Plant Physiology, December 1999, Vol. 121, pp. 1247–1255, www.plantphysiol.org © 1999 American Society of Plant Physiologists

Evidence for an Inorganic Carbon-Concentrating Mechanism in the Symbiotic Dinoflagellate *Symbiodinium* sp.[1]

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The presence of a carbon-concentrating mechanism in the symbiotic dinoflagellate *Symbiodinium* sp. was investigated. Its existence was postulated to explain how these algae fix inorganic carbon (C_i) efficiently despite the presence of a form II Rubisco. When the dinoflagellates were isolated from their host, the giant clam (*Tridacna gigas*), CO_2 uptake was found to support the majority of net photosynthesis (45%–80%) at pH 8.0; however, 2 d after isolation this decreased to 5% to 65%, with HCO_3^- uptake supporting 35% to 95% of net photosynthesis. Measurements of intracellular C_i concentrations showed that levels inside the cell were between two and seven times what would be expected from passive diffusion of C_i into the cell. *Symbiodinium* also exhibits a distinct light-activated intracellular carbonic anhydrase activity. This, coupled with elevated intracellular C_i and the ability to utilize both CO_2 and HCO_3^- from the medium, suggests that *Symbiodinium* sp. does possess a carbon-concentrating mechanism. However, intracellular C_i levels are not as large as might be expected of an alga utilizing a form II Rubisco with a poor affinity for CO_2.

Dinoflagellates of the genus *Symbiodinium* (=zooxanthellae) are known for their role in a number of symbiotic associations with mainly tropical marine invertebrates, including corals, clams, and sea anemones (Trench, 1987). The algae are either intra- or intercellular and generally associated with the digestive system of the host. The host therefore has a major influence on the supply of inorganic carbon (C_i) to the symbiont. Following carbon fixation by the zooxanthellae, much of the photosynthate is exported to the host and can contribute up to 100% of the host's energy requirements (Klumpp et al., 1992). The supply and fixation of carbon therefore has a major influence on the symbiosis.

In the giant clam (*Tridacna gigas*) zooxanthellae are found in tubules emanating from the stomach (Norton et al., 1992). These are in close proximity to the hemal sinuses, which contain hemolymph, the clam's blood supply. The hemolymph is the immediate source of nutrients for the zooxanthellae and its composition is affected by the photosynthetic rate of the dinoflagellates resulting in a diurnal variation in a number of parameters (Fitt et al., 1995, D. Yellowlees, personal communication). Thus, during photosynthesis the hemolymph [C_i] can drop from 1.8 to 0.8 mM, with a concomitant increase in pH from 7.3 in the dark to 8.2 at high light levels. During high rates of photosynthesis the hemolymph is supersaturated with O_2, as bubbles are present in hemolymph samples removed from the sinuses. The fluctuations in C_i and pH are probably greater in the tubules themselves, but no measurements have been reported to date.

Recently, we reported that *Symbiodinium* sp. possesses a form II Rubisco (Whitney et al., 1995), which had previously been reported only in prokaryotic anaerobic, nonsulfur purple bacteria. Like other form II enzymes, dinoflagellate Rubisco has a relatively low discrimination ratio (S_{rel}) between CO_2 and O_2 (Jordon and Ogren, 1981). Apart from dinoflagellates, all form II enzymes are found in anaerobic bacteria, in which a low S_{rel} value has no physiological significance. Whitney and Andrews (1998) reported an S_{rel} of approximately 35 for the form II Rubisco from *Amphidinium carterae*, a related free-living dinoflagellate. While this is the highest reported S_{rel} for a form II Rubisco, it is still 40% lower than any form I enzyme. Whitney and Andrews (1998) concluded that this S_{rel} value would allow dinoflagellates to maintain a positive photosynthetic carbon balance; however, the ratio of oxygenation to carboxylation would utilize light energy very inefficiently. This raises the question of how do dinoflagellates, with a form II Rubisco, survive in an aerobic environment and, in the case of *Symbiodinium* sp., export significant amounts of photosynthate? One possible mechanism for overcoming the limitations of a form II Rubisco in a potentially unfavorable CO_2/O_2 ratio environment would be the utilization of a carbon-concentrating mechanism (CCM). This would increase internal CO_2 concentrations and minimize the effect of the oxygenation reaction of Rubisco.

A significant number of algae and cyanobacteria have been shown to actively accumulate C_i internally by utilizing a CCM (for review, see Badger et al., 1998). These elevated internal C_i levels allow algae to grow in C_i-limiting environments, produce higher carbon fixation rates, and also reduce the energetically wasteful oxygenation reaction of Rubisco.

There is circumstantial evidence that *Symbiodinium* sp. does possess a CCM; it has both internal and external carbonic anhydrase (CA) (Yellowlees et al., 1993) and appears capable of both HCO_3^- and CO_2 utilization. Zoox-

[1] This work was supported by an Australian Research Council grant (D.Y.) including a Ph.D. scholarship (W.L.).

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anthellae isolated from corals utilize predominantly HCO_3^- (Goiran et al., 1996), while those from giant clams appear to utilize CO_2 (Yellowlees et al., 1993). Whether this is due to the different environment within the host or different zooxanthellae strains is not known. Zooxanthellae also exhibit changes in photosynthetic characteristics after isolation from a host, with a decrease in both P_{max} and $K_{0.5}$ photosynthesis, suggesting that there might be changes in the C_i supply to Rubisco (W. Leggat, personal observation).

In addition, the fresh water dinoflagellate *Peridinium gatunense* has been found to acquire a CCM under C_i-limiting conditions and can maintain internal C_i concentrations between 7- and 80-fold above external levels (Berman-Frank and Erez, 1996; Berman-Frank et al., 1998).

This study was designed to determine if the symbiotic dinoflagellate possesses a CCM and whether the characteristics of the CCM change with time after isolation from the giant clam. We report here the results of our study into the uptake and accumulation of C_i by *Symbiodinium* sp. The results indicate that zooxanthellae do possess a CCM; however, intracellular C_i concentrations are not as high as might be expected of an alga utilizing a form II Rubisco for photosynthetic carbon fixation.

