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Is Coral Bleaching due to the Instability of the Zooxanthellae Dark Reactions?

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Abstract

Coral reefs are dominated by symbioses between marine invertebrates (e.g. corals, clams, sea anemones, sponges) and the dinoflagellate *Symbiodinium*. Increases in seawater temperature linked to global warming have lead to the phenomenon known as "bleaching", involving the disassociation of these symbioses and mass mortalities of the invertebrate host. Mass bleaching events have been linked to a thermal lesion in the photosynthesis of *Symbiodinium*, either at photosystem II or in the dark reactions of photosynthesis. Examination of two of the proposed lesion points, the carbon concentrating mechanism (CCM) and form II Rubisco, indicate that, over the temperature range examined, the CCM is not disrupted in *Symbiodinium* isolated from giant clams, however it was not possible to determine if Rubisco is the point of thermal lesion resulting in bleaching. Maximum photosynthetic rates were measured between 28 and 31°C and declined as temperature was increased. Despite a low correlation between Rubisco activity, oxygen evolution and temperature, Rubisco activity declined with increasing temperature.

Keywords: Bleaching, zooxanthellae, Rubisco, carbonic anhydrase, corals, clam

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Abbreviations
BTP, 1,3-bis(Tris[hydroxymethyl]methylamino) propane
CCM, carbon concentrating mechanism
CPBP, 2'-carboxypentitol 1,5-bisphosphate
Epps, N-2-hydroxyethylpiperazine-N'-3-propane sulfonic acid
GBRMPA, Great Barrier Reef Marine Park Authority
HSP, heat shock protein
PMSF, phenylmethylsufonyl fluoride
Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase
RuBP, ribulose 1,5-bisphosphate

1. Introduction

Mass bleaching events of coral reefs, linked to increasing seawater temperature, are now considered to be one of the major threats to the survival of tropical reefs (Hughes et al., 2003). Bleaching in symbiotic marine invertebrates occurs when zooxanthellae (=*Symbiodinium*) are expelled from their symbiotic host or lose their photosynthetic pigments, leading to a whitening of the host tissue. The loss of zooxanthellae results in a reduction in the exported photosynthate and therefore the host's energy supply. Thermal bleaching has now been observed in almost all marine invertebrates found in symbiosis with *Symbiodinium* (e.g. corals, clams, anemones, sponges, hydroids).

Bleaching events are correlated with stress that leads to a breakdown of the symbiosis (Brown, 1997; Hoegh-Guldberg, 1999; Fitt et al., 2001) and may affect either or both of the symbiotic partners. Studies have shown that the major cause of mass bleaching events is elevated sea surface temperatures slightly above long-term summer maxima (Glynn, 1993; Brown, 1997). Mass bleaching events can lead to significant losses of symbiotic marine invertebrates; for example in the 1998 bleaching event up to 90% mortality of corals was recorded on reefs in the Indian Ocean (Wilkinson et al., 1999) while on the Great Barrier Reef approximately 87% of inshore reefs suffered some bleaching (Berkelmans and Oliver, 1999). In 2002, the worst bleaching in the recorded history of the Great Barrier Reef occurred with 65% of all corals being bleached and approximately 5% dying (P. Marshall, GBRMPA, personal communication). Data suggests that the frequency of mass-bleaching events is increasing in line with increasing seawater temperatures and global warming, and some models based upon current thermal thresholds suggest that they may occur annually by 2050 (Hoegh-Guldberg, 1999). These bleaching events are now considered to be one of the major threats to the survival of coral reefs worldwide (Hughes et al., 2003).

Despite a recognized link between elevated temperature and mass-bleaching events, the molecular mechanisms of the thermal lesion and symbiosis disassociation are not clearly understood. Initially Iglesias-Prieto et al. (1992) proposed that elevated temperatures lead to an inhibition of zooxanthellae photosynthesis at temperatures above 30°C, possibly due to changes in the lipid characteristics of the thylakoid membranes. The subsequent reduction in the transfer of photosynthetically fixed metabolites to the host resulted in a disruption of the symbiosis and bleaching. The theory of thylakoid disruption was subsequently expanded upon by Iglesias-Prieto (1997) and Warner et al. (1996), who observed a decreased photosynthetic yield suggesting a thermal lesion at, or near, photosystem II (PSII) using pulse-amplitude-modulated (PAM) chlorophyll fluorescence.

In a further study Warner et al. (1999) demonstrated a temperature-dependent loss of PSII activity and a concomitant decrease in the concentration of the PSII reaction centre protein D1, supporting the theory that PSII was the primary site of thermal lesion in zooxanthellae.

