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Quenching of superoxide radicals by green fluorescent protein

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Abstract

Green fluorescent proteins (GFP) are widely used *in vivo* molecular markers. These proteins are particularly resistant, and maintain function, under a variety of cellular conditions such as pH extremes and elevated temperatures. Green fluorescent proteins are also abundant in several groups of marine invertebrates including reef-forming corals. While molecular oxygen is required for the post-translational maturation of the protein, mature GFPs are found in corals where hyperoxia and reactive oxygen species (ROS) occur due to the photosynthetic activity of algal symbionts. In vitro spin trapping electron paramagnetic resonance and spectrophotometric assays of superoxide dismutase (SOD)-like enzyme activity show that wild type GFP from the hydromedusa, *Aequorea victoria*, quenches superoxide radicals ($O_2^{\bullet -}$) and exhibits SOD-like activity by competing with cytochrome *c* for reaction with $O_2^{\bullet -}$. When exposed to high amounts of $O_2^{\bullet -}$ the SOD-like activity and protein structure of GFP are altered without significant changes to the fluorescent properties of the protein. Because of the distribution of fluorescent proteins in both the epithelial and gastrodermal cells of reef-forming corals we propose that GFP, and possibly other fluorescent proteins, can provide supplementary antioxidant protection.

Keywords: GFP; Superoxide radical; Coral; EPR; Spin trapping; Capillary electrophoresis

1. Introduction

Green fluorescent protein, GFP, is a spontaneously fluorescent protein composed of ~238 amino acids (~27 kDa as a monomer) that absorbs blue light and re-emits it as green fluorescence [1,2]. The fluorescent chromophore originates from an internal Ser–Tyr–Gly sequence that is post-translationally modified in the presence of molecular oxygen to form a fluorescent ring structure that is maximally excited at either 395 nm or 475 nm and emits at 505–515 nm [1–3]. GFP from the hydromedusa *Aequorea victoria* has been sequenced and is now widely used as a reporter gene for transcriptional studies at the molecular level while mutants from *A. victoria* and other cnidarians with different fluorescent emission properties from blue to red have also been described [3]. GFP has also been identified in a variety of non-symbiotic and symbiotic cnidarians including reef-forming corals [4–8] where the protein is not coupled to a bioluminescent system and its function(s) remains unclear. It has been proposed that GFP and their homologues improve the efficiency of photosynthesis or provide photoprotection for the symbiotic dinoflagellates (zooxanthellae) [9]. Recent data, however, have not supported a role for GFP as an accessory pigment for photosynthesis [7,10] and other observations argue against a direct photoprotective role. For instance, the bathymetric distribution of GFP in two common species of coral from the Caribbean did not change significantly with depth [7], and non-bioluminescent, azooxanthellate, anthozoa in low light habitats [8] were found to contain fluorescent proteins. Despite these results, photoprotection is thought to be the primary function of these proteins [5,9,11].

Symbiotic cnidarians also experience hyperoxia from photosynthetically produced oxygen by their zooxanthellae [12,13]. While this is an ideal environment for the post-translational maturation and chromophore formation of this protein [3], it also imposes hyperoxic conditions that lead to the production of high concentrations of ROS as there is a direct relationship between the concentration of oxygen and ROS

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formation [14,15]. In response to this hyperoxia and ROS formation, both the coral host and its symbiotic zooxanthellae exhibit robust antioxidant defenses in the form of higher specific activities of enzymes such as SOD, catalase, and ascorbate peroxidase [15–18]. Simultaneous exposure to solar radiation and thermal stress in shallow tropical waters during physiological hyperoxia increases the production of ROS and can lead to a phenomenon known as coral bleaching where the algal symbionts are expelled from the host tissues as part of a stress response [17]. Several lines of evidence have shown that oxidative stress and subsequent apoptosis or necrosis are the underlying cellular causes of the bleaching phenomenon [16–18]. In these same corals, GFPs can be found in high concentration (>1.0 mg cm^2 coral surface area) within the host tissues of scleractinian corals [7,11]. During a recent study on the distribution and function of GFPs in Caribbean corals it was suggested that GFP may have the ability to quench ROS [7].