MATERIALS AND METHODS

Isolation of Zooxanthellae and Culturing of Algae

Giant clams (*Tridacna gigas*) were obtained from the Australian Centre for International Agricultural Research Giant Clam Project (James Cook University Orpheus Island Research Station, Queensland, Australia) and transported to the open-air aquarium at James Cook University (Townsville). Clams were acclimatized there for at least 3 weeks before experiments were commenced. Clams were then either killed at Townsville or flown to the Australian National University (Canberra, ACT, Australia) before being killed. Zooxanthellae were isolated by blending the mantle of a freshly killed clam in 0.45 μm of filtered seawater. The homogenate was then strained through two layers of cheesecloth and zooxanthellae pelleted by centrifugation at 600*g* for 2.5 min at 25°C. The zooxanthellae were then washed four more times in filtered seawater before they were suspended in filtered seawater at a cell density of approximately 2×10^6 cells mL^{-1} and cultured with a photon flux density of 100 μE m^{-2} s^{-1}.

A culture of *Amphidinium carterae* (CS-21) was obtained from the Commonwealth Scientific and Industrial Research Organization Culture Collection of Microalgae (Hobart, Australia) and grown in G media (Loeblich, 1975) at 25°C at a photon flux density of 100 μE m^{-2} s^{-1}. The algae were harvested during log-phase growth.

MS Measurements

Steady-State Photosynthesis and C_i Fluxes

All experiments were conducted at 28°C in CO_2-free artificial seawater medium containing 428 mM NaCl and 25 mM 1,3-bis(Tris[hydroxymethyl]methylamino) propane (BTP) (pH 7.0 or 8.0). Experiments were conducted as previously described in Badger et al. (1994). An O_2 electrode chamber was connected to a mass spectrometer via a gas-permeable membrane. The mass spectrometer was sequentially focused on masses 44 (CO_2) and 32 (O_2), and the changes in the concentrations recorded. Estimations were made of the HCO_3^- concentration by calibrating at acidic, buffered, and basic pH. Estimates of the flux of CO_2, O_2, and HCO_3^- into the cell were made using the equations of Badger et al. (1994).

Zooxanthellae were pelleted at 600g for 2.5 min, and resuspended in CO_2-free artificial seawater medium, so that the Chl *a* concentration was between 4 and 10 μg Chl *a* mL^{-1}, as determined by the method of Jeffrey and Humphrey (1975). Artificial seawater medium (5 mL, pH 7.0 or 8.0) was placed in the electrode chamber with 5 μL of acetazolamide (final concentration in cuvette of 50 μM), and 200 μL of zooxanthellae suspension was added. The cuvette was then illuminated (approximately 500 μE m^{-2} s^{-1}) for 2 min to acclimatize the algae before the light was shut off and the cuvette was purged with N_2 until the $[O_2]$ was approximately 100 μM. $NaHCO_3$ was then added and dark measurements taken until equilibrium was achieved. The cells were then illuminated at a saturating light level (500 μE m^{-2} s^{-1}) (Chang et al., 1983; Iglesias-Prieto and Trench, 1994) and readings taken until steady state was reached. Dark-light cycles were repeated with increasing HCO_3^- concentrations.

$H^{13}C^{18}O_3^-$ Exchange

These experiments were also conducted in the mass spectrometer using methods similar to that of Palmqvist et al. (1995). This method gives qualitative information about the presence of a CCM. The loss of ^{18}O from CO_2 was measured by monitoring the CO_2 masses 49 ($^{13}C^{18}O_2$), 47 ($^{13}C^{18}O^{16}O$), 45 ($^{13}C^{16}O_2$), and 44 (CO_2). The log enrichment of the ^{18}O fraction in $^{13}C^{18}O_2$ was calculated using the equations of Palmqvist et al. (1995):

$$\text{Log enrichment} = \log\left(\frac{100 \times [49]}{[49] + [47] + [45]}\right)$$

Experiments were conducted in 4 mL of artificial seawater medium (pH 8.0) to which was added $H^{13}C^{18}O_3^-$ (final concentration 1 mM), and the uncatalyzed exchange was allowed to equilibrate (2 min). Zooxanthellae (100 μL) was then added (approximately 5 μg Chl *a* mL^{-1}) and left in the dark for 3 min until equilibrium was established. They were then exposed to light for 3 min (500 μE m^{-2} s^{-1}), followed by 5 min of darkness. In acetazolamide (AZA) and ethoxyzolamide (EZA) treatments, the inhibitor was added before the labeled bicarbonate to a final concentration of 50 and 500 μM respectively.

Photosynthetic O_2 Exchange

All experiments were conducted at 28°C in CO_2-free artificial seawater medium containing 428 mM NaCl and 25 mM BTP (pH 8.0). This media was maintained CO_2 free

by purging with CO_2-free air, and was degassed under vacuum prior to use in the O_2-exchange assays. Cells were maintained in a concentrated suspension at room temperature prior to use. O_2-exchange assays were conducted in a 4-mL cuvette attached to a mass spectrometer via a Teflon semipermeable membrane. A similar method and calculations have been previously described (Canvin et al., 1980; Furbank et al., 1982). The assay and measurements involve the introduction of $^{18}O_2$ into reaction medium that has been depleted of $^{16}O_2$. This was done after the introduction of cells, using a small bubble of $^{18}O_2$ above the reaction medium. During both the dark and light periods, changes in mass 32 ($^{16}O_2$) and mass 36 ($^{18}O_2$) were continuously monitored, and the rate of change in the concentration of these species was used to calculate gross O_2 evolution, gross O_2 uptake, and net O_2 evolution. Assays were conducted with a cell density of 2 to 4 μg Chl *a* mL^{-1}, and an O_2 concentration of 250 to 350 μM (higher at the end of the experiment due to net O_2 evolution). Light was provided at the top of the cuvette at 500 $\mu E\ m^{-2}\ s^{-1}$ through a fiber-optic light source. Experiments were performed by adding cells to the cuvette and waiting for a steady-state dark value. Light was then switched on and C_i was added sequentially, allowing 4 to 7 min for a steady-state rate to be achieved at each C_i concentration.

