at random from the 682 database entries and subjected to the same analysis, homologies were found with other oxygenase sequences. There is also some biochemical evidence that luciferases do not operate as oxygenases. In Table 1, the turnover rates of a number of oxygenases are presented. In general, these rates are much higher than known turnover rates for luciferases and photoproteins reacting with imidazolopyrazine substrates, where turnover rates range from a minimum of 1 for aequorin to a maximum of 1600 min^{-1} for *Vargula* luciferase. These differences between the two groups indicate that luciferases are distinct from oxygenases. Therefore, the genetic and biochemical properties of luciferases suggest that it is improbable that any of the known luciferases have been derived from any of the oxygenase families described thus far.

The role of oxygen is therefore not that of a specific cofactor of a specialised enzyme, but is most probably related to the high energy consumption of bioluminescent reactions: the production of electronically excited states able to emit in the visible range of the spectrum requires large amounts of energy. For example, the emission of the blue photons (e.g. 470 nm) that are emitted by the majority of bioluminescent marine animals consumes 255 J mol^{-1}. In comparison, ATP hydrolysis only produces 30.5 J mol^{-1}, which is insufficient to generate visible light. In this context, the breakdown of a peroxide bond appears to be an ideal source of energy for the reaction and is therefore the main mechanism operating in bioluminescent reactions.

Table 1. *Turnover rates of oxygenases*

Enzyme	Turnover number (min^{-1})
Pyrocatechase	1776
Protocatechuate oxygenase	43120
Metapyrocatechase	38500
3,4-Dihydrophenyl-acetate 2,3-dioxygenase	14140
Prorocatechuate 4,5-dioxygenase	23550
Steroid 4,5-dioxygenase	6120
Tryptophan 2,3-dioxygenase	1200

From Nozaki (1978).

Luciferins as the evolutionary core of bioluminescent systems

Current evidence suggests that in many instances it is the luciferins themselves that have shaped the evolution of bioluminescent phenomena and that the central role of the luciferases is not that of efficient oxygenases, but rather to optimise the environment for luciferin chemiluminescence and to reduce unproductive quenching of the excited state emitter by surrounding molecules.

Coelenterazine (Fig. 1A) is an imidazolopyrazinone luciferin central to marine bioluminescent phenomena in general, and it illustrates the importance of the luciferin in the evolutionary history of bioluminescence. This type of luminescent substrate is found in fishes, protozoans, coelenterates, molluscs and chaetognaths (Campbell and Herring, 1990; Haddock and Case, 1993; Rees *et al.* 1990, 1992), and it seems likely that the vast majority of bioluminescent marine organisms rely on this molecule. Another imidazolopyrazinone luciferin is that found in the ostracod *Vargula*, which is also formed of three groups connected to a fused imidazolone-pyrazine nucleus (Fig. 1B). The occurrence of similar chemistries in phylogenetically distant organisms may in many cases be the result of dietary transfer of luciferin through trophic chains (Cormier *et al.* 1967; Thompson *et al.* 1987, 1988*a*,*b*; Thompson and Rees, 1995). In contrast, there is little to no convincing evidence for the lateral transfer of luciferases. In fact, as the sequences of more luciferases become known, cross-phylum homologies have yet to be uncovered. Among some coelenterates, the luminescent Ca^{2+}-binding photoproteins appear to be derived from a common calmodulin-like ancestor (Inouye *et al.* 1985), but the origins of other marine luciferases and their imidazolopyrazinone substrates remain more obscure.

E. N. Harvey (1952) likened the random distribution of bioluminescence to the pattern produced by throwing wet sand onto a diagram of a phylogenetic tree. One could consider the wet sand as a spray of coelenterazine randomly leading to the production of ecologically useful light on a few branches. In what ways is coelenterazine particularly suited for the generation of proto-bioluminescence? This molecule possesses an electronic structure with natural chemiluminescent properties which can be spontaneously expressed in aprotic solvents such as dimethylformamide in the presence of oxygen. Furthermore, imidazolopyrazinones can also luminesce in aqueous solutions in the presence of reactive forms of oxygen, such as singlet oxygen or the superoxide anion (Nakano *et al.* 1986; Suzuki, 1993; Lucas and Solano, 1992; Ghislain *et al.* 1995). While different luciferins exist with much lower spontaneous chemiluminescent yields than coelenterazine, it is very likely that this property allowed the development of coelenterazine-based bioluminescent mechanisms in so many