In contrast to these results, Jones et al. (1998), also using PAM data, proposed that damage to PSII might be a secondary effect with the initial site of damage, as in higher plants, being the dark reactions of photosynthesis. Inhibition of the dark reactions would lead to a decreased flow of electrons to the Calvin cycle and subsequent over-reduction of the electron transport chain and damage to PSII (sink limitation). They suggested that the initial thermal lesion might occur as a result of damage to either the Rubisco or the carbon concentrating mechanism (CCM).

Dinoflagellates, including *Symbiodinium*, are unique amongst oxygenic phototrophs as some utilize a form II Rubisco for carbon fixation (Morse et al., 1995; Whitney et al., 1995), as opposed to the usual form I Rubisco found in all other eukaryotic phototrophs. Previously it was thought that form II Rubisco was restricted to anaerobic purple non-sulfur bacteria, as the enzyme's poor ability to discriminate between CO_2 (photosynthesis) and O_2 (photo-respiration) would not support adequate photosynthetic carbon fixation in aerobic phototrophs. Based upon the poor CO_2/O_2 discrimination of dinoflagellate Rubisco it was concluded that they could only efficiently fix carbon if they possess a CCM that would increase the intracellular concentration of CO_2 around Rubisco (Whitney and Andrews, 1998). A CCM was subsequently found in *Symbiodinium* (Leggat et al., 1999).

Characterisation of *Symbiodinium* Rubisco showed that this enzyme is extremely labile upon cell lysis (Whitney and Yellowlees, 1995) and was therefore an obvious candidate in the breakdown of the photosynthetic capacity of zooxanthellae during bleaching. As suggested by Jones et al. (1998) another possible candidate may be the zooxanthellae CCM that enables the dinoflagellate to efficiently fix CO_2 (Leggat et al., 2002).

The experiments described here were designed to determine whether, as suggested by Jones et al. (1998), Rubisco or the CCM are the primary lesion point for thermal damage in mass bleaching events. To examine this we report the first experiments on the *in vivo* thermal tolerance of the carbon concentrating mechanism and Rubisco in *Symbiodinium*.

2. Materials and Methods

Clams were chosen as the source of *Symbiodinium* (= zooxanthellae) for these experiments as, unlike corals, they provide large quantities of alga needed to conduct the enzymatic assays utilised here. In addition the "clades" found in clams are also found in other symbiotic organisms, including corals (Baillie et al., 2000). *Tridacna gigas* were obtained from the intertidal reef flat of Pioneer Bay at Orpheus Island, Australia (18°37'S; 146°30'E) and transported to the James Cook University open-air aquarium where they were maintained in a flow through system exposed to ambient light and temperature. Clams were acclimatized for at least 2 weeks before being used in experiments. Separate populations of *Symbiodinium* sp. were isolated from the mantle of four *T. gigas* individuals. The clams were killed by cutting the adductor muscle and removing the tissue from the shell. The mantle was cut into 3 cm^3 sections and blended in 0.45 µm filtered sea water (FSW). The homogenate was then passed through 2 layers of cheesecloth to remove any large tissue pieces, and the filtrate centrifuged (600 g, 2.5 min). The pelleted *Symbiodinium* cells were washed 3 or more times in FSW (600 g, 2.5 min) before being suspended in either FSW or the appropriate artificial seawater.

Measurement of photosynthetic O_2 production

Isolated *Symbiodinium* were suspended in FSW at a cell density of approximately 1×10^6 cells ml^{-1} and kept at 28°C and used within 10 hours of isolation. 5×10^7 cells were then taken and slowly warmed (~0.2°C per min), equilibrated to various temperatures (28°C, 29°C, 30°C, 32°C, 35°C, 36°C, 37°C, 40°C) and maintained at temperature for up to 2 to 4 hours under constant illumination at 200 µE m^{-2} s^{-1} with shaking (100 rpm). Cells were then pelleted (600 g, 2 min), resuspended in artificial seawater (ASW, 428 mM NaCl, 25 mM BTP, pH 7.0) and placed at 28°C for 15 min before measuring photosynthesis and respiration in a Hansatech oxygen electrode.

Kinetic parameters were determined as per Leggat et al. (2000). All assays were conducted at 28°C. Data was analysed by ANOVA and Tukey's Post-Hoc analysis using Statistica V6.