Silicone Oil Centrifugation

This experiment used a method adapted from that of Badger et al. (1980). Freshly isolated or cultured zooxanthellae or *A. carterae* were pelleted (200*g*) and resuspended at a density of approximately 15 μg Chl *a* mL^{-1} in a solution of 25 mM BTP and 428 mM NaCl (pH 7.0 or 8.0) that had been bubbled with CO_2-free air for 2 d. The zooxanthellae suspension was then placed in an O_2 electrode chamber (Hansatech Instruments, King's Lynn, UK) at light levels of 500 $\mu E\ m^{-2}\ s^{-1}$ until O_2 production had ceased and the cells had reached their CO_2 compensation point. Killing solution (20 μL of 2 N KOH and 10% MeOH) was added to 400-μL plastic microfuge tubes (Eppendorf Scientific, Westbury, NY), and 50 μL of silicone oil (approximately 2:1, 200/20, Wacker Chemie, Munich) was overlaid. The zooxanthellae suspension (200 μL) was then added.

The tubes were illuminated at a photon flux density of approximately 500 $\mu E\ m^{-2}\ s^{-1}$. $H^{14}CO_3^-$ of known specific activity was added so that the final concentration was between 20 and 2,000 μM. Cells were incubated at each C_i concentration for 15 to 20 s, after which the tubes were centrifuged at 15,000*g* for 15 s to pellet the zooxanthellae. The tubes were snap-frozen and the bottom layer containing the zooxanthellae and the killing solution cut from the tube and resuspended in 500 μL of 0.1 N NaOH. The resuspended solution (200 μL) was added to 200 μL of 0.1 N NaOH or 200 μL of 0.2 N HCl. The acidic sample was placed in a fume hood and heated to 60°C for 1 h so that any unfixed ^{14}C was evolved. BSC scintillation fluid (4 mL, Amersham-Pharmacia Biotech, Uppsala) was added to both the acid and basic samples and the ^{14}C counted in a liquid scintillation counter (Wallac 1410, EG&G Wallac, Turku, Finland). The basic samples were assumed to contain both the fixed and unfixed ^{14}C in the cell, while the acidic sample contained only the fixed C_i. Corrections were made for any extracellular ^{14}C.

Calculation of Intracellular Volume and pH

Total extracellular and intracellular volumes were calculated using ^3H_2O and either [^{14}C]mannitol or [^{14}C]dextran (ICN, Costa Mesa, CA). Intracellular pH was measured using 5,5-dimethyl-[2-^{14}C]oxazolidine 2,4-dione (Badger et al., 1980).

RESULTS

Measurement of Steady-State Photosynthesis and C_i Fluxes before and after Isolation

An initial characterization of C_i uptake processes in the zooxanthellae can be approached by deriving estimates for the ability of the cells to utilize both CO_2 and HCO_3^- as carbon sources for photosynthesis. Mass spectrometry techniques (Badger et al., 1994) enable measurements of the net fluxes of CO_2, HCO_3^-, and O_2 into and out of the cell under steady-state photosynthesis conditions. This approach shows that freshly isolated zooxanthellae assayed at pH 8.0 predominantly take up CO_2 from the external media but are also capable of some HCO_3^- uptake (Fig. 1). Net CO_2 uptake is able to support between 45% and 80% of net photosynthesis, with the contribution increasing at higher levels of C_i. Conversely, the contribution of net HCO_3^- uptake declines from 55% to 20% over this same range.

After 2 d of isolation, the rates and patterns of C_i uptake changed significantly to increase the capacity for HCO_3^-

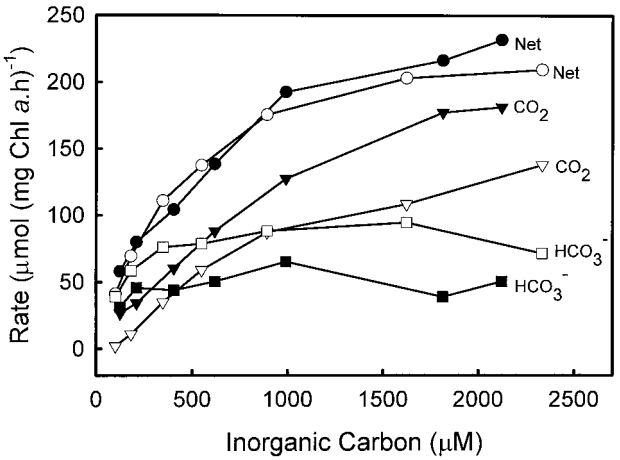

Figure 1. Comparison of net photosynthetic rate (●, ○), CO_2 uptake (▼, ▽), and HCO_3^- uptake (■, □) for *Symbiodinium* sp. freshly isolated from the giant clam *T. gigas* (black symbols) and a 2-d-old culture (white symbols) with differing C_i concentrations. Assays were conducted in 25 mM BTP (pH 8.0), 428 mM NaCl, and 50 μM AZA at 28°C with a photon flux density of 500 $\mu E\ m^{-2}\ s^{-1}$. Data were collected using the mass spectrometry disequilibrium technique described in "Materials and Methods."