organisms. A further advantage of imidazolopyrazinones is the spectrum of their chemiluminescent emission. The wavelength of light emission is a highly critical parameter in the marine environment. Because of the differential absorption properties of sea water, the signal will have an optimal range if it peaks as closely as possible to the maximally transmitted wavelength. This will allow luminescent signals to be propagated over long distances and maximise the efficiency of camouflage provided by ventral counterillumination (Clarke, 1963; McAllister, 1967; Denton *et al.* 1985). The spectral energy distribution of coelenterazine chemiluminescence at physiological pH (monoanion form) peaks at 470–480 nm (Hori *et al.* 1973; Qi *et al.* 1991), wavelengths that are maximally transmitted through oceanic sea water (Jerlov, 1976).

Among bioluminescent organisms with an imidazolopyrazinone luciferin, several observations suggest that the luminescent spectrum is mainly dependent on the structure of the imidazolopyrazinone itself, with the luciferase exerting only a minor influence. Structural modifications of the coelenterazine molecule and pH markedly modify the chemiluminescent emission spectrum (Qi *et al.* 1991; McCapra and Chang, 1967; McCapra and Manning, 1973; Hori *et al.* 1973; Teranishi and Goto, 1990) and bioluminescent emission spectra (Qi *et al.* 1991; Shimomura *et al.* 1988; 1989; Hart *et al.* 1979). In contrast, *in vivo* spectra resulting from the catalytic oxidation of native luciferins appear to be unaffected by luciferase structure. Many bioluminescent organisms, using either coelenterazine or *Vargula* luciferin, but with apparently unrelated luciferases, have very similar emission spectra all peaking near the chemiluminescence maximum of the monoanion form. Although similarities in present-day emission spectra could also be the result of similar selection pressure, it is noteworthy that the few coelenterazine-based systems emitting light with different spectral characteristics (i.e. longer wavelengths) do so by transferring the energy liberated by the excited species to a fluorescent protein which acts as a secondary emitter (Ward, 1979) rather than by modifying the structure of the enzyme as in other groups. For example, the emission spectrum in luminous beetles can be shifted over more than 30 nm by single mutations in the amino acid sequence of the luciferase (Wood *et al.* 1989). As energy-transfer mechanisms seem to be required for very limited spectral shifts in imidazolopyrazinone bioluminescence (e.g. 10 nm in *Renilla*), the adaptability of these luciferins for emitting light of different wavelengths appears to be rather limited. This low spectral adaptability might have been a key factor in the very successful utilisation of coelenterazine by marine organisms. Indeed, variations in the colour of light do not seem to be widely used as an intra- and interspecific recognition parameter in marine organisms. Differential wavelength absorption by sea water would gradually alter the spectral properties of the emitted light over its journey to the receivers, thus making it impossible for the latter to rely on this parameter for emitter identification. By situating the peak of emission near the wavelength of optimal transmission, the spectral quality of the signal would remain the same over most of its journey towards potential receivers while allowing communication over longer distances. The pattern of the bioluminescence display and flash kinetics are more probably recognition parameters in marine bioluminescence. Thus, the low adaptability of emission spectra produced by imidazolopyrazinones which would probably be disadvantageous in a terrestrial environment, where the colour of the light can be a significant recognition criterion, could have been a fundamental factor in the success of coelenterazine-based marine bioluminescence.

The particular chemiluminescent properties of imidazolopyrazinones discussed above would theoretically have allowed the development of luciferases from a variety of precursors. Serum albumin has been shown to catalyse the chemiluminescence of both coelenterazine and *Vargula* luciferin (Campbell and Herring, 1990; Rees and Thompson, 1993), whereas lysozyme also possesses some luciferase-like activity with *Vargula* luciferin (E. M. Thompson, unpublished data). Although these luciferase-like activities are apparently less efficient than present-day luciferases, these observations suggest that the constraints for an enzyme to increase the efficiency of the chemiluminescent oxidation of imidazolopyrazinones would be reduced. In this context, it is of no surprise that several isoforms of *Vargula* luciferase and aequorin have been described (Thompson *et al.* 1989; Blinks and Harrer, 1975). In fact, a major role of the luciferase may be to create an optimal hydrophobic environment for the chemiluminescent reaction and to reduce unproductive quenching and spectral spreading *via* collision of the excited state emitter with other molecules. It has been suggested that the role of *Vargula* luciferase could simply be to provide a hydrophobic environment and to initiate the reaction with oxygen by removing a proton from the luciferin (Goto, 1968). *Vargula* luciferin is a chemiluminescent emitter in micelle solutions (Goto and Fukatsu, 1969), such that a corresponding luciferase would only require a hydrophobic pouch accessible to the luciferin and oxygen. Such structural plasticity for the luciferase could have been a determinant factor in the development of imidazolopyrazinone bioluminescence. Furthermore, chemiluminescence accompanying the reaction of imidazolopyrazinones with reactive forms of oxygen could also have led to the appearance of luciferases based on a controlled production of reactive oxygen species, a mechanism that exists in a variety of cells (Williams, 1985).

