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Symbiosis-induced adaptation to oxidative stress

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Summary

Cnidarians in symbiosis with photosynthetic protists must withstand daily hyperoxic/anoxic transitions within their host cells. Comparative studies between symbiotic (*Anemonia viridis*) and non-symbiotic (*Actinia schmidti*) sea anemones show striking differences in their response to oxidative stress. First, the basal expression of SOD is very different. Symbiotic animal cells have a higher isoform diversity (number and classes) and a higher activity than the non-symbiotic cells. Second, the symbiotic animal cells of *A. viridis* also maintain unaltered basal values for cellular damage when exposed to experimental hyperoxia (100% O_2) or to experimental thermal stress (elevated temperature +7°C above ambient). Under such conditions, *A. schmidti* modifies its SOD activity significantly. Electrophoretic patterns diversify, global activities diminish and cell damage biomarkers increase. These data suggest symbiotic cells adapt to stress while non-symbiotic cells remain acutely sensitive. In addition to being toxic, high O_2 partial pressure (P_{O_2}) may also constitute a preconditioning step for symbiotic animal cells, leading to an adaptation to the hyperoxic condition and, thus, to oxidative stress. Furthermore, in aposymbiotic animal cells of *A. viridis*, repression of some animal SOD isoforms is observed. Meanwhile, in cultured symbionts, new activity bands are induced, suggesting that the host might protect its zooxanthellae *in hospite*. Similar results have been observed in other symbiotic organisms, such as the sea anemone *Aiptasia pulchella* and the scleractinian coral *Stylophora pistillata*. Molecular or physical interactions between the two symbiotic partners may explain such variations in SOD activity and might confer oxidative stress tolerance to the animal host.

Key words: cnidarians, zooxanthellae, symbiosis, oxidative stress, hyperoxia, thermal stress, SOD.

Introduction

Molecular oxygen (O_2) levels within the tissues of photosynthetic plants undergo cyclic variation from nocturnal hypoxia to diurnal hyperoxia. Interestingly, this phenomenon is not restricted to plant cells but also occurs in symbiotic associations between animals and photosynthetic protests, such as cnidarians and dinoflagellates. In these associations, respiration of the host and the symbiont leads to hypoxia at night, while in the daytime tissues are hyperoxic due to photosynthesis (D'Aoust et al., 1976; Dykens and Shick, 1982; Harland and Davies, 1995; Richier et al., 2003). Although O_2 has a quite low toxicity, it can be transformed and reduced into reactive oxygen species (ROS; Halliwell and Gutteridge, 1999). In tissues, imbalance between ROS production and antioxidant defense leads to cellular damage *via* lipid peroxidation, protein oxidation and DNA degradation. Among enzymatic factors, superoxide dismutases (SOD) constitute the first line of antioxidant defence (Fridovich, 1995). SOD enzymes are widely observed in biology: even in organisms lacking other antioxidant enzymes, such as catalase or peroxidase, SOD is expressed without exception (Asada et al., 1977). Plants display 4–8 different SOD isoforms (Alscher et al., 2002) while only two to three isoforms tend to be present in animals (Halliwell and Gutteridge, 1999). We have previously shown that in the associations *Anemonia viridis*/*Symbiodiniu* sp. (sea anemone/zooxanthellae) and *Stylophora pistillata*/*Symbiodinium* sp. (coral/zooxanthellae), animal host cells possess a large diversity of SOD isoforms, similar to that described in plants (Richier et al., 2003). On non-denaturing gels, *A. viridis* has at least seven different SOD activity bands that belong to the three well-known classes of SOD: CuZnSOD, MnSOD and FeSOD. Moreover, some of them have unusual characteristics. For example, some MnSODs are extramitochondrial and may be cytosolic. Furthermore, the FeSOD class is observed not only in photosynthetic cells but also in animal cells (Richier et al., 2003). This suggests an evolutionary adaptation of antioxidant defenses of symbiotic cnidarians to O_2 production by the symbiont, comparable with what has occurred in plants. Numerous other adaptations to the symbiotic lifestyle have already been highlighted in cnidarians as well as in other CO_2-

fixing invertebrates. For example, one of the most important benefits acquired by the host is the absorption of organic carbon synthesized by the symbiont. To ensure this source of organic carbon the host has evolved mechanisms of inorganic carbon absorption (Furla et al., 1998; De Cian et al., 2003) and concentration (Allemand et al., 1998; Leggat et al., 2002). These are unique to Metazoans but nonetheless similar to mechanisms described in plants and micro- and macro-algae.

The aim of the present study was twofold: (1) to determine, the role of symbiosis in determining oxidative stress tolerance; and (2) to assess the role of the symbiont in the expression of specific SOD isoforms in animal compartments. We have compared the effect of environmental stress in symbiotic and non-symbiotic species of temperate sea anemones. The Mediterranean sea anemone, *A. viridis*, was chosen as the symbiotic model by virtue of the easy separation of its two animal cells layers (ectoderm and endoderm) and the isolation of the symbiont cells (Bénazet-Tambutté et al., 1996). *Actinia schmidti* was chosen as the non-symbiotic model. Both animals have already been used for comparative study (Harland et al., 1990) on the basis of their common classification in the Actiniidae (Shick, 1991) and its relevance is also justified by their close location in the same habitat. Lipid peroxidation and protein oxidation were chosen as biomarkers of oxidative stress, and SOD activity as biomarker of antioxidant defence. High P_{O_2} tolerance was analysed during endogenous and experimental hyperoxia (60–100% O_2 saturation). To test whether O_2 tolerance promotes resistance to other stress, we also studied the effect of elevated temperatures. Interactions between host and symbionts were observed through the expression of SOD isoforms, using two symbiotic sea anemones (*A. viridis* and *Aiptasia pulchella*) and the scleractinian coral *S. pistillata*. SOD activities of symbiotic and aposymbiotic specimens, as well as of freshly isolated zooxanthellae (FIZ) and cultured zooxanthellae (CZ) were analysed.