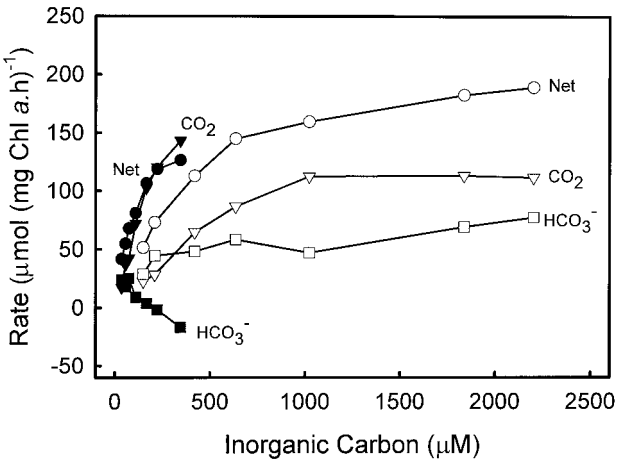

Figure 2. Comparison of net photosynthetic rates (●, ○), CO_2 uptake (▼, ▽), and HCO_3^- uptake (■, □) for *Symbiodinium* sp. at pH 7.0 (black symbols) and pH 8.0 (white symbols) after being in culture for 1 d. Assays were conducted in 25 mM BTP and 428 mM NaCl with 50 μM AZA at 28°C with a photon flux density of 500 μE m^{-2} s^{-1}. Data was collected using the mass spectrometry disequilibrium technique described in "Materials and Methods."

uptake. The contribution of net CO_2 uptake declined to 5% to 65%, while HCO_3^- increased to 95% to 35% over the same range of C_i concentrations. This change in HCO_3^- uptake indicates the induction of a HCO_3^- uptake system that allows utilization of a greater proportion of the available C_i, particularly at limiting [C_i]. Following isolation, the maximum net photosynthetic rate decreased from 300 ± 20 to 260 ± 10 μmol mg^{-1} Chl *a* h^{-1} (Fig. 1), while the $K_{0.5}$ (C_i) was found to be 640 ± 100 and 500 ± 50 μM, respectively. A similar, although larger decrease has previously been observed after isolation (W. Leggat, personal observation).

Flux measurements at pH 7.0 of zooxanthellae isolated for 1 d (Fig. 2) show that there is less HCO_3^- uptake capacity at this pH, where CO_2 is a more dominant species. Although HCO_3^- does support 40% of photosynthesis at the most limiting [C_i], this rapidly declines as C_i increases. At half-saturating [C_i], the net HCO_3^- uptake supports only 10% of net photosynthesis. The $K_{0.5}$(C_i) at pH 7.0 was 99 ± 9 μM compared with 433 ± 41 μM at pH 8.0. This indicates that $K_{0.5}$(CO_2) declines from around 16 μM to 9 μM from pH 7.0 to 8.0, supporting the occurrence of some increased HCO_3^- uptake, but clearly indicating that CO_2 uptake plays a significant role in photosynthesis at both pH values. The net photosynthesis (P_{max}) declined at pH 7.0 from 230 ± 10 (pH 8.0) to 160 ± 5 μmol mg^{-1} Chl *a* h^{-1}. The negative HCO_3^- uptake values at pH 7.0 in this figure are most likely an artifact due to the errors involved in the calculation of this value. The main significance of this is that HCO_3^- uptake is low compared with CO_2 uptake.

Light-Stimulated CA Activity

In searching for evidence for the operation of a CCM, one of the key processes common to both algae and cyanobacteria is active C_i transport, taking both CO_2 and HCO_3^- from outside the cell and placing it in contact with localized regions of CA inside the cell. This enables the generation of CO_2 from accumulated HCO_3^- so that [CO_2] can be elevated around Rubisco (Badger and Price, 1992). A robust way that this can be measured is through monitoring light-stimulated inorganic exchange processes using ^{18}O-enriched C_i species (Palmqvist et al., 1994). The active uptake of C_i species promotes the loss of ^{18}O to unlabeled water due to internal CA activity, and this can be measured by examining the changes in isotopic enrichment of the CO_2 species.

Figure 3 shows the results of such experiments. Control experiments and those with 50 μM AZA showed similar patterns (Fig. 3). The initial rapid decrease in enrichment with the addition of zooxanthellae is due to access of external CO_2 to internal CA. This rate declines as steady-state equilibrium is reached. Upon illumination, there was a distinct light-stimulated decline in enrichment due to light-activated CA activity. AZA (50 μM), which inhibits external CA (Miyachi et al., 1983; Moroney et al., 1985), did not have any effect on the enrichment pattern in the dark or the light, suggesting that there may be little external CA activity associated with these cells, and that it does not inhibit active uptake of C_i.

However, 500 μM EZA, which inhibits both internal and external CA, eliminated light-stimulated CA activity and active C_i uptake (Fig. 3). Patterns similar to those found with AZA and EZA have been observed in *Chlamydomonas reinhardtii* and *Scenedesmus obliquus* (Palmqvist et al., 1995). The effects of EZA are most readily interpreted as being

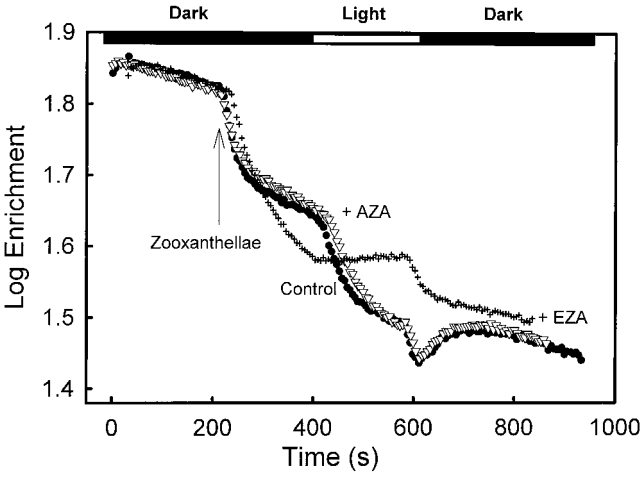

Figure 3. Effect of dark/light periods and CA inhibitors on the log enrichment (percentage $^{13}C^{18}O_2$) of C_i in the medium during assays of *Symbiodinium* sp. Assays were conducted in a water jacketed cuvette (28°C) connected to a mass spectrometer. Freshly isolated cells were added to the cuvette (final concentration of 0.125 μg Chl *a* mL^{-1}) containing 4 mL of CO_2-free media (25 mM BTP, pH 8.0, and 428 mM NaCl) and $H^{13}C^{18}O_3^-$ (1 mM) that had achieved chemical equilibrium. After 3 min of darkness, the cuvette was illuminated at a photon flux density of 500 μE m^{-2} s^{-1} for 3 min, followed by another 5 min of darkness, as indicated by the bar at the top of the graph. Where appropriate, the CA inhibitors AZA (▽; final concentration 50 μM) or EZA (+; final concentration 500 μM) were added prior to the addition of the cells. Data are presented as log enrichment as per the formula detailed in "Materials and Methods."