In vitro measurements of Rubisco

Symbiodinium cells were incubated at different temperatures as described above. After the cells were equilibrated back to 28°C they were centrifuged for 2 min at 2,500 g and the pellet resuspended in 3–4 ml of ice-cold extraction buffer (50 mM Epps-KOH pH 7.8, 25 mM $MgCl_2$, 25 mM $NaHCO_3$, 1 mM EDTA, 1 mM PMSF). Aliquots were removed for cell counting (40 µl).

The remaining *Symbiodinium* in extraction buffer were lysed by a single pass through a pre-chilled French Press (18 kPa). An aliquot (40 µl) was removed for cell counting using a haemocytometer to determine percent lysis (always >95%). The lysate was immediately mixed with 0.05 g of polyvinyl-polypyrrolidone and centrifuged for 30 s at 13,000 g (4°C). The supernatant was assayed for protein content using a dye binding assay (BioRad) and an aliquot (200 µl) processed for Western blot analysis by denaturing and separating the proteins using Bis-Tris-buffered 4–12% NuPAGE SDS gels according to manufacturer specifications (Novex, San Diego). The proteins were transferred onto nitrocellulose using a Bio-Rad mini-trans-blot electrophoretic transfer cell and the membranes probed with antibodies raised in mice against *Symbiodinium* Rubisco (Whitney and Yellowlees, 1995). Blots were then probed with an appropriate secondary antibody conjugated to horseradish peroxidase (Dakopatts, Denmark) and immunoreactive proteins visualized using 3,3'-diaminobenzidine (DAB) and H_2O_2 (Harlow and Lane, 1988).

The RuBP dependent carboxylase activity in the extract was measured by diluting an aliquot of *Symbiodinium* soluble protein three-fold into assay buffer (50 mM Epps-KOH pH 7.8, 25 mM $MgCl_2$, 20 mM $NaH^{14}CO_3$, 1 mM EDTA) at 25°C to which RuBP (0.26 mM final concentration) had just been added. After 1 min at 25°C, the reaction was terminated by adding 0.5 vol of 50% formic acid and heating at 80°C. When dry, water (200 µl) was added and then 5 ml of scintillant (BCS, AP Biotech, Sydney). The incorporation of $^{14}CO_2$ into acid stable contents was measured in a Wallac 1410 Liquid Scintillation Counter (AP Biotech).

The amount of catalytically viable Rubisco active sites was determined by the enzymes ability to bind the tight binding inhibitor ^{14}C-CABP (carboxyarabinitol 1,5-bisphosphate) according to Ruuska et al. (1998). *Symbiodinium* soluble protein extract (100 µl) was incubated for 5 min at 25°C before adding ^{14}C-CPBP (75 µM final concentration; specific activity 98,901 cpm $nmol^{-1}$), which is the non-purified form of CABP. After incubating a further 30 min on ice, unbound ^{14}C-CPBP was separated from that bound to Rubisco by gel filtration (Ruuska et al., 1998) and the content of viable Rubisco active sites was calculated from the ^{14}C-inhibitor bound protein fraction measured in a scintillation counter.

3. Results

Effect of temperature on photosynthesis and the CCM in Symbiodinium from a giant clam

The maximum photosynthetic rates at saturating C_i (P_{max}) and affinity for inorganic carbon ($K_{0.5}(C_i)$) were measured in *Symbiodinium* (= zooxanthellae) cultured between 28°C and 37°C to determine if the CCM was affected. There was a general decrease in P_{max} as temperature increased, indicating a loss in photosynthetic capacity. However the $K_{0.5}(C_i)$ was unaffected (Fig. 1). This value would be expected to increase if the CCM was impaired as a greater external C_i concentration would be required to maintain the same photosynthetic rate. A reduced P_{max} with increasing temperature has previously been observed in cultured *Symbiodinium* (Iglesias-Prieto et al., 1992). These data indicate that over the temperature range examined here the *Symbiodinium* CCM is not affected, suggesting the reduction in photosynthetic performance is due to a lesion elsewhere in the photosynthetic apparatus.

Differential susceptibility to temperature stress in Symbiodinium populations in clams

Photosynthethetic rate measurements were conducted on a number of *Symbiodinium* populations from different clams. During the experiments we observed that *Symbiodinium* isolated from two different clams had different thermal tolerances (Fig. 2). In *Symbiodinium* from one clam (population 2) the highest photosynthetic rate was found at 29°C and had significantly declined at 36°C (Tukey HSD, $p = 0.0007$), losing 70% of activity. Although in *Symbiodinium* population 3 the 36°C sample was also significantly different from the 29°C sample (Tukey HSD, $p = 0.006$) the photosynthetic rate had declined by only 20%. In both *Symbiodinium* populations, respiration was only affected at temperatures above 37°C. Unfortunately it was not possible to examine if there was a genetic difference between the two populations of *Symbiodinium*.