In this paper we combine EPR spin-trap measurements with assays of SOD-like activity, fluorescence excitation/emission spectra, and capillary electrophoresis to demonstrate that wild type GFP from the hydromedusa *Aequorea victoria* quenches $O_2^{\bullet -}$ radicals, has SOD-like activity, and does not alter the fluorescence properties of GFP during the quenching process. In conjunction with other enzymatic and non-enzymatic antioxidant defenses [17,19,20], we suggest that GFP could provide corals additional antioxidant properties by quenching $O_2^{\bullet -}$.

2. Materials and Methods

2.1. Measurements of $O_2^{\bullet -}$ spin-trap adducts

Electron paramagnetic resonance (EPR) spectra of the 5-ethoxycarbonyl-5-methyl-1-pyrroline-N-oxide (EMPO) and 5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide (DEPMPO) adducts of $O_2^{\bullet -}$ are specific and well described [21,22]. EPR spectra were measured on 40 μl samples in 1.0 mm (i.d.) quartz capillary contained in a 2.0 mm (i.d.)×4 mm (o.d.) quartz tube using a Bruker EleXsys E-500 spectrometer. The X-band EPR signals of the EMPO-OOH or DEPMPO-OOH adducts were recorded at room temperature immediately after the addition of the last reagent in the presence or absence of GFP (13.8 or 18.5 μM) (CLONTECH recombinant GFP #8360-2 from *Aequorea victoria*) or egg albumin (14 μM) in 50 mM phosphate buffer, pH 7.4, 0.5 mM diethylenetriamine pentaacetic acid (DTPA), 0.2 mM hypoxanthine, 50 mM EMPO or DEPMPO, and 0.3 U ml^{-1} xanthine oxidase (EC 1.1.3.22). All chemicals were of reagent grade (Sigma-Aldrich); EMPO and DEPMPO were purchased from AXXORA, LLC, San Diego, CA. Typical spectrometer parameters were: microwave power 20 mW; modulation amplitude 0.5 G; time constant 163.8 ms; sweep time 83.89 s and the signal averaged two times.

2.2. Fluorescence excitation/emission spectra

Fluorescence emission and excitation spectra were measured at room temperature on a Varian Cary Eclipse fluorometer using excitation and emission wavelengths of 395 nm and 509 nm, respectively, in the presence of GFP (0.37 μM) in 50 mM Tris–HCl buffer, pH 8.0, with and without 0.2 mM hypoxanthine, and 0.1 U ml^{-1} xanthine oxidase.

2.3. Superoxide dismutase-like activity

The ability of *Aequorea victoria* GFP to quench superoxide radicals and exhibit SOD-like activity was assayed spectrophotometrically as described by Oyanagui [23] using the nitrite method with GFP concentrations ranging from 2.4 to 61.7 nM. Briefly, $O_2^{\bullet -}$ is generated (hypoxanthine (1 mM), and 0.85 U ml^{-1} xanthine oxidase) and oxidizes hydroxylamine (1 mM) to nitrite which is detected using a diazo method in sodium borate buffer with 0.5 mM EDTA at a pH of 8.2 at 25 °C in a total volume of 3 ml. The degree of inhibition of nitrite formation is measured at 550 nm on a spectrophotometer (Hewlett Packard HP 8453). A standard curve was prepared using commercially available SOD (Sigma bovine erythrocyte Cu/Zn SOD). Similar results were obtained when the experiment was repeated using an OxisResearch SOD assay kit (SOD-525) containing DTPA to suppress any SOD-like activity that may arise from trace metal ions (data not shown).

