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PHOTOSYNTHETIC UTILIZATION OF INORGANIC CARBON IN THE ECONOMIC BROWN ALGA, *HIZIKIA FUSIFORME* (SARGASSACEAE) FROM THE SOUTH CHINA SEA[1]

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The mechanism of inorganic carbon (C_i) acquisition by the economic brown macroalga, *Hizikia fusiforme* (Harv.) Okamura (Sargassaceae), was investigated to characterize its photosynthetic physiology. Both intracellular and extracellular carbonic anhydrase (CA) were detected, with the external CA activity accounting for about 5% of the total. *Hizikia fusiforme* showed higher rates of photosynthetic oxygen evolution at alkaline pH than those theoretically derived from the rates of uncatalyzed CO_2 production from bicarbonate and exhibited a high pH compensation point (pH 9.66). The external CA inhibitor, acetazolamide, significantly depressed the photosynthetic oxygen evolution, whereas the anion-exchanger inhibitor 4,4′-diisothiocyano-stilbene-2,2′-disulfonate had no inhibitory effect on it, implying the alga was capable of using HCO_3^- as a source of C_i for its photosynthesis via the mediation of the external CA. CO_2 concentrations in the culture media affected its photosynthetic properties. A high level of CO_2 (10,000 ppmv) resulted in a decrease in the external CA activity; however, a low CO_2 level (20 ppmv) led to no changes in the external CA activity but raised the intracellular CA activity. Parallel to the reduction in the external CA activity at the high CO_2 was a reduction in the photosynthetic CO_2 affinity. Decreased activity of the external CA in the high CO_2 grown samples led to reduced sensitiveness of photosynthesis to the addition of acetazolamide at alkaline pH. It was clearly indicated that *H. fusiforme*, which showed CO_2-limited photosynthesis with the half-saturating concentration of C_i exceeding that of seawater, did not operate active HCO_3^- uptake but used it via the extracellular CA for its photosynthetic carbon fixation.

Key index words: **brown alga; carbonic anhydrase; CO_2; inorganic carbon; *Hizikia fusiforme*; marine macroalgae; photosynthesis**

Abbreviations: **AZ, acetazolamide; CA, carbonic anhydrase; C_i, inorganic carbon; DIDS, 4,4′-diisothiocyano-stilbene-2,2′-disulfonate; FW, fresh weight**

The dissolved gaseous CO_2, $[CO_2]_{aq}$, is only about 12 μM, being less than 1% of HCO_3^- in the air-equilibrated seawater (20° C, pH 8.2, salinity 35 psu). Photosynthesis of marine algae might be CO_2-limited because it diffuses slowly in water, being about 7000 times slower than in air. The photosynthetic oxygen evolution rates of many marine macroalgae, however, have been found to be faster than the theoretical maximum rates of CO_2 supply from the uncatalyzed spontaneous dehydration of HCO_3^- in seawater, suggesting the existence of photosynthetic utilization of HCO_3^- (Gao and McKinley 1994, Raven 1997). Although CO_2 can easily pass through biological membranes when there is a gradient in concentration, the ionic HCO_3^- cannot unless actively transported by some facilitating mechanisms (Beer 1994, Axelsson et al. 1995, Raven 1997, Raven and Brownlee 2001). Two basic processes have been proposed for HCO_3^- utilization by marine macroalgae. The first involves the catalysis of HCO_3^- dehydration by extracellular carbonic anhydrase (CA), supplying CO_2 to the cells, which could be suggested from the more or less complete inhibition of photosynthesis by the membrane impermeable CA inhibitor, acetazolamide (AZ) (Surif and Raven 1989, Haglund et al. 1992a,b, Johnston et al. 1992, Flores-Moya and Fernandez 1998, Mercado and Niell 1999). The second process comprises direct uptake of HCO_3^- across the plasma membrane facilitated by anion exchange proteins. It is accordingly insensitive to AZ and almost totally inhibited by the anion exchange inhibitor 4,4′-diisothiocyano-stilbene-2,2′-disulfonate (DIDS) (Axelsson et al. 1995, 1999, Larsson et al. 1997, Andría et al. 1999). Recently, the

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external CA-catalyzed conversion of HCO_3^- to CO_2 was suggested to be facilitated in the plasma membrane by a P-type H^+-ATPase (proton pump) in the brown algae *Laminaria saccharina* (Axelsson et al. 2000) and *L. digitata* (Klenell et al. 2002), the red alga *Coccotylus truncates* (Snoeijs et al. 2002), and the green alga *Cladophora glomerata* (Choo et al. 2002). The P-type H^+-ATPase was proposed (Schmid et al. 1996) and proved to be activated by blue light in *Laminaria* spp. (Klenell et al. 2002).