The relatively stable chemiluminescent emission spectrum of imidazolopyrazinone luciferins would have allowed mutations to occur in protoluciferase genes with negligible influence on the final emission spectrum. This would have reduced to some extent the constraints on protoluciferase evolution towards higher quantum yields. The yield (ϕ) of a chemiluminescent (CL) or bioluminescent (BL) reaction will depend on three factors as follows:

$$\phi_{BL} \text{ or } \phi_{CL} = \phi_C \times \phi_{FL} \times \phi_{EX},$$

where ϕ_C is the chemical yield of oxidised luciferin, ϕ_{FL} is the fluorescence quantum yield of the excited emitter and ϕ_{EX} is

the fraction of the product molecules that are in an electronically excited state.

The few known *in vitro* quantum yields for imidazolopyrazinone-based bioluminescent reactions range from 5 % in *Renilla* up to 30 % in *Vargula* (Campbell, 1988). Light intensity can be increased by (1) increasing the rate of reaction of luciferin with oxygen, (2) enhancing ϕ_{FL} of the oxyluciferin or (3) transferring the excitation to a secondary emitter with higher ϕ_{FL}. The very bright fluorescence of the photophores in the teleost *Porichthys notatus* with a spectrum different from that of the luminescence (Baguet and Zietz-Nicolas, 1979) suggests that the fish has found some way to increase ϕ_{FL} of the luciferin. It is likely that one role of the luciferase would be protection of the excited emitter from quenching by the solvent or other cellular constituents that could reduce ϕ_{FL} by (1) dissipating the excitation into vibrational energy (heat) or (2) transferring the energy to compounds with fluorescence properties emitting at wavelengths that are not ecologically valuable . The relatively high efficiency of imidazolopyrazinone chemiluminescence may have favoured a relatively early development of ecologically useful light emission with protoluciferases capable of fulfilling the above role. For example, Goto and Fukatsu (1969) have observed that the efficiency of *Vargula* luciferin chemiluminescence in basic diglyme was only five times less than in the luciferase-catalysed reaction. Increasing ϕ_{FL} by providing a protected hydrophobic pouch for the luciferin might account for the low turnover rates of imidazolopyrazinone luciferases, as this could reduce the diffusibility of both luciferin and oxyluciferin to/from the active site.

The origin of luciferins: the antioxidative hypothesis

The predominant role of the luciferin in the emergence of imidazolopyrazinone bioluminescence indicates that the evolutionary origins of this phenomenon are to be found in the history of the luminescent substrate rather than in that of the enzymes utilising it. The widespread distribution of coelenterazine in the marine environment suggests that it is unnecessary, perhaps even improbable, to postulate an origin in bioluminescent ancestor(s) species. The presence of coelenterazine in non-luminescent organisms and the ability of some of these to store coelenterazine (Shimomura, 1987) indicate possible nonluminescent roles for this molecule.