The ability of GFP to maintain superoxide quenching ability upon repeated additions of $O_2^{\bullet -}$ was assessed spectrophotometrically using the cytochrome *c* SOD assay as described by Oyanagui [23]. Briefly, cytochrome *c* (25 μM in 50 mM phosphate buffer, pH 7.8, 25 °C) reduction was monitored at 550 nm using a Varian Cary 50 spectrophotometer following repeated additions of hypoxanthine (6 μM) to the same sample in the presence of 0.1 U ml^{-1} xanthine oxidase. The spectrophotometric data were further analyzed with Origin 7.0 software (Microcal Inc.).

2.4. Capillary electrophoresis

Capillary electrophoresis (CE) and sodium dodecyl sulfate capillary gel electrophoresis (SDS-CGE) under native and denaturing conditions, respectively, were performed on a Beckman Coulter P/ACE MDQ capillary electrophoresis system. An eCap SDS 14-200 kit, SDS-coated capillary (50 cm long and 100 μm diameter), SDS 14-200 test mix containing seven proteins with molecular weight ranging from 14,200 to 205,000, SDS Orange G reference marker, SDS 14-200 gel buffer, and SDS sample buffer (0.12 M Tris–HCl and 1% SDS, pH 6) were purchased from Beckman-Coulter (Fullerton, CA). The standard mix was dissolved in 750 μl SDS 14-200 gel buffer and then diluted twice with de-ionized water. To a 200 μl standard sample, 10 μl of the reference marker Orange G was added and the solution vortexed for 2 min and suspended in boiling water for 10 min. The samples were then cooled for 10 min prior to running. Experimental GFP samples were prepared similarly except the final volume of the sample was 100 μl resulting in a final concentration of 27 μg protein 100 μl^{-1}. The SDS-CGE was run under reverse polarity conditions with detection at 214 nm. The voltage setting was 15 kV (or 300 V cm^{-1} for the 50 cm length of the capillary), and the separation time was set to 35 min. Superoxide radicals used in the SDS-CGE experiments were generated by the hypoxanthine/xanthine oxidase system in 0.1 M phosphate buffer, pH 7.2, using 0.1 mM hypoxanthine and 0.1 U ml^{-1} xanthine oxidase. To exclude the possibility that hydroxyl radicals would form and be responsible for the changes in the protein peaks observed by CE, 50 mM mannitol (a known hydroxyl radical scavenger) was added to some GFP samples. Similar results were obtained in the presence or absence of mannitol. For native CE, GFP samples (0.44 mg ml^{-1}) were prepared in 0.1 M phosphate buffer, pH 7.2. The separation time of the electropherograms was set to 30 min and borate buffer, pH 8.03, was used as the running buffer at a voltage of 10 kV (or 170 V cm^{-1} for 59 cm total length capillary).

3. Results

To demonstrate the ability of GFP to quench $O_2^{\bullet -}$ radicals, EPR experiments using two spin traps, EMPO and DEPMPO, were performed (Fig. 1). The stability of the spin-adducts and the well characterized electron spin resonance spectra make these compounds the best choice to trap ROS such as $O_2^{\bullet -}$ and hydroxyl radicals in biological systems [21,22,24,25]. Spectrum A of Fig. 1 is a control experiment where $O_2^{\bullet -}$ was produced by the hypoxanthine/xanthine oxidase generating system in phosphate buffer, pH 7.4 in the presence of EMPO. Spectrum B is a second control experiment obtained under the same conditions as spectrum A but in the presence of egg albumin used here as a control protein. A strong signal from trapped $O_2^{\bullet -}$ was observed in both controls (Fig. 1, spectra A