Materials and methods

Biological materials

Specimens of Mediterranean *A. viridis* (Forskål) and *A. schmidti* (Monteiro et al. 1997) were collected in Villefranche-sur-mer (France) and maintained in a closed-circuit seawater aquarium at 17.0±0.5°C. Aquarium illumination was provided by a metal halide lamp (HQI-TS 400 W; Philips, France) with a photosynthetic photon flux density of 250 μmol m^{-2} s^{-1} and a 12 h:12 h photoperiod. Aposymbiotic specimens were obtained from the public aquarium of the Oceanographic Museum of Monaco. Isolated zooxanthellae were cultured for 10 days in modified ASP 8A medium (Blank, 1987), heated to 17.0±0.1°C (Sanyo Growth Cabinet) and on a 12 h:12 h photoperiod using fluorescent tubes (36 W; Sanyo, Antony, France).

Specimens of the sea anemone *A. pulchella* were maintained at 17.0±0.1°C and illuminated under a constant irradiance of 175 μmol m^{-2} s^{-1}, using the same type of metal halide lamps on a 12 h:12 h photoperiod. Aposymbiotic anemones were maintained at the same temperature in continual darkness.

Microcolonies of *S. pistillata* were propagated in the Scientific Centre of Monaco (Tambutté et al., 1995) where they were maintained at 27±0.5°C and 38 PSU under the same type of metal halide lamps. Cultured zooxanthellae (*Symbiodinium* sp.) were obtained from a clonal culture originally provided by Robert Trench. The algal cultures were maintained in 500 ml screw-top polycarbonate Erlenmeyer flasks (Corning; Acton, MA, USA) in modified ASP-8A medium (Blank, 1987) at pH 8.2 and incubated at 26.0±0.1°C under an irradiance of 100 μmol photons m^{-2} s^{-1} provided by Sylvania Gro-Lux (Germany) and daylight fluorescent tubes, on a 12 h:12 h photoperiod. Stock cultures were transferred monthly.

Aposymbiotic specimens correspond to bleached cnidarians in which disruption of symbiotic association has been achieved by either ambient conditions in the public aquarium of the Oceanographic Museum of Monaco (*A. viridis*) or by long term (>1 month) incubation in darkness (*A. pulchella*).

Experimental designs

Experimental hyperoxia

Specimens of *A. viridis* and *A. schmidti* were first held (48 h) in an airtight bottle, then subjected to 10 h of 100% O_2 at 17.0±0.1°C under a constant irradiance of 250 μmol m^{-2} s^{-1}. O_2 saturation of the medium was achieved by bubbling pure O_2 in seawater and was monitored using a gas analyser (Radiometer Copenhagen ABL 30; Copenhagen, Denmark). At the end of the stress period, a minimum of 5–10 tentacles from at least four specimens of *A. viridis* and whole specimens of *A. schmidti* (N=4) were sampled for biomarker assays.

Thermal stress

Three aquaria, each containing one specimen of *A. viridis* and four of *A. schmidti* (designed for sampling over the kinetic), were heated from 17°C (control temperature) to 24°C (stress temperature) of +7°C over 2 h and maintained at this maximal temperature for 5 days. For biomarker assays, 5–10 tentacles from *A. viridis* and a whole specimen and/or tentacles of *A. schmidti* were sampled from each aquarium day 0 (control condition) and after 1, 2 and 5 days of consecutive thermal stress.

Tissue extractions

Unless otherwise specified, all chemicals were obtained from Sigma. Each extract was prepared at 4°C in a different medium appropriate to the analysis.

Anemonia viridis

The three cellular compartments [ectoderm, endoderm and freshly isolated zooxanthellae (FIZ)] were extracted according to Richier et al. (2003) thus avoiding any contamination between FIZ protein and the endodermal host cell. ‘Total extract’ corresponds to the protein extract from whole tentacle (Richier et al., 2003) separated from the FIZ by centrifugation (1,000 ***g*** for 3 min). For extracts prepared under dark conditions, tentacle separation and protein extraction were

conducted under far red light to avoid photosynthesis during the extraction steps.

Actinia schmidti

The whole animal was frozen in liquid nitrogen and powdered in a mortar. Subsequent steps were performed in the extraction medium (Richier et al., 2003) with 800 µl g^{-1} of animal tissue (frozen mass). The crude extract of *A. schmidti* tissues was obtained after sonication (6×10 s) and centrifugation (12,000 ***g*** for 5 min). The supernatant was used for subsequent assays. Extraction was also conducted on *A. schmidti* tentacles to confirm whether a discrepancy exists between whole animal and tentacle analysis.

Aiptasia pulchella

The whole animal was homogenized in a Potter-Elvehijm tissue grinder containing chilled extraction medium at 4°C, sonicated (6×10 s) and centrifuged (12,000 ***g*** for 5 min). Supernatant was collected and reserved for analysis, which constitutes the cytosolic fraction. Freshly isolated zooxanthellae (FIZ) extraction was made according to Richier et al. (2003).

Stylophora pistillata

Coral microcolonies were frozen in liquid nitrogen and powdered in a mortar. Subsequent steps were performed in extraction medium containing 50 µl g^{-1} of whole coral tissue (Richier et al., 2003). FIZ extraction was performed as previously described (Richier et al., 2003).

Chlorophyll measurement

Chlorophylls *a* and c_2 were extracted from whole tissues of symbiotic and aposymbiotic specimens of *A. viridis* and *A. pulchella* in 90% acetone and measured according to Jeffrey and Humphrey (1975).