due to an inhibition of both C_i transport processes and internal CA. This leads to a situation in which external C_i species are in passive equilibrium with the internal compartment. When light activates photosynthesis under these conditions, there is a competition between Rubisco and C_i hydration processes for CO_2, which actually reduces the exchange of label from CO_2, causing the enrichment to rise rather than fall. This pattern is also typical of algal species that appear to lack a CCM (Palmqvist et al., 1995). The rapid decline in the dark in the presence of EZA is somewhat anomalous and not readily explained. However, this feature was present in all experiments that were conducted using EZA as an inhibitor.

Similar experiments were also conducted on zooxanthellae that had been cultured for 2 d, and the results obtained were similar to those of freshly isolated zooxanthellae (data not shown).

Silicone Oil Centrifugation

Although mass spectrometry allows estimates to be made of C_i uptake, it does not provide information about the intracellular C_i concentration (C_{int}). However, silicone oil centrifugation provides estimates of both the intracellular pool size and the pH, which influences the equilibrium concentration of C_i within the cell. When measurements were made of the C_{int} and fixed carbon at both pH 7.0 and 8.0, the P_{max} at pH 7.0 and 8.0 was 144 ± 7 and 142 ± 9 μmol C fixed mg^{-1} Chl *a* h^{-1}, respectively, while the $K_{0.5}$ (C_i) was 90 ± 10 and 600 ± 100 μM (data not shown). The internal pH of the zooxanthellae was estimated as 7.62 ± 0.09, while the total intracellular volume of the cells in the assay was 0.12 ± 0.05 μL. At pH 7.0, freshly isolated zooxanthellae had an internal C_i/external C_i (C_{int}/C_{ext}) between 1.5 and 3.2 times what would be expected if only passive diffusion of C_i into the cells had occurred (Fig. 4; Table I). At pH 8.0 the results were similar, with the C_{int} between 2.2 and 4.6 times what would be expected after passive diffusion (Fig. 4, Table I). C_{int}/C_{ext} was measured for 5 d after isolation, over this period C_{int}/C_{ext} increased to a maximum of 24.2 (7 times passive diffusion) after 1 d and decreased to a maximum of 14.4 (4.2 times passive diffusion) 5 d after isolation (Table I).

The internal C_i was also determined for the free-living dinoflagellate *A. carterae*. The C_{int}/C_{ext} ratio was similar to that found for *Symbiodinium*, between 7.3 and 26.4 (Table I).

Photosynthetic O_2 Uptake

One of the key roles of the operation of a CCM in photosynthetic organisms is to suppress the oxygenase activity of Rubisco and thus reduce the deleterious effects of O_2 on photosynthesis. This may be particularly so for zooxanthellae possessing a form II Rubisco with low S_{rel} and a potentially high oxygenase activity (Whitney and Andrews, 1998). Therefore, it was of interest to examine the photosynthetic O_2 uptake associated with photosynthesis and the effects of C_i limitation on potential oxygenase activity.

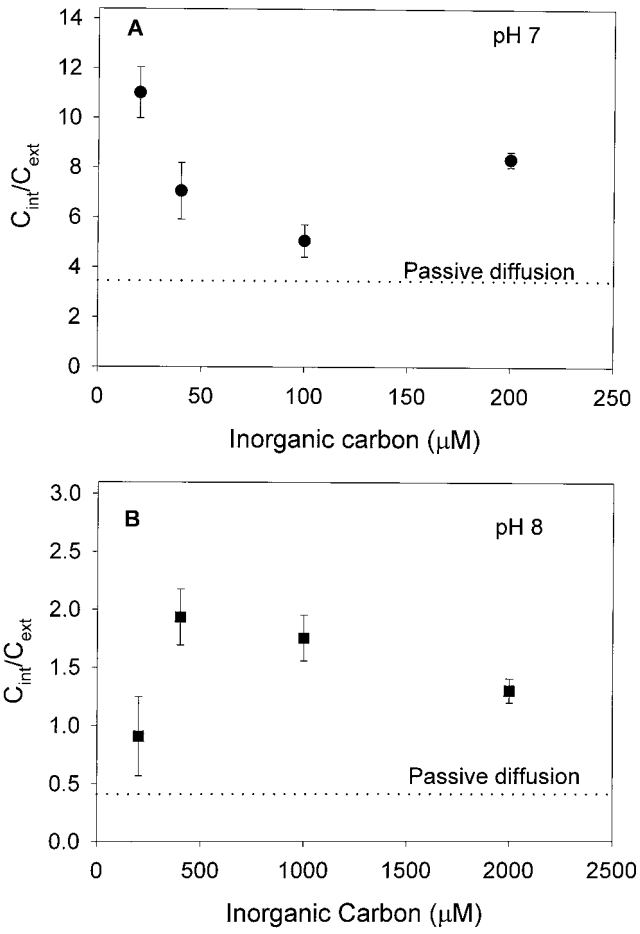

Figure 4. The effect of external C_i on C_{int}/C_{ext} at pH 7.0 (A) and 8.0 (B) for *Symbiodinium* sp. freshly isolated from *T. gigas*. Passive diffusion indicates the C_{int}/C_{ext} expected if only passive diffusion of CO_2 into the cell was occurring, assuming an intracellular pH of 7.62. Assays were conducted using silicone oil centrifugation in 25 mM BTP and 428 mM NaCl at a photon flux density of 500 μE m^{-2} s^{-1} using $H^{14}CO_3^-$ as a substrate as in "Materials and Methods." Error bars represent SES (n = 3).