Rubisco activity and catalytic viability

In *Symbiodinium*, Rubisco activity declines rapidly following cell lysis (Whitney and Yellowlees, 1995). Consequently, both CO_2 and Mg^{2+} (required for activation of the Rubisco active site) were included in the extraction buffer and activation of the enzyme was performed on ice to minimize activity loss. From three separate *Symbiodinium* protein preparations it was established that 5 min activation on ice gave the maximum RuBP dependent carboxylase

activity measurements when assayed for 1 min at 25°C. The same activating conditions were found to be optimal for measuring the content of Rubisco active sites capable of binding ^{14}C-labelled CPBP, a tight binding inhibitor of all Rubiscos. Incubating the activated extracts with ^{14}C-CPBP for at least 30 min on ice was required for maximal recovery of radioactivity in the ^{14}C-CPBP-protein fraction following gel filtration.

The ability of *Symbiodinium* Rubisco to bind ^{14}C-CABP and catalyse the carboxylation of substrate RuBP was similarly affected when cultured between 28°C and 40°C. In general, the activity and catalytic viability of Rubisco (determined by the ability of an active site to bind ^{14}C-CABP) decreased with increasing temperature in two *Symbiodinium* populations isolated from separate clams (Figs. 3a,b). In both populations, the amount of viable Rubisco and extractable activity increased at temperatures around 37°C before rapidly declining between 38–40°C (Figs. 3a,b). The reason for this anomaly is unknown, although it was obtained on a number of occasions and was not reflected in the *in vivo* measurements of the photosynthetic rates (Fig. 2).

A poor correlation was observed between photosynthetic activity measurements made *in vitro* (Rubisco carboxylase activity) and *in vivo* (oxygen evolution). On a cell number basis, no correlation was observed when the data from the two *Symbiodinium* populations were compared (r^2 = 0.0497 for the pooled data). This poor correlation is largely attributed to the large discrepancy in photosynthetic rate measurements. For example, at 29°C carboxylase activities of 4.3 and 13.8 nmol CO_2 fixed $(10^6 \text{ cells})^{-1}$ h^{-1} for *Symbiodinium* populations 2 and 3, respectively, are 40 and 120 times lower than the corresponding rate measured from oxygen evolution (Fig. 2). Notably, the three-fold difference in carboxylase activity measurements between the two *Symbiodinium* populations is largely attributable to a similar difference in the amount of catalytically viable Rubisco in the cells (1.3 and 3.1 pmol Rubisco large subunit sites $(10^6 \text{ cells})^{-1}$ at 29°C for populations 2 and 3, respectively).

A close correlation was found between the amount of Rubisco capable of binding ^{14}C-CABP and the amount of detectable carboxylase activity in cell free extracts from two *Symbiodinium* populations (r^2 = 0.93, Fig. 3c), yielding a catalytic turnover (k_{cat}) rate of 1.2 ± 0.2 s^{-1} (n=17) for *Symbiodinium* Rubisco. However, this rate is clearly an underestimate of that *in vivo* since accurate measurement of carboxylase activity is impaired by the catalytic instability of *Symbiodinium* Rubisco following cell lysis. In contrast, quantifying Rubisco content in *Symbiodinium* grown at 28–29°C by ^{14}C-CABP binding is not noticeably impaired by cell lysis (Whitney and Yellowlees, 1995; Whitney, S.M, unpublished). Therefore, from ^{14}C-CABP Rubisco content measurements and *in vivo* photosynthetic rate (oxygen evolution) measurements at 29°C, k_{cat} rates of 140 and 48 s^{-1} were calculated for *Symbiodinium* Rubisco in *Symbiodinium* populations 2 and 3, respectively.

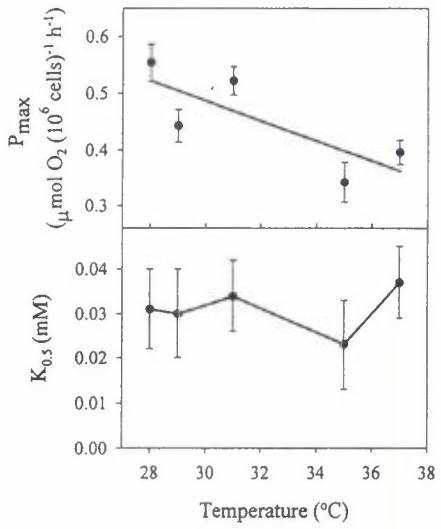

Fig. 1.