Hizikia fusiforme (Harv.) Okamura (Sargassaceae, Phaeophyta) is an economically important species in China, Korea, and Japan, being cultivated and used for food and alginate generation. Although its reproductive biology (Park et al. 1995, Hwang et al. 1999, Ruan and Xu 2001) and cultivation techniques (Hwang et al. 1997, Li 2001) have been studied, the photosynthetic physiology of *H. fusiforme* is poorly understood. The present work aims to clarify the mechanism of inorganic carbon (C_i) acquisition by this alga and how its plasticity may affect the use of C_i sources for photosynthesis.

MATERIALS AND METHODS

Material and culture conditions. *Hizikia fusiforme* (Harv.) Okamura was collected from lower intertidal rock during low tide in February and March 2002 along the coast of Nanao, Shantou, China (23°20′N, 116°55′E). The thalli were cleaned of visible epiphytes and attached sediments. Only the individuals without receptacles were selected and were transported to the laboratory in an insulated cooler (5° C) within 4 h. The samples were maintained for 3 days in a glass aquarium containing filtered natural seawater (salinity 33 psu) enriched with 45 μM $NaNO_3$ and 2.5 μM NaH_2PO_4 before culture in CO_2 chambers (Conviron, EF7, Winnipeg, Manitoba, Canada) at 20° C and 150 μmol photons $\cdot m^{-2} \cdot s^{-1}$ (fluorescent illumination, 12:12-h light:dark cycle) with 20 (low), 360 (ambient), and 10,000 (high) ppmv CO_2 in aeration. The low CO_2 level was obtained by passing ambient air through 5 M NaOH solution, and the high CO_2 concentration was maintained with CO_2-enriched air by mixing pure CO_2 and ambient air. The seawater was renewed by 50% every other day. The samples were grown with the varied levels of CO_2 for 6–8 days before being used for experiments.

Assay of CA activity. The CA activity was assayed by the potentiometric method according to Haglund et al. (1992a). The time required for a drop of 0.4 pH units was measured at 2° C, using a cuvette containing 6 mL buffer (40 mM Tris, pH 8.4, 5 mM EDTA-Na, 25 mM isoascorbic acid, 25 mM mercaptoethanol). Thalli of about 0.25 g fresh weight (FW) were cut into segments of about 0.8-cm length with a sharp razor blade and were washed two times with the buffer before placed in the cuvette. The reaction was initiated by injecting 1 mL CO_2-saturated distilled water (2° C). Total CA activity was determined as the activity in the crude extract of about 50 mg fresh samples homogenized in the buffer. The enzyme activity was estimated as follows: $EU = 10 \times (T_b/T_e - 1)$, where T_b and T_e are the times in seconds for the pH drop without and with the algal sample, respectively.

pH-drift experiment. A total of 1.0 g FW of *H. fusiforme* thalli was immersed in 20 mL filtered seawater in sealed glass vials. The vials were then maintained in an incubator at 145 μmol photons $\cdot m^{-2} \cdot s^{-1}$ and 16° C. The pH changes in the seawater were recorded with a pH meter (420A, Orion, Boston, MA, USA). The pH compensation point was determined as the point where pH no longer increased (Maberly 1990).

Photosynthetic measurements. CO_2-free seawater was prepared according to Gao et al. (1993). The biological buffers (Sigma, St. Louis, MO, USA) were used as final concentrations of 20 mM to adjust the pH in addition to using 0.5 M NaOH and HCl. Varied pH levels were obtained with MES (pH 6.5), HEPES (pH 7.5), Tris (pH 8.2 and 9.0), and CAPS (pH 10.0). Although Tris buffer has been shown to inhibit the photosynthetic carbon uptake by the marine brown macroalga *Laminaria saccharina* (Axelsson et al. 2000) and the seagrass *Zostera marina* (Hellblom et al. 2001), it did not affect the photosynthetic O_2 evolution of *H. fusiforme* (Table 1).