Recent data (Nakano *et al.* 1986; Suzuki, 1993; Sugioka *et al*., 1986; Takahama, 1993; Lucas and Solano, 1992; Ghislain *et al.* 1995) have shown that imidazolopyrazinones react not only with molecular oxygen but also with highly reactive derivatives of oxygen such as the superoxide anion ($O_2^{\cdot -}$), singlet oxygen ($^1O_2$) and HOCl. It is now well established that these reactive oxygen forms (ROS) can oxidise many cellular components, such as lipids, proteins and DNA, and initiate cellular lysis or induce mutations in the genes (Halliwell and Gutteridge, 1990). As these radicals are known to be produced in intracellular metabolic reactions of all aerobic cells, these cells have developed a variety of protective mechanisms. Detoxification mechanisms include enzymes, such as superoxide dismutase, catalase and peroxidases, which convert oxygen derivatives into less toxic forms and antioxidant substances, such as ascorbic acid, β-carotene and α-tocopherol, that have high affinities for ROS and annihilate their destructive potential (Slater *et al.* 1987). It is remarkable that measurements of the rate constants of the reaction between coelenterazine, $^1O_2$ and $O_2^{\cdot -}$ revealed values of $2.86\times10^8\,mol^{-1}\,l\,s^{-1}$ and $1.2\times10^5\,mol^{-1}\,l\,s^{-1}$, respectively (Ghislain *et al.* 1995; B. de Wergifosse, unpublished results). These values are similar to those of other known antioxidants such as ascorbic acid and α-tocopherol (Table 2). It should be noted that both $O_2^{\cdot -}$ and $^1O_2$ are considered as primary molecular species in oxygen toxicity (Cadenas, 1989). The reactivity of imidazolopyrazinones with ROS suggests that coelenterazine could be a physiologically relevant antioxidative agent.

In an attempt to demonstrate this, we have studied the ability of this luciferin to protect human fibroblasts (MRC-5) subjected *in vitro* to tert-butyl-hydroperoxide, a widely used free radical initiator. The results clearly demonstrate that coelenterazine can prevent cellular mortality induced by oxidative stress at concentrations as low as $100\,nmol\,l^{-1}$ (Fig. 3). Furthermore they show that the protection conferred by coelenterazine considerably surpasses that of the vitamin E analogue trolox (O. Noiset, in preparation). *In vitro* tests of the chain-breaking activity of coelenterazine in delaying the oxidation of linoleate when submitted to a radical initiator demonstrated a greater efficacy for coelenterazine than for α-tocopherol (Table 3). After the onset of oxidation, coelenterazine was able to lower the propagation rate of oxidation while α-tocopherol could not. Thus, it is most likely that coelenterazine can neutralise a wider

Table 2. *Rate constants of coelenterazine, the amino acids constituting the proposed tripeptide precursor and two important cellular antioxidants with singlet oxygen ($^1O_2$) and superoxide anion ($O_2^{\cdot -}$)*

Quencher	k_q with $^1O_2$ ($mol^{-1}\,l\,s^{-1}$)	k_q with $O_2^{\cdot -}$ ($mol^{-1}\,l\,s^{-1}$)	References
Coelenterazine	2.86×10^8	1.2×10^5	B. de Wergifosse, (unpublished data)
Ascorbic acid	1.6×10^8	2.7×10^5	Rougée and Bensasson (1986); Nishikimi (1975)
α-Tocopherol	6.7×10^8	2×10^5	Bielski *et al*. (1985)
L-Tyrosine	–	<10	Bielski *et al*. (1985)
L-Phenylalanine	–	<0.36	Bielski *et al*. (1985)

k_q, rate constant.

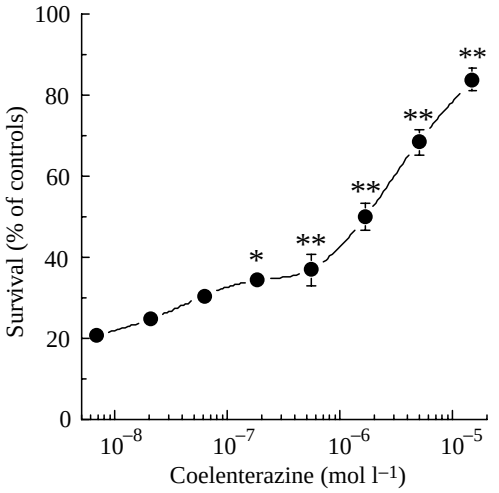

Fig. 3. Cytoprotective effect of coelenterazine on human fibroblasts (MRC-5) subjected to oxidative stress induced by 10^{-4} mol l^{-1} tert-butyl-hydroperoxide. Survival is expressed as a percentage of that measured in control cells unexposed to the stress-inducing agent. Values are means ± S.E.M. (N=6). Asterisks indicate values siginificantly different from the value in the absence of coelenterzine; *P<0.05, **P<0.01.