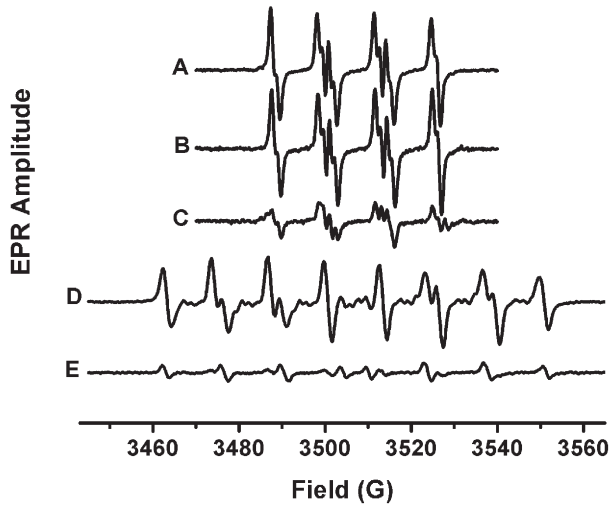

Fig. 1. EPR of EMPO-OOH adduct in buffer alone as a control (A), in the presence of egg albumin (14 μM) (B), and in the presence of 13.8 μM GFP (C). EPR of DEPMPO-OOH adduct in the absence (D) and presence (E) of GFP (18.5 μM). $O_2^{\bullet -}$ were generated by the hypoxanthine/xanthine oxidase system in 50 mM phosphate buffer at pH 7.4, 0.5 mM DTPA and 50 mM EMPO or DEPMPO.

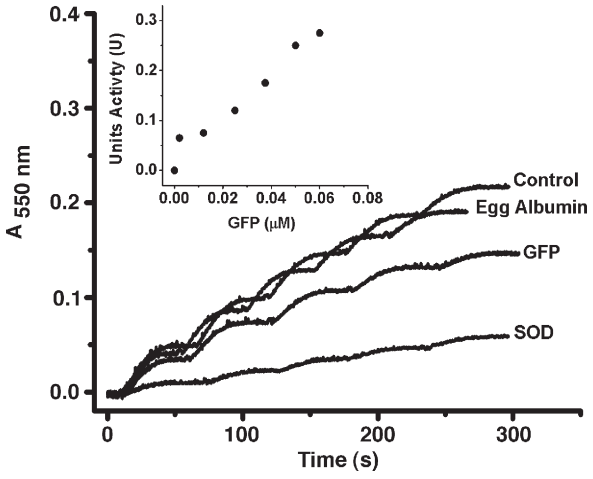

Fig. 2. Reduction of cytochrome *c* by $O_2^{\bullet -}$ generated using the hypoxanthine/xanthine oxidase system and inhibition by SOD or GFP. Conditions: 25 μM cytochrome *c*, 0.1 mM EDTA, 1300 U ml^{-1} catalase, 6 μM hypoxanthine per injection (stars denote injection times), and 0.1 U ml^{-1} xanthine oxidase. Kinetics were performed in the presence of either egg albumin (1.5 μM), GFP (1.5 μM) or Cu/Zn-SOD (1.5 U ml^{-1}, 0.015 μM) from bovine erythrocytes (Sigma, EC 1.15.1.1) Inset: Superoxide quenching activity of *Aequorea victoria* GFP at increasing protein concentrations (2.4 to 61.7 nM) and a constant 100 nM ($O_2^{\bullet -}$).

and B) indicating that egg albumin does not quench $O_2^{\bullet -}$ and inhibit the formation of the EMPO-OOH adduct. In contrast, the EPR amplitude of EMPO-OOH is greatly attenuated (64%) in the presence of GFP (Fig. 1, spectrum C). Similar results were obtained in the presence of the spin trap DEPMPO where GFP attenuated the EPR signal by 77% (Fig. 1). These spin trapping results indicate that the purified recombinant GFP protein reduces the amount of $O_2^{\bullet -}$ radicals available for reaction with the two spin traps (EMPO and DEPMPO).