Thiobarbituric acid assay

Malondialdehyde (MDA) has been identified as the product of lipid peroxidation that reacts with thiobarbituric acid to give a red chromophore absorbing at 532 nm. Measurements followed the slightly modified method of Janero and Burghardt (1989). Cytosolic fractions (see 'Tissue extraction' method), containing 75 µg and 150 µg of protein, were analysed for *A. viridis* and *A. schmidti,* respectively. The absorbance was read at 532 nm using a microplate apparatus. MDA concentration of the sample was calculated using an extinction coefficient of 1.56×10^5 mol^{-1} l^{-1} cm^{-1}.

Protein carbonylation

Carbonyl content of the cytosolic fraction was measured using both the ELISA assay and spectrophotometry was used according to Buss et al. (1996). A total of 4 mg ml^{-1} of protein in both bovine serum albumine (BSA) standard curves (0–100% reduced BSA) and analysed extracts. The ELISA assay used biotin-conjugated rabbit IgG polyclonal antibody raised against dinitrophenylhydrazine component (anti-DNP 1:3000; Molecular Probes, Inc, Cergy-Pontoise, France) and streptavidine biotinylated horseradish peroxidase (1:2500; Amersham International; Orsay, France).

SOD activity

Both qualitative (native gel) and quantitative (spectrophotometry) analyses were used to study SOD activity.

Qualitative analysis

SOD isoforms in each tissue compartment were monitored by 8% non-denaturing polyacrylamide gel electrophoresis and nitro-blue tetrazolium (NBT) staining as described by Beauchamp and Fridovich (1971). Specific inactivation of enzyme activity was determined by soaking gels in 10 mmol l^{-1} H_2O_2 (inhibitor of both Fe- and CuZnSOD) or 10 mmol l^{-1} KCN (inhibitor of CuZnSOD) 30 min prior to the staining steps. Visualized SOD activity bands were named in function of genus and species relative to the studied organism (AsSOD for SOD activity bands relative to *A. schmidti*).

Quantitative analysis

Activity was measured after preparing each sample in a modified extraction medium (50 mmol l^{-1} potassium phosphate, 0.1 mmol l^{-1} EDTA; method by McCord and Fridovich, 1969). This method is based on the reduction of ferricytochrome *c* by O_2^-, generated during the sequential oxidation of xanthine by xanthine oxidase. The reaction mixture contained 10 µmol l^{-1} ferricytochrome *c*, 50 µmol l^{-1} xanthine, 20 µg ml^{-1} catalase and 50 nmol l^{-1} xanthine oxidase to produce a rate of reduction of ferricytochrome *c* at 550 nm of 0.025 absorbance unit per min. Measurements were made after adding a determined quantity of protein to 1 ml of reaction solution at 25°C. In control conditions, standard curves were generated for total extract of each sea anemone (*A. viridis*, *A. schmidti* and *A. pulchella*) and the three compartments (ectoderm, endoderm and FIZ) of *A. viridis* to determine the quantity of protein resulting in 50% inhibition of cytochrome *c* reduction. In this context, the amount of superoxide dismutase required to inhibit of 50% the rate of reduction of cytochrome *c* is defined as 0.33 unit of activity. Absorbance was measured at 550 nm.

Presentation of results

Results are presented as means ± S.E.M. and normalized by the protein content of each fraction. Protein content was determined by the Biorad protein assay using BSA as the standard protein. The results were validated by one-way ANOVA with Fisher *post-hoc* test or Student's *t*-test and were considered statistically significant when $P<0.05$.

Results

SOD activities in the symbiotic (A. viridis) *and non-symbiotic* (A. schmidti) *temperate sea anemones*

Electrophoresis under non-denaturating conditions (Fig. 1A) shows the pattern of SOD activities in animal extracts

(ectodermal plus endodermal cells) of the symbiotic sea anemone *A. viridis*. At least five activity bands are observed, corresponding to the MnSOD and CuZnSOD classes as previously described (Richier et al., 2003). A FeSOD class was also detected in previous analyses, but only in the enriched endodermal cell extracts. Under the same conditions, the non-symbiotic organism (*A. schmidti*) displays only three activity bands (Fig. 1B). This observation is more consistent with conditions described in most animal cells. *A. schmidti* isoforms were characterized by a KCN gel incubation test as two CuZnSOD forms (AsSODa and AsSODb) and one MnSOD form (AsSODc). Spectrophotometric measurements carried out on animal fractions of both actiniid indicate reveal a 1.8-fold higher SOD activity in *A. viridis* with 10.5 U mg^{-1} of protein in animal fraction (ectoderm and endoderm), than in *A. schmidti,* with 5.83±1.39 U mg^{-1} protein (Table 1).

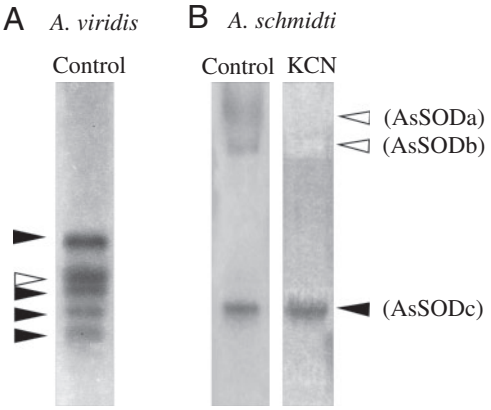

Fig. 1. SOD electrophoretic patterns in symbiotic (*Anemonia viridis*) and non-symbiotic species (*Actinia schmidti*). (A) SOD activity in *A. viridis* animal (ectoderm plus endoderm) extract and (B) *A. schmidti* total extract in the absence (control) and in the presence of KCN (10 mmol l^{-1}) revealed on native PAGE (8%) and by NBT staining. Arrows indicate pharmacological identification of bands: MnSOD (black arrows), CuZnSOD (open arrows). Cytosolic fractions containing a quantity of 150 μg of protein was loaded in each well. Similar results were obtained in four to 10 independent experiments for *A. schmidti* and *A. viridis* specimens, respectively.