range of ROS than previously suspected and that alkyl, alkoxyl or peroxyl radicals can be very efficiently quenched by the imidazolpyrazinone. In addition to a high affinity for ROS, an efficient antioxidant is usually characterised by the ability to produce stable, relatively unreactive, derivatives with oxygen. This is most often achieved by reactions between two activated molecules of the antioxidant. However, combination of two radical species is probably not the main antioxidative mechanism for imidazolopyrazinones. Instead, the high instability of the excited species would lead to rapid disintegration into its oxidised products. The postulated reaction mechanism of imidazolopyrazinones with ROS is similar to that of the bioluminescent reaction and includes the formation of a dioxetanone intermediate, which breaks down to excited oxyluciferin and CO_2 (Nakano *et al.* 1986). The return of the excited product to the ground state is very fast, taking approximately 10^{-12} to 10^{-9} s (Hart *et al.* 1979). Through this mechanism, the destructive energy of the ROS would be rapidly dissipated, with little possible transfer of the excited state to neighbouring molecules in a chain-like reaction. Most of the energy would be dissipated as heat, while another, much smaller, amount (less than 1 %) would lead to the emission of light. When the reactivity of ROS with the amino acids that were proposed (McCapra and Roth, 1972) to cyclise to produce coelenterazine (Phe, Tyr) is compared with that with coelenterazine, the rate constant of the reaction with $O_2^{\cdot -}$ is 10^6 to 10^7 times smaller in the initial constituents than in the final product (see Table 2). Thus, the cyclisation of the tripeptide leads to a product with quenching properties that were completely absent in the starting constituents.

If the scavenging properties of imidazolopyrazinones are involved in the detoxification of ROS, enzymes could possibly catalyse the reaction and increase the efficiency of the quenching. The intervention of enzymes catalysing the oxidation of an electron acceptor by highly reactive oxygen species is a well-known process. Glutathione peroxidase catalyses the reaction of glutathione with H_2O_2 (Mills, 1957), and lactoperoxidase can utilise a wide range of reducing compounds, such as ascorbic acid (Chance *et al.* 1979). This could also be the case for coelenterazine, as recent work by Mitani *et al.* (1994) has revealed that imidazolopyrazinones can act as electron donors in the elimination of H_2O_2 by peroxidase. Interestingly, albumin, which shows some luciferase-like activity with both *Vargula* luciferin and coelenterazine, also quenches ROS (Halliwell and Gutteridge, 1990). Thus, the possibility exists that imidazolopyrazinones may constitute secondary acceptors of radicals formed in the reaction of proteins or other substances with ROS.

Table 3. *Chain-breaking activity of the α-tocopherol trolox C and coelenterazine as measured by the lag time (latency) after the addition of AAPH and the rate of propagation after the onset of peroxidation*

	Controls	Trolox C, 2.5 μmol l^{-1}	Coelenterazine, 2.5 μmol l^{-1}
Latency (min)	0.26±0.28[a]	23.25±0.81[b]	75.30±1.20[c]
Propagation rate (munits A_{234} min^{-1})	13.37±0.53[a]	11.99±0.41[a]	7.33±0.40[b]

The test used emulsified linoleate incubated at 37 °C with 1.6 mmol l^{-1} AAPH [azo-bis (2-amidinopropane hydrochloride)] as described by Saija *et al.* (1995).

Values are means ± S.E.M. (N=3).

Means with different superscripts are statistically different (P<0.001).

The deep sea: an environment with reduced oxidative stress

The results obtained with coelenterazine suggest that the molecule may participate in the antioxidative arsenal of marine organisms. Recent reports suggest that marine organisms are exposed to high environmental concentrations of potentially deleterious oxygen derivatives. High concentrations of superoxide anion and hydrogen peroxide are present in sea water (Van Baalen and Marler, 1966; Zika *et al.* 1985). Because superoxide anion is formed photochemically and then decays predominantly to H_2O_2 (Petasne and Zika, 1987), these two oxygen derivatives are abundant in the upper layers of the oceans and their concentration decreases with increasing depth (Zika *et al.* 1985). In coastal waters at mid-day, the rate of superoxide production can reach 5×10^{-7} mol l^{-1} h^{-1} and its steady-state concentration 2×10^{-8} mol l^{-1} (Petasne and Zika, 1987); the concentration of H_2O_2 in the upper waters (10^{-7} to 2×10^{-7} mol l^{-1}) is also higher than in deeper water layers (Van Baalen and Marler, 1966; Petasne and Zika, 1987; Zika *et al.* 1985). Algae involved in red tides have also been shown to

produce very high levels of $O_2^{\cdot -}$ at a rate approximately 100 times higher than in activated mammalian phagocytes. These radicals may be in part responsible for the significant damage inflicted by the algae on fish tissues (Tanaka *et al.* 1992; Oda *et al.* 1992). This also suggests that organisms that form red tides have highly developed antioxidative defences.