In the cytochrome *c* SOD assay for superoxide quenching, repeated aliquots of hypoxanthine were added to the reaction mixture in the presence of xanthine oxidase to produce increments of $O_2^{\bullet -}$. No difference in the initial rates of ferricytochrome *c* reduction by $O_2^{\bullet -}$ was observed whether egg albumin (used as a protein control) was present or not (Fig. 2), indicating that egg albumin does not compete with cytochrome *c* for reaction with $O_2^{\bullet -}$ nor does it exhibit SOD-like activity. In contrast, when GFP was present, a constant 40% inhibition of the rate of ferricytochrome *c* reduction relative to the controls was observed with each addition of hypoxanthine compared to 75% inhibition in the presence of bovine erythrocyte SOD at a 100-fold lower protein concentration (Fig. 2). As long as small increments of ~1–2 $O_2^{\bullet -}$ per GFP are generated per addition, GFP maintains SOD-like quenching activity over the course of five successive additions of $O_2^{\bullet -}$. When ratios of ~6–10 $O_2^{\bullet -}$ per GFP are used, the initial SOD-like activity corresponding to ~40% inhibition of cytochrome *c* reduction is also observed for the first addition whereas successive $O_2^{\bullet -}$ additions showed rates similar to those of the control (data not shown). These results suggest that the protein is altered under conditions of high $O_2^{\bullet -}$ /protein ratios (more below).

In the nitrite reduction SOD assay, the $O_2^{\bullet -}$ quenching activity of GFP increased linearly with increasing protein concentration, consistent with the protein having SOD-like activity under these conditions (Fig. 2, inset). The SOD-like activity of GFP is estimated to be ~60 U mg $protein^{-1}$ from the nitrite reduction assay, a value somewhat higher than that from the cytochrome *c* assay (~20 U mg $protein^{-1}$) but typical of the dependence of the measured activity on the assay employed. These values are significantly lower than the SOD activities of mammalian SODs generally [26] but comparable to those observed in the zooxanthellae of corals and other photoautotrophic organisms [27–29].

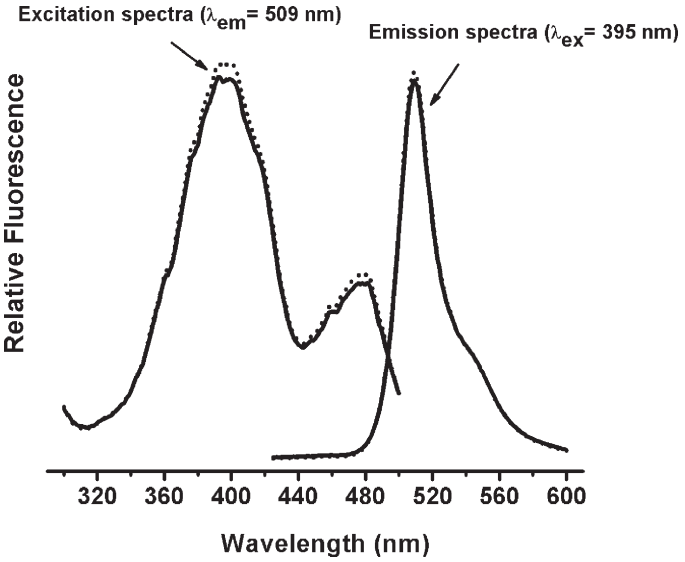

Fig. 3. Fluorescence excitation/emission spectra of GFP before (dotted line) and after (black line) exposure to $O_2^{\bullet -}$. Conditions: GFP (0.37 μM) in 50 mM Tris–HCl buffer, pH 8.0, with or without 0.2 mM hypoxanthine, and 0.1 U ml^{-1} xanthine oxidase.