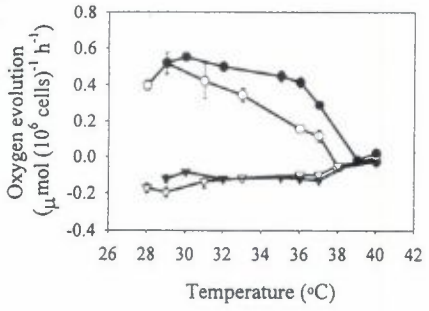

Fig. 2.

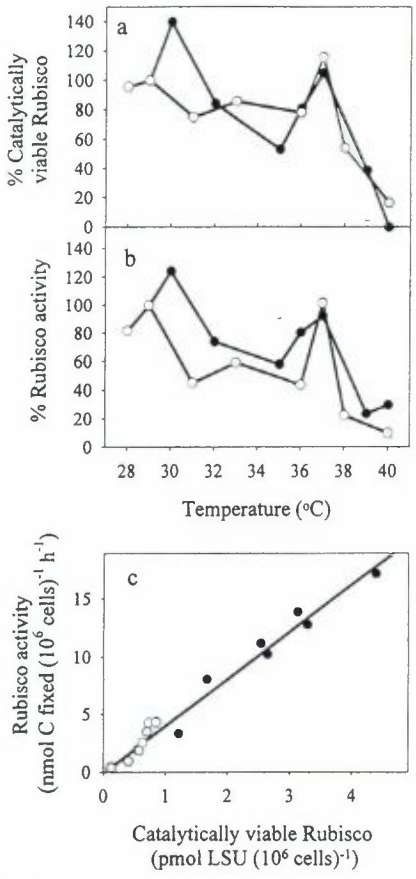

Fig. 3.

Figure 1. Effect of temperature on photosynthesis in zooxanthellae population 1 isolated from *T. gigas*. Replica *Symbiodinium* inoculums were incubated in water baths at the different temperatures for 1.5 h with illumination (200 μE m^{-2} s^{-1}) and then re-equilibrated at 28°C for 15 min before measuring P_{max} and $K_{0.5}(C_i)$. Error bars represent standard deviation standard errors calculated using the program Regression v1.23 (Blackwell Scientific Publishers, Oxford, UK).

Figure 2. Effect of temperature on *Symbiodinium* populations 2 and 3 isolated from different individuals of *T. gigas*. Rates were measured in the presence of 0.5 mM C_i. Open symbols are from population 2 and closed symbols are from population 3. Circles represent net photosynthetic rates and triangles represent respiration rates. Error bars represent standard deviation (n=2).

Figure 3. Effect of temperature on viable Rubisco content and activity measured *in vitro*. The amount of (a) catalytically viable Rubisco large subunits (LSU: re- the ability to bind ^{14}C-CABP) and (b) carboxylase activity in *Symbiodinium* cell free extracts measured following rapid extraction (see methods). Data are expressed as a percentage of that measured at 29°C. (c) Comparison of Rubisco carboxylase activity and viable enzyme content measured in (a) and (b) (r^2 = 0.93). Population 2 (open symbols) and population 3 (closed symbols).

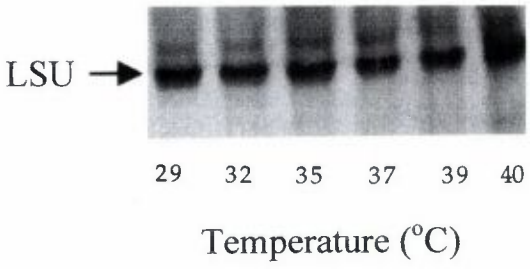

Figure 4. Western blot analysis on the influence of temperature on *Symbiodinium* Rubisco large subunit (LSU, 52 kDa) content and integrity. Soluble cellular protein (10 µg) from *Symbiodinium* cultured for two hours at the temperatures shown were analysed by western blots probed with antibodies raised to *Symbiodinium* Rubisco (see Methods).

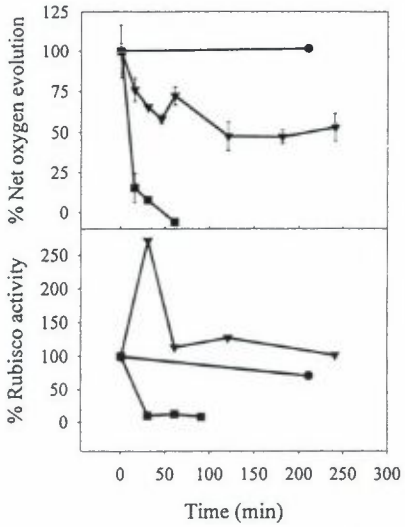

Figure 5. Effect of incubation time on (a) net oxygen evolution and (b) Rubisco carboxylase activity for a fourth population of *Symbiodinium*. Samples were incubated at 28°C (circle), 36°C (triangle) and 40°C (square). Error bars represent standard deviation (n=2). Rates are expressed as a percentage of those measured initially at 28°C.