Figure 5 shows the response of both gross and net O_2 fluxes to varying inorganic carbon at pH 8.0. In control cells, gross O_2 uptake near the C_i compensation point represented about 30% of the maximum O_2 evolution rate at saturating C_i. O_2 uptake was stimulated by the light, increasing from around 50 in the dark to 130 μmol mg^{-1} Chl *a* h^{-1} at the lowest C_i concentration. Increasing C_i actually stimulated O_2 uptake reactions up to around 0.5 mM C_i, increasing O_2 uptake to 45% maximum O_2 evolution. A stimulation of O_2 uptake in the light by C_i has been seen in higher plants (Canvin et al., 1980) and has been interpreted as being due to an activation of Rubisco by CO_2, leading to increased oxygenase activity. Cells were treated with EZA to inhibit CCM activity and induce increased C_i limitation. EZA-treated cells showed a decreased affinity for C_i, and the P_{max} photosynthesis may have also been reduced. Despite an increased C_i limitation in EZA-treated cells, O_2 uptake was actually decreased at

Table I. *Comparison of intracellular and extracellular inorganic carbon concentration of Symbiodinium sp. and A. carterae calculated using silicone oil centrifugation at differing pH values*

Assays were conducted in 25 mM BTP and 428 mM NaCl at a photon flux density of 500 μE $m^{-2}s^{-1}$.

Species	Culture Conditions	External pH	C_i External	C_{int}/C_{ext}	Estimated C_{int}/C_{ext} for Passive Diffusion[a]	C_{int}/C_{ext} / Estimated C_{int}/C_{ext}
			μM			
Symbiodinium sp.	Freshly isolated	8.0	200	0.9 ± 0.3	0.41	2.2 ± 0.7
			400	1.9 ± 0.2	0.41	4.6 ± 0.5
			1,000	1.7 ± 0.2	0.41	4.1 ± 0.5
			2,000	1.3 ± 0.1	0.41	3.2 ± 0.2
	Freshly isolated	7.0	20	11.0 ± 1.0	3.47	3.2 ± 0.3
			40	7.1 ± 1.1	3.47	2.0 ± 0.3
			100	5.1 ± 0.6	3.47	1.5 ± 0.2
			200	8.4 ± 0.3	3.47	2.4 ± 0.1
	1-d-old Culture[b]	7.0	20	24.2 ± 5.1	3.47	7.0 ± 1.5
			40	23.0 ± 4.5	3.47	6.6 ± 1.3
			100	16.5 ± 1.4	3.47	4.8 ± 0.4
			200	12.9 ± 1.2	3.47	3.7 ± 0.3
	5-d-old Culture[b]	7.0	20	14.4 ± 2.4	3.47	4.1 ± 0.1
			40	11.8 ± 1.7	3.47	3.4 ± 0.5
			100	9.0 ± 0.9	3.47	2.6 ± 0.3
			200	6.8 ± 0.7	3.47	2.0 ± 0.2
A. carterae	Cultured[c]	7.0	20	7.3 ± 2.6	ND	ND
			40	26.4 ± 7.7	ND	ND
			100	17.4 ± 2.2	ND	ND
			200	20.5 ± 5.8	ND	ND

[a] Estimated C_{int}/C_{ext} assuming only passive diffusion of CO_2 into the cell and an intracellular pH of 7.62. [b] *Symbiodinium* sp. was isolated from *T. gigas* and maintained in filtered seawater (0.45 μm). [c] *A. carterae* was grown in G media (Loeblich, 1975) and harvested in log phase growth.

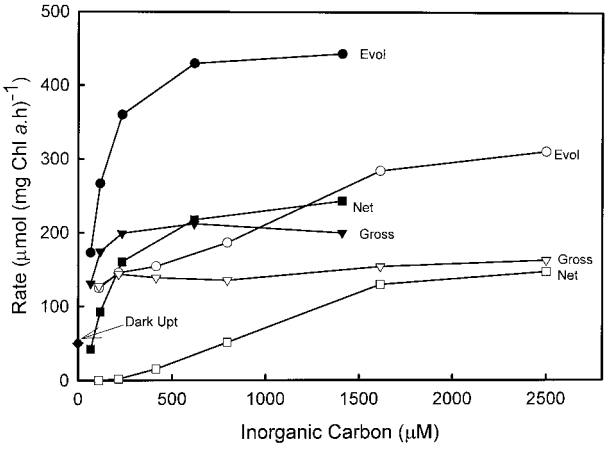

Figure 5. Photosynthetic O_2 exchange of freshly isolated *Symbiodinium* sp. in response to external C_i. The experiments were conducted as described in "Materials and Methods" at a cell density of 2.2 μg Chl *a* mL^{-1} and a photon flux density of 500 μE m^{-2} s^{-1}. Shown are values for gross O_2 evolution (Evol), gross O_2 uptake, and net O_2 evolution. C_i responses are shown for both control (black symbols) and plus 500 μM EZA (white symbols) added just before cells for each treatment, together with a value for O_2 uptake measured in the dark.

limiting C_i compared with control cells, and again there was evidence for stimulation by increasing C_i. In a number of experiments with control cells, stimulation of O_2 uptake by increasing C_i was always observed, and the maximum O_2 uptake capacity varied between 35% to 45% of maximum O_2 evolution at saturating C_i.

It is possible that the zooxanthellal Rubisco may have a low affinity for O_2, as is the case for cyanobacterial and non-green algal form I Rubiscos (Jordon and Ogren, 1981). Thus, the response of O_2 uptake to varying O_2 was examined and is shown in Figure 6. Both close to the C_i compensation point and at near-saturating [C_i], there was no stimulation of O_2 uptake by increasing O_2 from 0.1 to 0.5 mM.