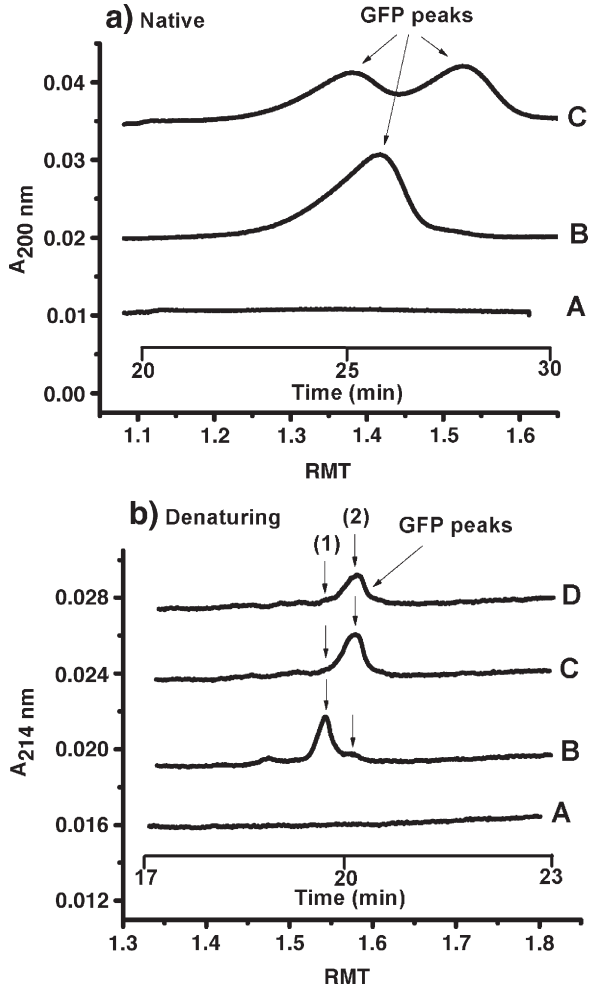

Fig. 4. (a) Electropherograms of GFP (0.44 μg μl^{-1}) in the presence and absence of $O_2^{\bullet -}$ (A) Buffer control, (B) GFP alone in buffer, (C) GFP + $O_2^{\bullet -}$. Conditions: 0.1 M phosphate buffer, pH 7.2, 0.4 mM DTPA, 0.17 mM hypoxanthine and 0.1 U ml^{-1} xanthine oxidase. (b) Electropherograms of GFP (0.27 μg μl^{-1}) under denaturing conditions. (A) Buffer control, (B) GFP alone in buffer, (C) GFP + $O_2^{\bullet -}$ + mannitol, (D) GFP + $O_2^{\bullet -}$ but no mannitol. Arrows denote the conversion of species (1) to (2) following $O_2^{\bullet -}$ addition. Conditions: 0.1 M phosphate buffer, pH 7.2, 50 mM mannitol, 0.1 mM hypoxanthine and 0.1 U ml^{-1} xanthine oxidase. In order to correct for the run-to-run variations, the data were normalized by dividing the migration times for each sample by the migration time of the phosphate buffer peak in native CE or by that of Orange G in SDS-CGE to give a relative migration time (RMT).

One of the critical questions regarding the quenching activity of GFP is the fate of the protein after $O_2^{\bullet -}$ quenching. One assessment of function, and therefore structure, of fluorescent proteins is the measurement of fluorescence excitation/emission spectra [30]. The fluorescence excitation/emission spectra for a GFP sample exposed to $O_2^{\bullet -}$ shows the typical excitation maxima at 395 nm and 475 nm (Fig. 3) [3]. The shape of the excitation/emission spectra did not change and no apparent spectral shift or disappearance of the well-described excitation or emission maxima was observed (Fig. 3), a result indicating no significant change in the fluorochrome structure following exposure of the protein to high concentration of $O_2^{\bullet -}$ (~540 $O_2^{\bullet -}$ per GFP). The only subtle change was a small decrease in fluorescence intensity (Fig. 3). When these samples were further exposed to either dithionite or $FeSO_4$, the fluorescence disappeared and then returned upon exposure to air as previously described for the wild type protein exposed to reducing and oxidizing environments [31].