Response to environmental stress

Effects of experimental hyperoxia on SOD activities

Qualitative analysis of SOD activity on native gel revealed no change in SOD isoform expression within the animal compartments of *A. viridis* during endogenous (photosynthetic) hyperoxia (results not shown) or experimental hyperoxia (Fig. 2A). Spectrophotometric measurement shows a significant 1.5-fold increase in SOD activity in the ectodermal fraction. No change occurred in endodermal cells (Table 1).

In the zooxanthellae, qualitative analysis of SOD activities shows an unaltered electrophoretic pattern during hyperoxic stress. However, a significant 1.9-fold increase in activity was quantified by spectrophotometric measurements (Table 1).

After 10 h incubation at high P_{O_2}, *A. schmidti* expresses a new SOD activity band, AsSODd, identified as CuZnSOD by KCN native gel incubation (Fig. 2B). A weaker AsSODa activity band was observed under hyperoxia (Fig. 2B). Spectrophotometric measurements of total extract of *A. schmidti* show a slight decrease in SOD activity after 10 h incubation at high P_{O_2} (Table 1).

Effects of experimental hyperoxia on damage biomarkers

In the symbiotic sea anemone *A. viridis*, damage biomarkers (protein carbonyl or MDA concentration) detected no significant variation during either endogenous hyperoxia (daily light/dark cycle, results not shown) or experimental hyperoxia (Fig. 3A,B). By contrast, significant differences were observed in *A. schmidti* tissues during experimental hyperoxia. Protein carbonylation was sixfold higher in treated tissues than in control tissues (Fig. 3C). However, MDA values did not differ significantly after similar treatment (Fig. 3D). Basal values of damage biomarkers, either for MDA level or carbonyl content were higher in symbiotic specimens than in non-symbiotic specimens (Fig. 3).

Effects of thermal stress on SOD activities

The overall SOD pattern of *A. viridis* did not change in animal compartments even after 5 days of incubation at a temperature 7°C above normal (Fig. 4A–C). Quantitative measurements

Table 1. *SOD activities in the three compartments of* Anemonia viridis *(ectoderm, endoderm, FIZ) and in* Actinia schmidti *total extract**

	Control	Hyperoxic stress	Thermal stress		
	Normoxia	100% O_2 (10 h)	24 h	48 h	7d
A. viridis					
Ectoderm	1.36±0.16 *N*=10	1.9±0.19 *N*=9§	1.65±0.09 *N*=8	1.67±0.23 *N*=8	1.67±0.12 *N*=6
Endoderm	5.59±1.14 *N*=10	4.40±0.57 *N*=9	3.84±0.36 *N*=5	4.06±0.73 *N*=5	4.87±0.31 *N*=5
FIZ	6.11±2.11 *N*=12	11.7±0.41 *N*=4§	9.21±0.56 *N*=6‡	8.35±0.78 *N*=7†	8.62±0.68 *N*=5*
A. schmidti					
Whole tissue	1.94±0.46 *N*=10	1.55±0.06 *N*=6†	1.19±0.10 *N*=6§	1.35±1.16 *N*=5‡	1.26±0.13 *N*=3‡

*SOD activities given are U mg^{-1} of protein. Data represent means ± S.E.M. of mentioned replicates (*N*) and were considered significant when $P<0.05$ (one-way ANOVA with Fisher test; $^{\dagger}P<0.05$, $^{\ddagger}P<0.01$, $^{\S}P<0.001$).

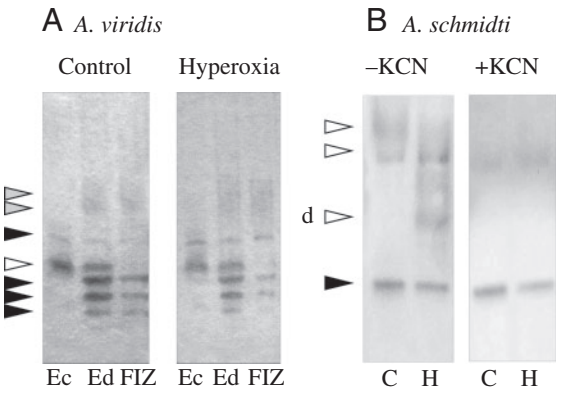

Fig. 2. Response of SOD activity to experimental hyperoxia. (A) SOD activity of the three separated compartments of *A. viridis* (Ec, ectoderm; Ed, endoderm; FIZ, freshly isolated zooxanthellae). (B) Total extract of *A. schmidti* in two conditions: control (C; air saturated) and hyperoxia (H_3; O_2-saturated seawater), revealed by native PAGE (8%) and NBT staining, either without KCN (–KCN) or with (+). Arrows indicate pharmacological identification of bands: MnSOD (black arrows), CuZnSOD (open arrows) and FeSOD (grey arrows). (d) Corresponds to an O_2-induced SOD isoform (AsSODd). Cytosolic fractions containing a quantity of 150 µg of protein was loaded in each well. These patterns were reproducible in four independent experiments for each species.