DISCUSSION

It has been hypothesized that as *Symbiodinium* sp. possess a form II Rubisco they must have a CCM that increases CO_2 at the site of carbon fixation. This study was designed to examine the C_i uptake and utilization of *Symbiodinium* sp. after isolation to determine if this hypothesis is true. We also examined the effect of culturing freshly isolated zooxanthellae in filtered seawater. This was prompted by two previous observations: that the mantle tissue of giant clams contain high levels of CA that may assist in supplying C_i to zooxanthellae in symbiosis (Yellowlees et al., 1993; Baillie and Yellowlees, 1998), and that zooxanthellae P_{max} and $K_{0.5}$ both decrease by approximately one-half after isolation (W. Leggat, personal observation).

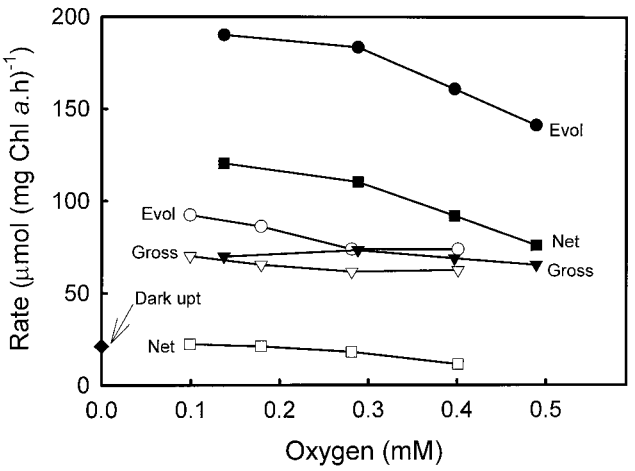

Figure 6. Photosynthetic O_2 exchange in freshly isolated *Symbiodinium* sp. in response to external O_2. The experiments were conducted as described in Figure 6 at a cell density of 4.7 μg Chl *a* mL^{-1}. Values for gross O_2 evolution (Evol), gross O_2 uptake, and net O_2 evolution are shown for each treatment, together with a value for O_2 uptake measured in the dark. O_2 responses are shown both in the presence of 1 mM added C_i (black symbols) and in the absence of added C_i (white symbols). Experiments were conducted from low to high O_2, and the O_2 concentration was increased between points by the introduction of a small bubble of $^{18}O_2$.

Freshly isolated zooxanthellae exhibit a number of characteristics of algae that possess a CCM: they are able to utilize HCO_3^- for photosynthesis (Fig. 1), they show light-stimulated CA exchange (Fig. 3), EZA decreases the affinity of photosynthesis for C_i (Figs. 3 and 5), they are able to accumulate a modest amount of internal C_i in excess of a passive accumulation (Table I), and their photosynthetic O_2 uptake shows few characteristics to suggest that there is substantial Rubisco oxygenase activity at limiting C_i (Figs. 5 and 6). These features will be discussed in further detail below.

CO_2 and HCO_3^- Uptake

Presently there is some conjecture about what form of C_i is utilized by zooxanthellae. Studies on zooxanthellae isolated from corals found that HCO_3^- was the C_i species taken up mostly by zooxanthellae (Goiran et al., 1996); however, studies on *Symbiodinium* sp. isolated from giant clams suggest that CO_2 is preferentially utilized (Yellowlees et al., 1993). Different clades of *Symbiodinium* sp. are known to populate different hosts (Rowan and Powers, 1991) and different environments within the one host (Rowan and Knowlton, 1995). This may account for observed differences in the photosynthetic characteristics of the zooxanthellae isolated from different hosts. We found that zooxanthellae from *T. gigas* do utilize CO_2 predominantly, with net CO_2 uptake supporting 45% to 80% of net photosynthesis at pH 8.0 when first isolated (Fig. 1), and the contribution increasing at higher C_i levels. The contribution of CO_2 is even more pronounced at pH 7.0 (Fig. 2). However, 2 d after isolation, net CO_2 uptake decreased to 5% to 65% over the same C_i range, while net HCO_3^- uptake increased (Fig. 1). With isolation there was also a concomitant decrease in P_{max} by 13% and $K_{0.5}$ by 23% (Fig. 1). These changes in C_i uptake and utilization would appear to be the result of the changes associated with moving from a symbiotic to a free-living lifestyle.

Light-Stimulated CA Activity

When given labeled $H^{13}C^{18}O_3^-$ as a C_i source, freshly isolated, aged, and cultured zooxanthellae displayed a light-stimulated CA activity that was inhibited by the EZA (Fig. 3). These data clearly show that light is able to stimulate the access of external C_i to internal CA activity, and is consistent with light-stimulated C_i uptake activities. Similar activities have been observed in green and non-green algae (Palmqvist et al., 1994, 1995; Badger et al., 1998) and cyanobacteria (Badger and Price, 1989) that possess CCMs.

Intracellular C_i Accumulation

The initial discovery of CCM activity in both cyanobacteria and green algae was associated with the demonstration that these cells could actively accumulate C_i in the light, and that this accumulated C_i was used to elevate internal [CO_2] around Rubisco. Thus, many attempts to demonstrate a CCM have relied on the ability to measure such accumulation in the light. The experiments conducted in this study with freshly isolated and cultured zooxanthellae (Fig. 4; Table 1) found that the internal C_i was between 1.5 and 4.6 times what would be expected if only passive diffusion of C_i into the cell was occurring. This was true at both pH 7.0 and pH 8.0. Internal C_i concentration measurements were also made for *A. carterae*, a non-symbiotic marine dinoflagellate, as a comparison. *A. carterae* was found to concentrate internal C_i approximately 25-fold more than the external C_i (Table I), which is slightly more than *Symbiodinium* sp.. These values are similar to those found by Burns and Beardall (1987) for other marine algae, in which C_{int}/C_{ext} values were between 5.5 and 8.3. Berman-Frank and associates have made the only other report of an intracellular pool for a dinoflagellate, finding that the *P. gatunense* have internal C_i levels between 7 and 80 times the external medium (Berman-Frank and Erez, 1996; Berman-Frank et al., 1998). However, this algae lives in freshwater and there were no estimates made of the intracellular pH, therefore, it is not possible to determine how much greater the C_{int} is compared with that which would be facilitated by passive diffusion.