CE experiments were therefore undertaken to determine whether GFP undergoes oxidative/reductive damage elsewhere on the protein following treatment with $O_2^{\bullet -}$. SDS-CGE has been shown to be an excellent tool for studying the integrity of proteins following exposure to oxidative reagents [32]. The electropherograms of GFP samples obtained under either native or denaturing conditions showed no change in the protein peak when GFP was exposed to only ~1–2 $O_2^{\bullet -}$ per GFP (data not shown) which is consistent with the retention of SOD-like activity as observed above. In contrast, when higher $O_2^{\bullet -}$ to protein ratios were used (~6–10 $O_2^{\bullet -}$ per GFP) an environment was produced in which the activity was degraded, and the electropherograms of GFP samples under both native and denaturing conditions were altered, indicating changes in charge and/or molecular weight of GFP (Fig. 4a, b). At least two new protein species were produced from the parent protein (Fig. 4a, see curve C for $O_2^{\bullet -}$ treated *versus* B for untreated GFP). The data demonstrate that a structural change(s) occur in the protein when it is exposed to high concentrations of $O_2^{\bullet -}$, a result in accord with the loss of SOD-like quenching activity observed in the kinetics data noted above when ratios of ~6–10 $O_2^{\bullet -}$ per GFP were used.

It is important to note that the fluorescence properties of GFP are not affected by the presence of large concentrations of $O_2^{\bullet -}$ (Fig. 3). This suggests that the fluorochrome within the β-barrel of the protein is still protected from damage despite the structural change(s) in the protein observed by CE (Fig. 4).

4. Discussion

This study is the first report showing that GFP can quench $O_2^{\bullet -}$. The mechanism of quenching is not apparent from the data in this study but appears to involve weak SOD-like activity at low $O_2^{\bullet -}$ concentrations while at higher concentrations quenching still occurs but at the expense of structural changes in the protein. The observation that there is no loss of activity, or fluorescence, when GFP is exposed to low fluxes of $O_2^{\bullet -}$ radicals, is important because low fluxes of $O_2^{\bullet -}$ are present in most organisms under normal conditions [20]. The modest SOD-like activity of GFP may well be compensated by its rather high abundance in corals, making it a significant contributor to the antioxidant defenses of the organism [7,11]. There is a continuing debate on whether $O_2^{\bullet -}$ radicals are actually a significant threat to cells since such a small fraction of O_2 reduction gives rise to $O_2^{\bullet -}$ and its reactivity at physiological pH is relatively low [20]. It has been shown, however, that although SOD reduces the steady state concentration of $O_2^{\bullet -}$ by several orders of magnitude, $O_2^{\bullet -}$ still has significant, and independent, potential to damage many cellular targets [33].

While no changes in fluorescence was observed when the protein was exposed to $O_2^{\bullet -}$, other species of ROS such as hydrogen peroxide or peroxynitrite anion are known to decrease

GFP fluorescence in the presence of mercaptoethanol, suggesting that the protein itself was irreversibly damaged [34]. Since peroxynitrite is very reactive under physiological conditions and formed by the reaction between nitric oxide ($NO^{\bullet}$) and $O_2^{\bullet -}$, the presence of high concentrations of GFP in corals would not only be useful in preventing the formation of hydroxyl radicals but also in preventing the formation of peroxynitrite since $NO^{\bullet}$ is known to occur in symbiotic cnidarians and nitric oxide synthase activity increases in corals experiencing thermal stress [35,36].

The present results do not allow us to identify the molecular origin of the SOD-like activity of GFP. Similarly, the location of the $O_2^{\bullet -}$ induced damage is unknown, but proteins in general can undergo a range of oxidative modifications that involve amino acid residues such as tyrosine, phenylalanine, tryptophan, cysteine and methionine, the latter two being particularly susceptible because of their accessible –SH and $-SCH_3$ groups [20,37]. Protein damage caused by hydroxyl radicals may also be involved [38,39] but our CE results (Fig. 4) in the presence and absence of mannitol, a known hydroxyl radical scavenger, argue against this.