These values are similar to those calculated from previous data for *Symbiodinium* (38 s^{-1}) and *Amphidinium carterae* (54 s^{-1}) (Whitney and Yellowlees, 1995).

Western blot analysis showed the decrease in Rubisco activity and catalytic viability with increasing temperature was not due to proteolytic degradation of the Rubisco large subunits. Blots of SDS treated and heat denatured total soluble protein from *Symbiodinium* cultured at different temperatures were separated by SDS PAGE and probed with antibodies raised against *Symbiodinium* Rubisco (Fig. 4). In contrast to the CABP binding measurements of Rubisco viability (Fig. 3a), immunoblot analyses showed no evidence of changes to the structural integrity or amount of the Rubisco large subunit in cells incubated at higher temperatures. Clearly, the damage to the Rubisco active site that impairs the viability and activity of *Symbiodinium* Rubisco at higher temperatures is not due to gross proteolytic degradation of the Rubisco large subunits.

Influence of temperature on photosynthetic activity over time

The influence of temperature on Rubisco carboxylase activity and oxygen evolution in *Symbiodinium* (population 4) incubated at different temperatures was examined over 250 min. Three temperatures that spanned the range previously examined were used (Fig. 5). At 28°C both the oxygen evolution rate and Rubisco activity were relatively unchanged. By contrast, when incubated at 36°C oxygen evolution gradually decreased by approximately half over 2 hours and maintained this rate over the next 2 hours. This pattern was not mirrored in the *in vitro* measurements of Rubisco carboxylase activity that had increased 2.5 fold after 30 min before returning to initial rates for the remaining 3.5 hours. At 40°C, both oxygen evolution and Rubisco activity rapidly decreased with little or no activity detectable by 30 min. Western blot analysis of *Symbiodinium* extracts again showed no discernable difference in the content or integrity of the Rubisco large subunit between time points and temperatures (data not shown). Clearly, western blot analyses cannot be used as a proxy for Rubisco activity or catalytic integrity in *Symbiodinium*.

4. Discussion

Despite the ecological and economic significance of mass-bleaching events, we still do not understand the underlying molecular mechanisms of thermal dysfunction of *Symbiodinium*. Two models have been proposed to explain the thermal dysfunction in *Symbiodinium*. In this paper, we address the question of whether either of the two thermal lesion points in the model proposed by Jones et al. (1998) operates during the earliest steps of thermal stress and bleaching.

Previous reports have demonstrated that temperatures above ~28°C disrupt photosynthesis of *Symbiodinium* when in symbiosis (Warner et al., 1996) and

reduce the P_{max} of cultured zooxanthellae from the jellyfish *Cassiopeia xamachana* (Iglesias-Prieto et al., 1992). The P_{max} and respiration rates obtained in this study are similar to those of Iglesias-Prieto et al. (1992) (approximately 0.3 and 0.075 μmol O_2 $(10^6$ cells$)^{-1}$ h^{-1}, respectively), although in contrast to this study they found net O_2 production was completely eliminated at 35°C. The CCM was one of the possible molecular sites of thermal lesion suggested by Jones et al. (1998). Despite this, the effect of temperature on the CCM, as measured by the alga's affinity for inorganic carbon ($K_{0.5}$), has not been examined nor its response to thermal stress. If this hypothesis were correct it would be expected that the $K_{0.5}$ for photosynthesis would increase with increasing temperature. This was not the case (Fig. 1), indicating that the site of damage to the *Symbiodinium* photosynthetic apparatus occurs elsewhere.

The second site for thermal damage proposed by Jones et al. (1998) was Rubisco. We have previously shown that *Symbiodinium* possess a form II Rubisco (Whitney et al., 1995; Rowan et al., 1996) that is catalytically unstable upon cell lysis but still able to tightly bind the inhibitor ^{14}C-CABP stoichiometrically, allowing the content of catalytically competent Rubisco to be quantified (Whitney and Yellowlees, 1995). To date there is no data available on the thermostability of *Symbiodinium* Rubisco, most likely because of the enzyme's instability which results in a discrepancy of ~100-fold between the photosynthetic rates measured *in vivo* (oxygen production) and *in vitro* (carboxylase activity) (Whitney and Yellowlees, 1995).