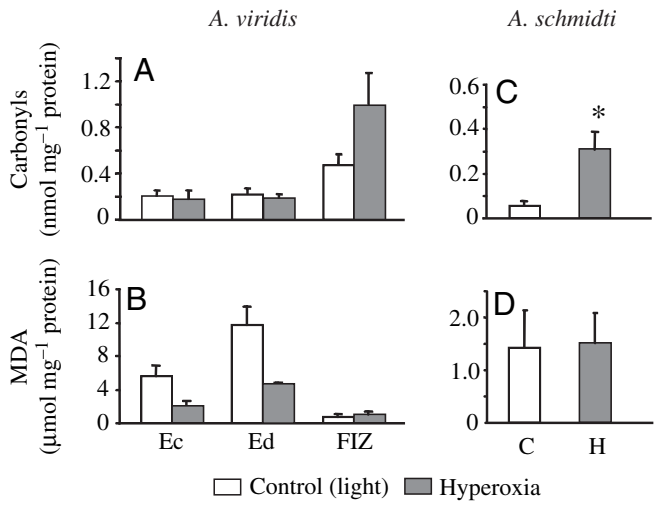

Fig. 3. Cellular damage in response to experimental hyperoxia. (A) Carbonyl and (B) MDA contents were measured in the three compartments of *A. viridis* (Ec, ectoderm; Ed, endoderm; FIZ, freshly isolated zooxanthellae) during the daytime. (C) Carbonyls and (D) MDA content were also measured in *A. schmidti* total extract during the daytime, for control (C) and hyperoxia-treated specimens (H). Cytosolic fractions containing a quantity of 100 and 150 µg of protein were analyzed for carbonyl and MDA content, respectively. Data are presented as means ± S.E.M. of four independent analyses. *Indicates significant differences between control and stressed specimens (Student's *t*-test $P<0.05$).

detected a slight increase after 24 h incubation in ectodermal and no significant change in endodermal animal cells (Table 1). In zooxanthellae, two or more SOD activity bands, identified as

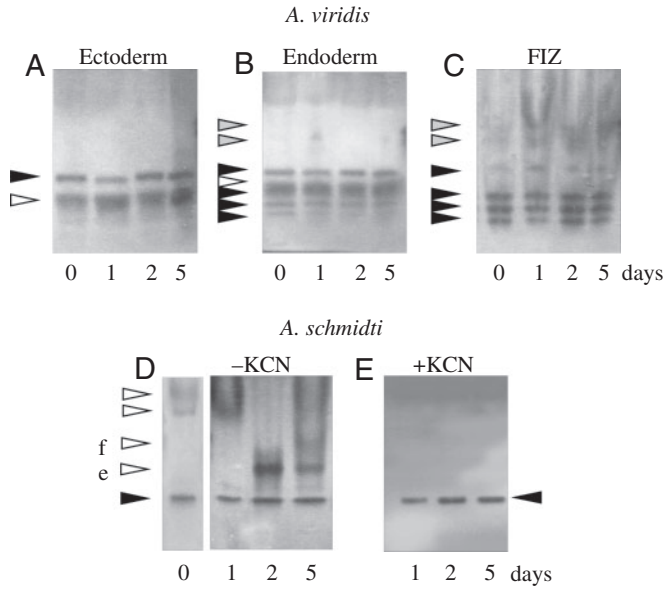

Fig. 4. Response of SOD activity to +7°C increase above ambient. SOD activities of the three compartment of *A. viridis* (A) ectoderm, (B) endoderm and (C) FIZ (freshly isolated zooxanthellae), (D,E) total extract of *A. schmidti* under control conditions 0 (17°C) and after 1, 2 and 5 days of incubation to elevated temperature (24°C), revealed by native PAGE (8%) and NBT staining, either without KCN (–KCN) or with (+KCN). Pharmacological identification of the bands is indicated by arrows: MnSOD (black arrows), CuZnSOD (open arrows) and FeSOD (grey arrows). 150 µg of protein was loaded in each well. These patterns were reproducible in four independent experiments for each species.

FeSOD, appeared as soon as the stress period begins. Quantitative analysis by spectrophotometry revealed a 1.5-fold increase in SOD activity in this compartment (Table 1).

A. schmidti SOD activities responded strongly to the experimental treatment (Fig. 4D). The CuZnSOD activity bands (AsSODa and AsSODb) disappeared after 2 days and a novel activity band (AsSODe) appeared (Fig. 4D). After 5 days of incubation, another CuZnSOD activity band (AsSODf) was expressed, and a high-molecular mass smear appeared (Fig. 4D). Pharmacological identification of SOD activity bands is presented in Fig. 4E. Parallel measurements by spectrophotometer show a global decrease in SOD activity during 5 days of incubation at elevated temperature (Table 1).

Effects of thermal stress on damage biomarkers

After 1 day of thermal stress, the symbiotic sea anemone had a threefold increase of carbonyl content in its three compartments (Fig. 5A). Under the same conditions, MDA content did not vary significantly (Fig. 5B).

In the non-symbiotic *A. schmidti*, carbonyl content responded immediately and significantly. A sixfold increase of the basal value occurred after one day of exposure (Fig. 5C). After 5 days, carbonyl content increased a tenfold relative to the control. Lipid peroxidation increased threefold after 5 days (Fig. 5D).

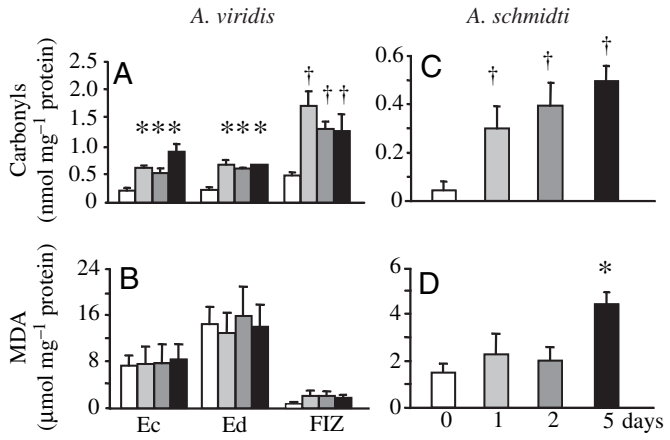

Fig. 5. Cellular damage in response to +7°C increase above ambient. (A) Carbonyls and (B) MDA content have been measured in the three compartments (Ec, ectoderm; Ed, endoderm; FIZ, freshly isolated zooxanthellae) of *A. viridis* and (C,D) in *A. schmidti* total extract. Control condition (white bars) and after 1 (grey bars), 2 (dark-grey bars) and 5 (black bars) days in +7°C seawater. Data are presented as means ± S.E.M. of four independent analyses. *Indicates significant differences between control and stress specimens (ANOVA, $^{*}P<0.05$; $^{\dagger}P<0.01$).