Thus, organisms living in the upper regions of the marine water column are bathed in a virtual 'sea of radicals' and must overcome the continuous threat posed by these oxidants. One solution would be to reduce cellular contact with sea water. However, as marine fishes are largely hypo-osmotic to their environment, water losses need to be compensated by drinking sea water, whereas marine invertebrates are iso-osmotic to sea water and free exchange is possible. These environmental parameters, combined with the high levels of unsaturated fatty acids in the tissues of marine organisms, render the possession of antioxidative capacities critical to their survival. Thus, the ability to use imidazolopyrazinones to reduce oxidative stress would have been advantageous to epipelagic organisms.

There are several lines of evidence that argue for the continued use of imidazolopyrazinones as antioxidants in present marine organisms. It is noteworthy that coelenterazine is distributed among all tissues of bioluminescent organisms rather than being restricted to the light-emitting organs. Several studies revealed that the highest coelenterazine content is not found in the photophores but in the digestive glands, the gonads and the skin (Shimomura *et al.* 1980; Rees *et al.* 1992; Rees and Thompson, 1994). Also, coelenterazine is present in the tissues of many non-luminescent marine organisms; the highest concentrations are generally detected in digestive glands, in the liver in fish and in the hepatopancreas in crustaceans (Young *et al.* 1979; Shimomura *et al.* 1980; Shimomura, 1987). These organs are well-known for their high rates of oxidative reactions. In mammals, the liver has been estimated to generate 24 nmol $O_2^{\cdot -}$ min^{-1} g^{-1}, and the steady-state levels of H_2O_2 and $O_2^{\cdot -}$ were estimated to be approximately 10^{-7} to 10^{-9} mol l^{-1} and 10^{-11} mol l^{-1}, respectively (Chance *et al.* 1979). Non-luminescent shrimp injected with coelenterazine were shown to retain the luciferin for weeks. Furthermore, they were able to synthesise a stabilised derivative, probably an enol-sulphate form, which was stored in the digestive gland (Shimomura, 1987). In cephalopods, high-intensity chemiluminescence comparable to that of bioluminescent species has been reported in various organs of non-luminescent species. This chemiluminescence, mainly located in the digestive glands of squids, has been linked to the presence of imidazolopyrazinones, presumably coelenterazine or its disulphate derivative (Young *et al.* 1979).

In deeper regions of the marine water column, the exposure to oxidative stress is considerably lower because of reduced light irradiance and lower oxygen levels. In the oxygen minimum layer, the concentration of oxygen can be as low as 2 % of that found near the surface. Furthermore, the reduction in the metabolic activity of pelagic fishes with increasing depth of occurrence (Childress, 1971; Torres *et al.* 1979) probably decreases the endogenous production of ROS. Our work on fishes demonstrates that in most tissues the activities of superoxide dismutase and glutathione peroxidase are well adapted to endogenous rates of oxidative metabolism, as measured by the activity of the aerobically poised enzyme citrate synthase (Janssens *et al.* 1997). The activity of the two detoxification enzymes decreases exponentially with increasing depth of occurrence. Therefore, organisms living in deeper waters would have lower constraints on antioxidative defences and could have developed mechanisms for harnessing the chemiluminescence of imidazolopyrazinones. The preferential association of imidazolopyrazinones with structures of the digestive system in many organisms (pyloric caeca in cardinal fishes, diverticules of the hepatopancreas in decapods) might possibly reflect the original need to oppose radicals as soon as they enter the body. Also, the disposition of the photophores in most species at sites exposed to sea water, i.e. digestive glands or the external surface of the body, may be related to the location of their antioxidative evolutionary precursors. Indeed, the skin and the digestive tract and its associated glands are tissues that undergo high oxidative stress in fish, as indicated by the elevated activities of glutathione peroxidase and superoxide dismutase in these tissues (Nakano *et al.* 1993*a*,*b*; Tappel *et al.* 1982; B. Janssens, unpublished observations).