It is reasonably presumed that hyperoxia and exposure to high fluxes of ROS has been a pervasive feature of coral biology since the Triassic period (250–300 mya) when the symbiosis with photosynthetic zooxanthellae arose [40]. Additionally, the GFP family of genes is believed to have evolved before the Cnidaria and the Bilateria separated over 700 mya [41] and that the green fluorescent protein is the ancestral state [42]. GFP may now be part of a comprehensive antioxidant defense system that includes high concentrations of DMS (dimethylsulphide) from dimethylsulphoniopropionate (DMSP) known to occur in corals [43] as both DMS and DMSP have been shown to quench singlet oxygen ($^1O_2$) and hydroxyl radicals, respectively [44]. Additionally, another potent antioxidant found in many cnidarians is the imidazole compound coelenterazine, a Ca^{2+} dependent luciferin used as a substrate for bioluminescence in many marine species [45]. There is remarkable similarity in the structure of coeleterazine and GFP [46], and like GFP in corals coeleterazine is found in several cnidarians without bioluminescense [45]. Both theoretical and experimental data suggest that the luciferin/luciferase enzyme system first evolved as a ROS scavenging system and then was co-opted (i.e., exapted) for light production [45]. No bioluminescent system has been described for scleractinian corals and the absence of bioluminescence but presence of GFP, and many fluorescent and non-fluorescent homologous proteins, begs the question of the evolution and role of GFP in scleractinian corals.

Some investigators favor multiple roles for GFP in addition to its commonly accepted role to increase the quantum yield of light production by converting the blue emission of bioluminescence into green, or other, wavelengths in hydroids, jellyfish, and some anthozoans (e.g., sea pens). These roles include augmenting photosynthesis, animal coloration, and photoprotection. There is no evidence to suggest the GFP can support photosynthesis and in all cases examined it is been shown to be biophysically impossible due to limitations by fluorescence resonance energy transfer (FRET). Both Mazel et al. [7] and Gilmore et al. [10] found, using different spectroscopic approaches, that chlorophyll fluorescence is not stimulated by exciting GFP within the tissues of several species of coral suggesting no coupling between the photoproteins. While fluorescent proteins have been suggested to contribute to the coloration of scleractinian corals [5], the contribution of fluorescence to animal coloration is highly variable and dependent on the spectral composition of the downwelling irradiance and the optical properties of the seawater. In some cases, fluorescence makes a contribution but in many, if not most, cases fluorescence is overwhelmed by reflectance [47]. The evidence for photoprotection is either negative [7] or weak [9]. Mazel et al. [7] showed that GFP in two common Caribbean corals is found principally in the epithelial cells, but that the bathymetric distribution of GFP in these corals did not change significantly with depth arguing against a direct photoprotective role. Salih et al. [9] proposed that fluorescent proteins filtered out harmful wavelengths in the ultraviolet portion of the spectrum as well as excessive irradiances of visible radiation. Their measurements of chlorophyll fluorescence quantum yields did show differences in the mid-day quantum yields of different color morphs of the coral *Acropora palifera* containing different fluorescent proteins. However, these results could just as easily be explained by differences in the degree of non-photochemical quenching in the zooxanthellae as opposed to the presence of different types of fluorescent proteins.

The data presented here clearly show that GFP can quench $O_2^{\bullet -}$. While elucidation of the detailed mechanism of this protective effect is beyond the scope of the present study, the data nevertheless suggest that the antioxidant properties of GFP could be an important supplement to the antioxidant defenses of symbiotic cnidarians under the constant hyperoxic conditions that these organisms experience during the daytime. It would be informative to ascertain whether the diversification of GFPs within the cnidarians, which has resulted in many fluorescent homologs exhibiting different fluorescence properties, and non-fluorescent homologs [3–6], also have the ability to quench $O_2^{\bullet -}$. The fact that corals are hyperoxic, produce large quantities of ROS, and require robust antioxidant protection is well documented [17]. It is also well known that GFP, and its homologs, are commonly found in all zooxanthellate corals examined to date. We propose that whatever the selective pressures were for the evolution of GFP 700 mya, that during the Triassic (250–300 mya) the evolution of the modern day symbioses between corals and zooxanthellae imposed a novel selective pressure, one of hyperoxia and subsequent increase in ROS formation, that then maintained the expression of GFP as part of a comprehensive antioxidant system.

Acknowledgements

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