In this study we observed a similar discrepancy with the Rubisco carboxylase activity measurements accounting for only 1–2.5% the activity expected from oxygen evolution measurements at 29°C. However, despite this discrepancy, a strong correlation was found between carboxylase activity and the amount of catalytically viable Rubisco extracted from *Symbiodinium* cells grown at the different temperatures (Fig. 3). This suggests that the small fraction of Rubisco's catalytic activity that can be measured upon cell lysis (i.e. ~1–2.5% of that measured *in vivo*) remains proportionally uniform with the amount of Rubisco that is catalytically viable.

Our data indicates that thermal stress of *Symbiodinium* perturbs the structural conformation of Rubisco's active site and that this is not accompanied by gross proteolysis of the enzyme. The only previous study to examine Rubisco and temperature reported a 10% decrease in Rubisco activity in cultured *Symbiodinium bermudense* isolated from the sea anemone *Aiptasia pallida* after 22 days at 31°C, compared to values of those cultured at 25°C (Lesser, 1996). With the shorter periods of heat stress of 2 h used here, we found that at temperatures between 29 and 36°C there was a general reduction in the amount of Rubisco capable of binding CABP and carboxylating RuBP in *Symbiodinium* from clams (Fig. 3). This is consistent with an increasing loss in the conformational integrity of the active site, possibly due to dissociation of the

Rubisco oligomeric structure or increased aggregation of the subunits. Over this temperature range whole cell photosynthetic rates in the three *Symbiodinium* populations studied were also perturbed (Figs. 1 and 2), although to a lesser extent in population 3. Curiously, in cells treated at ~37°C, the integrity of Rubisco's active site appears to improve as illustrated by a rise in both catalytically viable Rubisco and activity (Fig. 3). One possible explanation could be the temperature stress to the cells is sufficiently severe that heat shock protein (HSP) synthesis is sufficiently elevated that it rescues stress-damaged Rubisco from a structurally inoperable state. At temperatures above 37°C, Rubisco's structural integrity is irretrievably compromised resulting in a rapid decline in the viability and activity of Rubisco. Consistent with this apparent threshold of thermal stress is the rapid decline in whole cell photosynthesis measurements in *Symbiodinium* above 36°C (Fig. 2). This mirrors the rate of Rubisco's catalytic demise (Fig. 3). The induction of HSPs is a common response in living cells to heat stress. They function to prevent inappropriate association or aggregation of unfolded or partially unfolded proteins and, amongst other things, helps dissociate protein aggregates that have formed (Sun et al., 2002; Miernyk, 1999). A similar induction and role for HSPs in thermally stressed *Symbiodinium* is very likely. In the case of Rubisco, interaction with HSPs may help sustain structural integrity by either preventing or dissociating Rubisco aggregates. Clearly, future attempts might use proteomic approaches to investigate whether the conformational state of Rubisco in heat stressed zooxanthellae does change and which HSPs are up-regulated, particularly at temperatures between 36 and 40°C when perturbations in the health of the cells are obvious (Fig. 5). The use of non-denaturing PAGE, similar to that performed previously (Whitney and Yellowlees, 1995), to analyse cellular protein extracts from differentially heat stressed *Symbiodinium* might be useful in detecting differences in the oligomeric conformation of Rubisco and possibly identify changes in HSP complex production.

We are unable to conclude from the preliminary data presented here that the loss in Rubisco's catalytic activity in thermally stressed cells is the photosynthetic lesion point in *Symbiodinium* from clams. Future work will have to examine whether the catalytic destabilization of Rubisco measured here is not an artifact of post-lysis exposure of the enzyme to stress-induced disruptive cellular by-products (e.g. reactive oxygen species) that are otherwise compartmentalised away from Rubisco in the intact cell. The inclusion of additives (e.g. ascorbic acid, DTT, protease inhibitors) in the lysis buffer to neutralise the stress-induced by-products remains to be rigorously tested. Likewise, as nothing is known about the subunit architecture or regulation of dinoflagellate form II Rubiscos it is possible accessory proteins associated with maintaining and regulating its structure and activity might be