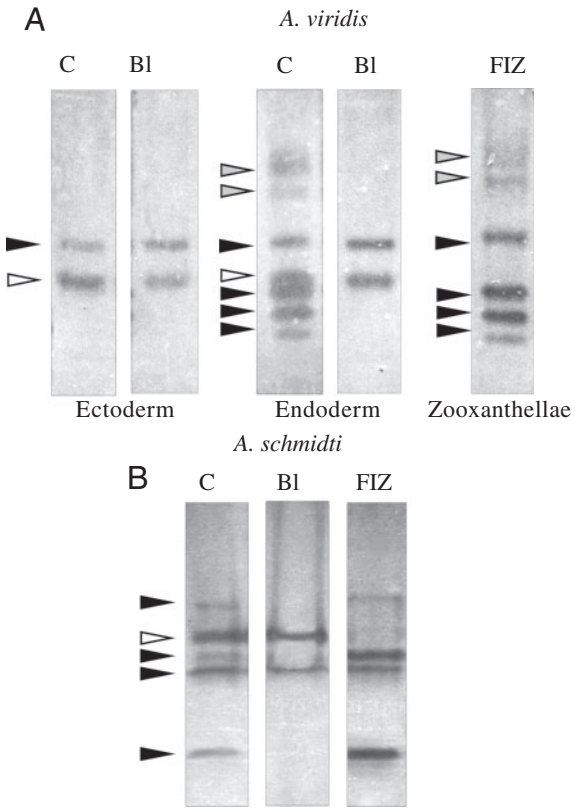

Fig. 6. SOD activity in associated and dissociated symbiotic partners. SOD activity in animal compartments and FIZ of *A. viridis* (A) in control (C) and bleached conditions (Bl) and in total extract and FIZ of *A. pulchella* (B) for control (C), bleached specimen (Bl) and (FIZ) extracts, is revealed by 8% native PAGE and NBT staining. 150 µg of protein was loaded in each well. Arrows indicate pharmacological identification of bands: MnSOD (black arrows), CuZnSOD (open arrows) and FeSOD (grey arrows). These patterns were reproducible in four independent experiments for each species.

Symbiotic regulation of SOD expression

Symbiont regulation of SOD expression within the host

Bleached specimens of *A. viridis* and *A. pulchella* exhibit a global decrease in SOD activities and a loss of some SOD activity bands (Fig. 6). Bleaching was confirmed by the measured a decrease in chlorophyll content (Table 2). The analysis of the three compartments (ectoderm, endoderm and FIZ) from *A. viridis* enabled a more precise localization of modifications in the SOD pattern (Fig. 6A). While the electrophoretic pattern for ectodermal cells were unchanged, the endodermal host cells lost its major SOD activity bands, representing MnSOD and FeSOD classes as identified by Richier et al. (2003). Spectrophotometric measurements confirm five- and threefold decreases of SOD activity in the ectodermal and endodermal compartments, respectively (Table 3). In the *A. pulchella* total extract, three SOD activity bands (pharmacologically identified as MnSOD) disappear from the bleached anemone (Fig. 6B). However a MnSOD band and a CuZnSOD band remain in both symbiotic and aposymbiotic specimens (Fig. 6A,B). The CuZnSOD class was neither observed in FIZ from *A. viridis* (Richier et al., 2003) nor in those of *A. pulchella*, *S. pistillata* and *Galaxea fascicularis* (results not shown). This would suggest an animal cell specificity of this isoform.

Table 2. *Chlorophyll concentration of tissue extracts from symbiotic and aposymbiotic specimens of* Anemonia viridis *and* Aiptasia pulchella

Symbiotic state	Chlorophyll $a+c_2$
A. viridis	
Symbiotic	1.20±0.33
Aposymbiotic	0.05±0.02
A. pulchella	
Symbiotic	13.11±1.95
Aposymbiotic	0.28±0.13

Chlorophyll concentrations are given as µg mg^{-1} (means ± S.E.M., N=5).

Host regulation of SOD expression within zooxanthellae

Fig. 7 compares the SOD patterns of FIZ and cultured zooxanthellae (CZ) from both *A. viridis* and *S. pistillata* symbiotic specimens. Despite their common genotype clade A (D. Forcioli, personal communication), FIZ and CZ showed specific electrophoretic patterns depending on their animal host origin. Native gels revealed that CZ from both cnidarians showed additional specific bands compared with their respective FIZ. These additional bands were identified as MnSOD in CZ from *A. viridis*, and as MnSOD and FeSOD in CZ from *S. pistillata*.

Table 3. *Global SOD activity in intact and bleached specimens of* Anemonia viridis*

	Control	Bleached
Ectoderm	1.36±0.16 *N*=10	0.05±0.03 *N*=8†
Endoderm	5.59±1.14 *N*=10	1.76±0.51 *N*=5†

*SOD activities given are U mg^{-1} of protein. Data represent means ± S.E.M. of mentioned replicates (*N*) and were considered significant when $P<0.05$ (Student's *t*-test, †$P<0.001$).