It is surprising that marine dinoflagellates do not show larger accumulation ratios considering they possess a form II Rubisco that may have poor affinity for CO_2. The levels obtained for C_{int} were comparable to those values previously found for other marine algae that utilize a form I Rubisco (Burns and Beardall, 1987). It may be expected that dinoflagellates would have to concentrate C_i to a greater extent than other algae to overcome the limitations of a relatively inefficient Rubisco. There may be a number of explanations for this. Recently, a thylakoid CA has been found to be central to the operation of the CCM in *C. reinhardtii* (Karlsson et al., 1995, 1998; Funke et al., 1997),

and this CA has been hypothesized to use thylakoid protons to convert HCO_3^- to CO_2 (Raven, 1997). If this is the case, then models of such a CCM indicate that proton-facilitated conversion of HCO_3^- to CO_2 may actually lead to a depletion of the internal C_i pool relative to passive accumulation, although CO_2 is still elevated within a localized region (Badger et al., 1998). Another possibility is that the measured accumulation of C_i may be in a specific region such as the stroma or even a subregion of the chloroplast. Thus, the actual level of C_i may be much higher in this subregion compared to the values estimated here on a whole-cell basis.

Due to the unstable nature of the dinoflagellate enzyme (Whitney and Yellowlees, 1995) the kinetic parameters of *Symbiodinium* sp. Rubisco are not known. It is therefore difficult to accurately model the CO_2 concentrations required within the cell to achieve the photosynthetic responses observed in this report. However, some estimates can be made by taking the kinetic properties of the form II Rubisco from *Rhodospirillum rubrum* and adjusting the $K_{0.5}(CO_2)$ to achieve an S_{rel} of 37, which was the value recently obtained for *A. carterae* (Whitney and Andrews, 1998). This adjustment can be made by simply reducing the $K_{0.5}(CO_2)$ to 50 to 60 μM in the presence of 21% O_2. A $K_{0.5}(CO_2)$ of 16 μM at pH 7.0 (Fig. 2) could therefore theoretically be produced by a 3- to 4-fold concentration of external CO_2 within the cell, while at pH 8.0 a 6- to 7-fold concentration could produce a $K_{0.5}(CO_2)$ of 9 μM (Fig. 2). More careful calculations will have to await a full kinetic characterization of dinoflagellate Rubisco.

Photosynthetic O_2 Uptake

The photosynthetic O_2 uptake displays characteristics that are not consistent with the presence of a large Rubisco oxygenase activity in these cells. Although there was considerable O_2 uptake capacity, representing some 35% to 45% of maximum O_2 evolution, it was not stimulated by increasing O_2 and was inhibited by limiting C_i concentrations (Figs. 5 and 6). The stimulation of O_2 uptake by increasing C_i may be interpreted as being due to activation of Rubisco by increasing internal CO_2, as seen with higher plants (Canvin et al., 1980), but the insensitivity to O_2 is not readily explained. Based on other form II enzymes and cyanobacterial form I Rubiscos with low S_{rel} values (Badger et al., 1998), it may be expected that the oxygenase activity from zooxanthellae may display a low affinity for O_2, thus exhibiting low oxygenase activity at ambient levels of O_2. The saturation of O_2 uptake by 0.1 mM O_2 at both low and high C_i concentrations is inconsistent with this and is more similar to an O_2 uptake reaction coupled to photosynthetic electron transport, such as the Mehler reaction (Badger, 1985). The photosynthetic O_2 uptake process in zooxanthellae requires further study before an adequate explanation is forthcoming.

A Zooxanthella CCM

Currently, the function of the pyrenoid in eukaryotic algae is not understood, but it has been hypothesized that it may play a role in CO_2 elevation, similar to carboxysomes in cyanobacteria. This hypothesis is based on changes in pyrenoid morphology when cells are transferred from high to low C_i conditions (Ramazanov et al., 1994), localization of Rubisco to the pyrenoid (Osafune et al., 1990), and a general correlation between the presence of pyrenoids and CCMs (Badger et al., 1998). All of these data suggest that the pyrenoid plays some role in C_i accumulation. Recently, electron microscopic examination has found that *Symbiodinium* sp. also has Rubisco localized within the pyrenoid (D. Yellowlees, personal communication); therefore, it is reasonable to hypothesis that zooxanthellal pyrenoids may also play a role in C_i accumulation.

Recent evidence and speculation suggest that there may be considerable diversity in the mechanistic operation of CCMs in algae. This diversity may include the extent to which a thylakoid-generated proton supply is coupled to the dehydration of HCO_3^-, as well as the need for a pyrenoid and pyrenoid-located CA to be present (see Raven, 1997; Badger et al., 1998). The evidence presented for a zooxanthella CCM in the present study indicates the presence of active C_i transport, a dependence on internal CA for efficient photosynthesis, and a suppression of photorespiratory O_2 uptake, as well as a well-developed pyrenoid. However, we found no substantial C_i accumulation. Such considerations would favor the operation of a CCM in which C_i is elevated only in a very localized region and/or protons are used to aid conversion of HCO_3^- to CO_2, leading to a depressed internal HCO_3^- concentration. Obviously, further research must be conducted to determine what type of CCM *Symbiodinium* sp. possesses. This includes localization of intracellular CA and further study on the characteristics of dinoflagellate Rubisco. This is currently hindered by the extremely unstable nature of the Rubisco after isolation (Whitney and Yellowlees, 1995). Investigations into zooxanthellae isolated from different hosts may also provide further information about zooxanthellae C_i accumulation.

ACKNOWLEDGMENT

We would like to thank G.D. Price for his expertise in analysis of data from the mass spectrometer and his helpful comments.

Received May 3, 1999; accepted September 4, 1999.

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