the targets of thermal lesion. For example form I Rubisco from higher plants is extremely heat stable, requiring temperatures in excess of 50°C to bring about loss of activity or significant conformational changes to the purified enzyme (Eckardt and Portis, 1997; Li et al., 2002; Crafts-Brandner and Salvucci, 2000). Despite this, in plants net photosynthesis is inhibited at higher temperatures. Recently the thermal lesion point in cotton and tobacco has been pinpointed to Rubisco activase functionality (Crafts-Brandner and Salvucci, 2000; Salvucci et al., 2001). This enzyme functions to activate Rubisco by promoting the dissociation of dead end complexes formed with tight binding sugar phosphates (Salvucci and Ogren, 1996; Andrews, 1996). However, Rubisco activase is heat labile (Eckardt and Portis, 1997; Crafts-Brandner and Salvucci, 2000) and it is suggested that under high leaf temperature Rubisco activation decreases, due to either the rate of Rubisco deactivation exceeding the activation capacity of Rubisco activase (Crafts-Brandner and Salvucci, 2000) and/or denaturation of the Rubisco activase (Salvucci et al., 2001). Whether an "activase-like" enzyme is associated with the dinoflagellate form II Rubisco is unknown, and attempts to demonstrate its presence in dinoflagellates have proved unsuccessful (Whitney, unpublished results).

One interesting feature determined from this study was the fast catalytic turnover (k_{cat}) value estimated for *Symbiodinium* Rubisco from *in vivo* photosynthetic rates. In addition to improvements in CO_2/O_2 specificity (compared to other Form II enzymes, Whitney and Andrews, 1998), the Rubisco from dinoflagellates appears to have evolved a catalytically faster version. Its turnover rates are at least 8-fold higher than that measured for the form II Rubisco from *Rhodospirillum rubrum* and ~4-fold higher than the highest k_{cat} measured for the form I Rubisco from a cyanobacterium (*Synechococcus*) (Badger et al., 1998). Notably, these rates will be overestimated if the form II dinoflagellate Rubisco does not bind CABP stoichiometrically as the enzyme content will be underestimated. However, this does not appear to be the case since measurements of Rubisco content (determined from CABP binding) in non-heat stressed dinoflagellate cells are in accord with the amount observed in cell extracts (Whitney and Yellowlees, 1995, data not shown) making the order of magnitude for error to be less than 0.5 fold. One advantage of a catalytically faster Rubisco would be less amount of the enzyme would be required by the cell, although this would also depend on the enzymes affinity for substrate CO_2 (i.e. the Michaelis-Menten constant for CO_2, $K_m(CO_2)$). Consistent with this, in *Symbiodinium* cells cultured at 29°C (populations 2 to 4) Rubisco was found to constitute only ~1 to 2% of the cellular protein, similar to that measured previously (Whitney and Yellowlees, 1995). Unfortunately, obtaining a measure of $K_m(CO_2)$ for *Symbiodinium* Rubisco to compare it with other Rubiscos is not possible because of the enzyme can not be purified in an active configuration.

Another interesting observation in this study was that *Symbiodinium* isolated from different clams exhibit different temperature tolerances. The population with the higher amount of extractable Rubisco activity and content (population 3) had a greater thermal tolerance (Fig. 2). As the clams were maintained side by side for in excess of 2 months it seems unlikely that these differences could be attributed to the past history of the zooxanthellae, although there is evidence of previous light history mediating the effects of thermal bleaching (Brown et al., 2002). Different thermal tolerances between *Symbiodinium* populations have previously been described from other symbiotic organisms (Warner et al., 1996, 1999) although not from clams. This difference in thermal stability suggests that some populations of *Symbiodinium*, and therefore their symbiotic partners, may be more resistant to bleaching events. Unfortunately it was not possible to determine if the populations were genetically different. In rice it has been found that thermal tolerant strains have protective mechanisms in place that involve a more thermostable Rubisco and less proteolytic degradation (Bose et al., 1999). In *Symbiodinium*, the form II Rubisco LSU is encoded by a multi-gene family in the nucleus whose translated products vary in amino acid sequence (Rowan et al., 1996). Whether differential expression of these genes and their dissimilar translated products lead to assembly of Rubisco oligomers with altered thermal tolerances or catalytic capabilities has yet to be determined.

5. Conclusion

The data presented here indicate the CCM in *Symbiodinium* is not the major cause of thermal inactivation of photosynthesis and the subsequent bleaching of symbiotic marine invertebrates, as discussed by Jones et al. (1998). However, the data do not discount the possible lesion point being associated with destabilization of the Rubisco active site, this will require further work. What can be gleaned from this work is that the aerobic lifestyle of *Symbiodinium* has coerced the evolution of a more efficient form II Rubisco whose catalytic turnover surpasses that of all other known Rubiscos by at least 4-fold. Clearly, comprehensive analysis on the structure, regulation and conformational stability of dinoflagellate Rubisco should be the targets of future work to determine if this is the site of thermal lesion in mass bleaching events.

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