The ability to produce light with ecological functions has appeared on several occasions during the course of evolution, as reflected by the many different chemistries found in existant bioluminescent organisms. Although it is unlikely that the functional shift from antioxidative towards bioluminescent roles that we propose in coelenterazine-based luminescent systems can be extended to all existing luminescent chemistries, it is noteworthy that either oxygen or its activated species is required in all luciferin–luciferase systems (Table 4).

Table 4. *Possible relationships between bioluminescent systems and antioxidative mechanisms*

Group/Genus	Oxygen species	Other links
Annelid		
Diplocardia	H_2O_2	
Harmothoë	$O_2^{\cdot -}$	Fe^{2+}
Balanoglossus	H_2O_2	Fe^{2+}
Bivalve		
Pholas	H_2O_2, $O_2^{\cdot -}$	Peroxidase activity of the luciferase
Gastropod		
Latia		Vitamin A analogue
Bacteria		
Vibrio	O_2, H_2O_2	Aliphatic aldehyde, catalase-like activity of the luciferase

Besides the direct involvement of activated species of oxygen, some systems are linked either by the catalytic activity of the luciferase towards reactive oxygen species, or by the involvement of cofactors linked to oxidative stress (see text for explanations and references).

Several systems have been shown to involve reactive oxygen species. Polynoidin, the luciferin found in the scale worm *Harmothoë*, can react with the superoxide anion and yield a light-emitting complex. This system appears to lack a luciferase, and the luminescence could be triggered by a controlled production of ROS, such as superoxide anion or the hydroxyl radical (Nicolas *et al.* 1982). The bioluminescence of the parchment worm *Chaetopterus variopedatus* may derive from a similar reaction (Shimomura and Johnson, 1968*a*). In the boring clam *Pholas dactylus*, luminescence can be triggered equally well by Fe^{2+} in the presence of O_2 or by the luciferase, thus indicating that ROS are involved in the reaction (Henry and Michelson, 1973; Henry *et al.* 1973, 1978). In the earthworm *Diplocardia longa*, H_2O_2, but not oxygen, is required for luminescence (Bellisario *et al.* 1972), which can also occur in the absence of luciferase when H_2O_2 is mixed with the luciferin (Rudie *et al.* 1981). *Latia neritoides*, the only bioluminescent animal spending its entire life in fresh water, possesses a luciferin very similar to vitamin A (Shimomura and Johnson, 1968*b*), thus indicating that it could have been derived from this antioxidative defence mechanism. Bacterial luciferin can be substituted by H_2O_2 in the reaction with luciferase, but the reaction has a low light yield, thus indicating that the primary function of the system could have been related to the detoxification of H_2O_2 in a catalase-like manner (Watanabe *et al.* 1993). This possible primitive function could have evolved later towards bioluminescence when bacteria started producing the long-chain fatty acid aldehyde tetradecanal. Interestingly, fatty acid aldehydes are the main product formed during the peroxidation of unsaturated fatty acids. These alcanals and alcenals are very reactive with the amino and sulphydryl groups of proteins and peptides, and some alcenals (e.g. 4-hydroxy-nonenals) are mutagenic (Eckl *et al.* 1993). Thus, it is possible that the luciferase might also have played or could still play a role in the detoxification of these toxic aldehydes and provide some protection to the cells. In order to test this hypothesis, we investigated the ability of bacterial luciferase to utilise aldehydes by the peroxidation of cellular lipids. Rat hepatocytes were treated with tert-butyl hydroperoxide for 2 h, and the production of malondialdehyde and the luminescence elicited with $FMNH_2$-bacterial luciferase were assayed in parallel in the supernatant. The results indicate that the intensity of the luminescence closely follows the amount of malonaldialdehyde produced, thus indicating that substrates for the luciferase are being released by cells when lipids are being oxidised (Fig. 4). This supports the idea that bacterial luciferase precursors or the present-day enzyme could be involved in the detoxification of the toxic metabolites (H_2O_2, aldehydes) generated when cells are subjected to oxidative stress. The production of aldehyde substrates for bacterial luciferase during the peroxidation of lipids has been demonstrated in cell-free extracts of the luminous bacterium *Vibrio harveyi* (Ismailov *et al.* 1994). However, whether the luciferase is involved in the protection of bacterial cells against oxidative stress remains to be investigated.