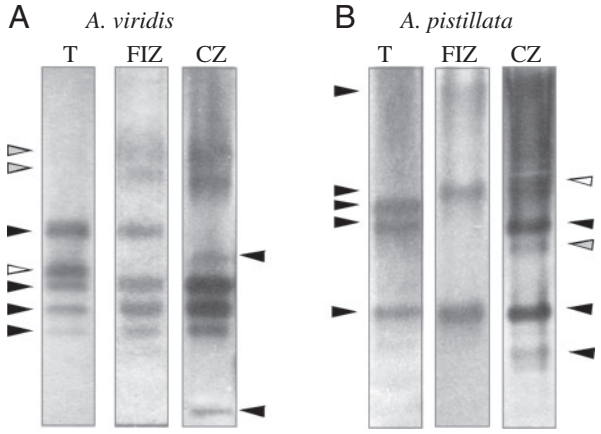

Fig. 7. Interactions of symbiotic partners in SOD expression. SOD isoforms of the whole symbiotic association (T), the cultured zooxanthellae (CZ) and the freshly isolated zooxanthellae (FIZ) of two symbiotic organism *A. viridis* (Mediterrannean sea anemone) and *Stylophora pistillata* (tropical scleractinian coral) have been revealed by 8% native PAGE and NBT staining. Each lane was loaded with 150 µg of protein. Arrows indicate pharmacological identification of bands: MnSOD (black arrows), CuZnSOD (open arrows) and FeSOD (grey arrows). These patterns were reproducible in four independent experiments on each species.

Discussion

This study suggests an effect of symbiosis on the antioxidant response of the animal hosts to environmental changes. Symbiotic sea anemones differ from non-symbiotic species both in their antioxidant defence (SOD) response and in the control of cellular damage when facing environmental stress.

Symbiotic adaptation to the hyperoxic state

Under control conditions, the SOD electrophoretic patterns are more diversified in animal cells living in association with photosynthetic dinoflagellates than in the non-symbiotic sea anemone. Three SOD classes (FeSOD, CuZnSOD and MnSOD) and up to seven activity bands were observed in *A. viridis* symbiotic host cells (Richier et al., 2003). Only two classes are generally present in animals (Halliwell and Gutteridge, 1999) as was observed for the non-symbiotic *A. schmidti*. This high diversity in the symbiotic example is similar to that observed in plants, which can display up to seven isoforms (Halliwell and Gutteridge, 1999; Alscher et al., 2002). A similar high diversity (six SOD activity bands) is observed in zooxanthellae, a symbiotic chlorophyll-containing protist. Furthermore, as has been previously observed (Shick and Dykens, 1985), the symbiosis-dependant SOD diversity in activity bands is associated with a global increase in SOD activity (Table 1). The comparison of SOD expression between these two sea anemones suggests that the diversification of SOD activity in animal host cell could be a consequence of the endosymbiont presence's rather than a unique feature of the phylum Cnidaria.

Naturally, dealing with oxidative stress while avoiding membrane damage represents an inherent challenge to all aerobic life forms. Lipid peroxidation and protein oxidation are universal responses (Ernster and Hochstein, 1994). Nevertheless, in the present study, both damage basal values and SOD activities remain unchanged during natural light-induced hyperoxia (results not shown). This suggests a host adaptation to symbiont photosynthesis, possibly involving a constitutive expression of several SOD activity bands, as is the case for plants (Gillham and Dodge, 1987). During daytime, however, symbiotic sea anemones have higher basal MDA and carbonyl concentrations than do the non-symbiotic organisms. This would implicate continuous ROS production from zooxanthellae photosynthesis (Dykens and Shick, 1984; Dykens et al., 1992). A high basal value for damage biomarkers has also been shown in photosynthetic organisms. For example, plant leaves are unable to escape photooxidative damage due to their exposure to bright light, while they produce O_2 (Asada and Takahashi, 1987).

To test whether natural transitions between hyperoxia and anoxia contribute to oxidative stress adaptation, both SOD activity and damage biomarkers were further analysed under experimental hyperoxia. In the non-symbiotic sea anemone *A. schmidti*, experimental hyperoxia (100% O_2) leads to qualitative changes in SOD. Evidence for this is the appearance of new isoforms (CuZnSOD activity band, Fig. 2B). However, such a high P_{O_2} seems to be deleterious for this antioxidant enzyme, since SOD activity decreased during the stress period (Table 1). Under conditions of elevated prooxidative challenge, depletion of antioxidant defenses has been reported as a common pathway of toxicity in several marine invertebrates (Winston and DiGiulio, 1991; Regoli and Principato, 1995). In the present study, an increase in cellular damage was observed as a 10-fold increase in protein oxidation (Fig. 3C). These results agree with those previously reported for animal cells (Ahmed et al., 2003).

By contrast, neither qualitative changes in SOD pattern (Fig. 2) nor significant increases in damage biomarkers (Fig. 3) were observed in the symbiotic sea anemone *A. viridis*, under imposed hyperoxia. Meanwhile, in response to the hyperoxic stress, SOD activity increased 1.6-fold relative to the control in the ectodermal compartment, presumably to counterbalance prooxidant period during hyperoxia. This differential response to hyperoxic stress in the two cnidarians supports the hypothesis of the influence of the photosynthetic protist on the animal cell to deal with ROS.

Does symbiosis contribute host adaptation to environmental stressors, such as increase temperatures? Similar to the

response to hyperoxic stress, *A. schmidti* responds to thermal stress with a decrease in global SOD activity and the expression of a novel stress-specific SOD isoform (the thermal stress-inducible isoform, CuZnSOD). Thermal stress on *A. schmidti* resulted in a time-dependent increase in both damage biomarkers (a threefold increase in MDA production and a 10-fold increase in protein oxidation) after 5 days under stress. By contrast, in the symbiotic species *A. viridis*, similar thermal stress had no effect on lipid peroxidation or resulted in less than 3.5-fold increase in protein oxidation after 5 days (Fig. 6) No modification of the SOD activity pattern occurred. In conjunction with the SOD activity response to hyperoxic stress, the symbiotic sea anemones also have a progressive increase in ectodermal compartment during thermal stress.

This comparative study demonstrates that, while the non-symbiotic species appeared sensitive to thermal stress, the sympatric symbiotic species was tolerant. These observations suggest that the symbiotic state plays a role in host cell adaptation to thermal stress. A similar relationship has already been demonstrated in associations between fungi and plants. In this symbiosis, the host plant acquires a thermotolerance because the fungal endophyte produces cell wall melanin that may dissipate heat and/or complex with oxygen radicals generated during heat stress. Alternatively, the endophyte may act a 'biological trigger' allowing symbiotic plants to activate stress-response systems more rapidly and strongly than non-symbiotic plants. Furthermore, this mutualism may involve other benefits (e.g. nutrient acquisition by the fungus; Redman et al., 2002). Moreover, a recent study on the Mediterranean symbiotic sponge *Petrosia ficiformis* shows comparable adaptation, with antioxidant changes in response to photosynthetically produced ROS (Regoli et al., 2004).

The present results support the hypothesis that environmental stress seems to enhance antioxidant defenses to limit and/or avoid cellular damage in the symbiotic *A. viridis*. However, in the non-symbiotic sea anemone, SOD responds differently to stress. The qualitative analysis shows diversification of SOD isoforms, and quantitative analysis shows repression of global activity that might lead to an increase in damage biomarkers (Figs 3C,D, and 5C,D). Regoli et al. (2000) reported similar responses to oxidative stress in the symbiotic demosponge *Petrosia ficiformis*.

Our results also show that different isoforms of CuZnSOD appear stress-sensitive in the non-symbiotic sea anemone *A. schmidti*. New isoforms are expressed following hyperoxia (AsSODd) or thermal stress (AsSODe, AsSODf), and certain isoforms are repressed following high P_{O_2} (AsSODa) or thermal increase (AsSODa and AsSODb). Although no qualitative changes are observed in *A. viridis*, quantitative modifications for CuZnSOD are still possible given that total SOD activity increased during the experiment. This last result supports several recent field studies on tropical scleractinian corals (Brown et al., 2002; Downs et al., 2002) that document an increase in total SOD content by ELISA assay following thermal stress.

Following thermal or hyperoxic stress, zooxanthellae seem to adapt to O_2 variation in a manner comparable to the animal cells of *A. viridis.* Global SOD activities increase (Table1) and cellular damages are minimized (Figs 3A,B, and 5A,B). After 5 days of incubation at 25°C, the increases in SOD activity observed by spectrophometry may be correlated with the appearance on native gels of FeSOD isoforms (Fig. 4C).

Host–symbiont interactions in antioxidant defence

One of the key questions arising from these results is whether SOD activity is sensitive to the symbiotic state. To answer this question, we compared dissociated partners (aposymbiotic animal and cultured zooxanthellae) and intact symbiotic associations. As expected, we observed a consistent decrease in SOD activity (in aposymbiotic tissue extracts of *A. viridis* and *Aiptasia pulchella*). Interestingly, activity bands common to host and symbionts disappeared. Furthermore, the effect of dissociation of the symbiosis is observed not only in the endodermal compartment but also in the ectodermal compartment (which never harbours zooxanthellae). SOD activity decreased fivefold upon bleaching.

Decrease of host defence upon bleaching may be the result of a decrease of oxidative stress following the reduction in the photosynthetic process ('oxidative stress-regulated SOD expression') or a consequence of disruption of the symbiotic association ('symbiosis-regulated SOD expression'). These possibilities will be tested in further experiments. Similarly, a lower SOD activity in aposymbiotic cells compared with symbiotic one was also described in the symbiotic sea anemone *Anthopleura elegantissima* by Dykens and Shick (1982) and Dykens (1984), who originally proposed that photosynthetic hyperoxia necessitates higher antioxidant defenses in the host.

Cultured zooxanthellae, revealed a persistent increases in SOD activity and the appearance of new activity bands. Similar increases in antioxidant defence (SOD and catalase) in cultured zooxanthellae compared with FIZ were also observed in another symbiotic sea anemone, *A. pallida,* by Lesser and Shick (1989). Cultured zooxanthellae may, therefore, require additional endogenous antioxidant protection compared with the *in hospite* state and may depend on the animal to play a protective role. This conclusion is in agreement with Brown et al. (2002), who suggested such protection in the host *Goniastrea aspera* following thermal stress.

This work provides evidence for an acute resistance against oxidative stress of the symbiotic species of sea anemone compared to the non-symbiotic one. This resistance could be the result of the presence of the symbiont that acclimates the animal cell to high P_{O_2} on a daily basis. The greater diversity of isoforms induced by the presence of the photosynthetic symbionts within animal tissue could contribute to this adaptation. Hyperoxic adaptation may also be a preconditioning step that could prevent cellular damage during thermal stress, implying that symbiosis may confer host resistance to multiple stress. Moreover, interactions at the antioxidant defence level between both symbiotic partners could also be a basis for protecting cells following environmental changes. As a result of physical interactions or possible molecular communication between both

species, the presence of the zooxanthellae may induce increases in SOD activity and may contribute to the adaptation to stress encountered by the animal partner. Further molecular characterization of common SOD isoforms shared by two phylogenetically different species would yield important information concerning the origin of the unusual SOD diversity present within animal tissues (endoderm) and its relevance to the evolution of antioxidant enzymes in both host and symbionts.

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