Thus, the imidazolopyrazinone-based bioluminescent

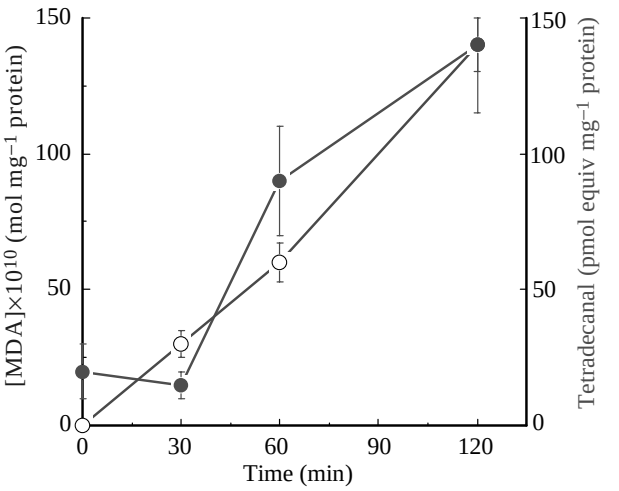

Fig. 4. Catabolic activity of bacterial luciferase towards fatty acid aldehydes produced during lipid peroxidation (A. Coutelier and J.-F. Rees, unpublished results). Rat hepatocytes were treated for 2 h in the presence of 10^{-2} mol l^{-1} tert-butylhydroperoxide. Malondialdehyde (MDA), an oxidation product of unsaturated fatty acids, was assayed in the culture medium (○). In parallel, the luminescence elicited by mixing a sample of the medium with bacterial luciferase and $FMNH_2$ (according to the method of Morse *et al.* 1986) was measured and expressed in pmol tetradecanal required to elicit the same luminous intensity (●).

systems that have been characterised to date may reflect to some extent the evolutionary history of bioluminescence. If imidazolopyrazinone luciferases have evolved from oxygenase ancestors, as has been proposed, it is curious that no significant homologies have been uncovered among the different luciferases that have been cloned to date or with the more than 600 known oxygenase sequences in the SwissProt database. If there was an ancestral oxygenase, the total lack of homology in the latter case is difficult to explain in view of the rather central role of oxidative metabolism. Given the high reactivity of oxygen as an electron acceptor, it is not surprising to find rather high rates of catalysis among a number of oxygenases. However this contrasts sharply with the consistently low substrate turnover rates of the imidazolopyrazinone luciferases that have been characterised thus far. Rather, it seems that the most important role of these luciferases was to provide a hydrophobic cage in order to increase the quantum yield of the chemiluminescence derived from imidazolopyrazinone oxidation and to prevent quenching or spectral degradation of this emission as a result of the collision of the excited state emitter with other proteins or cellular components. This would be consistent with the derivation of luciferases from a variety of unrelated protein precursors. Substrate turnover is not limited by the actual oxidation of the imidazolopyrazinone luciferin. Instead, the low turnover rates are likely to be a compromise in order to produce a bioluminescent emission of reasonable quantum efficiency and high spectral quality, while perhaps sacrificing some initial source intensity which could be achieved through faster substrate turnover. In this regard, it is of interest that in the *Vargula* reaction, which currently has

the highest known turnover rate for an imidazolopyrazinone luciferase, the enzyme and substrate are secreted into the surrounding sea water where the concentration of spectrally degrading quenchers is likely to be lower than in an intracellular reaction with a higher macromolecular density.

This model proposes that it was the antioxidant origins and chemiluminescent properties of imidazolopyrazinones that were the foundation of this type of marine bioluminescent system. Luciferases were accessories derived from a variety of precursors in the eventual adaptation of this weak chemiluminescence to biologically useful light production. Further information on the identification of organisms that are the sources of imidazolopyrazinone production and the sequencing of luciferases in different marine groups will yield important data for the testing and refinement of the proposed model.

This work was supported by grants from the Fonds National de la Recherche Scientifique (FNRS), the Fonds de la Recherche Fondamentale Collective (convention 2.4536.96) and the Fonds de Développement Scientifique of the University of Louvain. M.D. and J.-F.R. are respectively junior research assistant and research associate of the FNRS, B.d.W. and B.J. are supported by fellowships from the Fonds pour la Formation à la Recherche dans l'Industrie et dans l'Agriculture (FRIA). We are very grateful to Dr Thérèse Wilson for her comments on the manuscript.

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