Photosynthetic rates were measured as O_2 evolution by using a Clark-type oxygen electrode (YSI model 5300, Yellow Spring, OH, USA) at 600 μmol photons $\cdot m^{-2} \cdot s^{-1}$ and 20° C. The irradiance was provided by a halogen lamp, and the temperature was maintained constant by using a cooling circulator (Cole Parmer, Chicago, IL, USA). Segments of *H. fusiforme* were incubated in seawater at 100 μmol photons $\cdot m^{-2} \cdot s^{-1}$ and 20° C for at least 1 h before the measurement in an attempt to minimize the effect of cutting. About 0.3 g of fresh samples were transferred to the O_2 electrode chamber containing 8 mL of the CO_2-free seawater, which was magnetically stirred. Samples were allowed to photosynthesize to deplete the remaining C_i in the cells and the bulk medium until no oxygen evolution was observed. A known quantity of 200 mM $NaHCO_3$ solution was injected into the chamber to obtain the desired levels of C_i.

AZ (Sigma) was used as an inhibitor of extracellular CA (Haglund et al. 1992a,b), and DIDS (Sigma) was used to inhibit the direct uptake of HCO_3^- by the algal cells (Axelsson et al. 1995, 1999). AZ or DIDS solutions were added into the chamber to final concentrations of 200 and 400 μM, respectively. Stock solution of AZ (50 mM) was prepared in 0.05 M NaOH and that of DIDS (50 mM) by dissolving it into distilled water.

Calculation of the maximum rate of CO_2 supply from uncatalyzed HCO_3^- dehydration. The theoretical maximum rates of CO_2 production derived from spontaneous (uncatalyzed) dehydration of HCO_3^- in the seawater were calculated according to Miller and Colman (1980) and Matsuda et al. (2001), provided that the alga consumed bulk CO_2 at a rate causing it to approach zero. The rate of such a conversion ($d[CO_2]/dt$) could be described by the following equations:

$$d[CO_2]/dt = K_1 \times [DIC]/A + (K_3 \times [DIC] \times [H^+]/K_{H_2CO_3})/A,$$

$$A = 1 + [H^+]/K_1 + K_2/[H^+],$$

where K_1 and K_3 are the rate constants for the reactions $HCO_3^- \rightarrow CO_2 + OH^-$ and $H_2CO_3 \rightarrow CO_2 + H_2O$, respectively. $K_{H_2CO_3}$ and K_2 correspondingly represent the coeffi-

TABLE 1. Net photosynthetic rates (μmol $O_2 \cdot g^{-1}$ FW $\cdot h^{-1}$) of *Hizikia fusiforme* in natural seawater containing 0.55 or 2.2 mM C_i after addition of Tris buffer (final concentration 20 mM).

	Control	Tris buffer
pH 8.2		
0.55 mM C_i	7.7 ± 0.8	8.0 ± 1.5
2.2 mM C_i	21.8 ± 2.8	23.4 ± 3.2
pH 9.0		
2.2 mM C_i	12.3 ± 2.5	11.5 ± 1.1

Values are means ± SD ($n = 3$).

cients for the reactions $H^+ + HCO_3^- \leftarrow\rightarrow H_2CO_3$ and $H^+ + CO_3^{2-} \leftarrow\rightarrow HCO_3^-$.

Statistics. The data were expressed as means ± SD. Statistical significance of the data was tested with analysis of variance or *t*-test, with the significant level set at 0.05.

RESULTS

CA activities were detected potentiometrically with both the thalli and homogenates of *H. fusiforme* grown at 20 (low), 360 (ambient), and 10,000 (high) ppmv CO_2 in aeration (Table 2), indicating the presence of both extracellular and intracellular CA regardless of the CO_2 background in culture. However, the extracellular and intracellular CA activities were reduced by about 60% in the samples grown at high CO_2 compared with at low CO_2.

The rates of measured photosynthetic oxygen evolution exceeded those of theoretical maximum CO_2 supply derived from uncatalyzed dehydration of HCO_3^- at different levels of pH in seawater containing either 1.0 (Fig. 1) or 2.0 mM C_i (Table 3), regardless of the CO_2 concentrations in culture. In the presence of AZ, with the extracellular CA being inhibited, the theoretical rates of CO_2 supply exceeded the photosynthetic CO_2 demands at pH 8.2 but not at pH 9.0 (Table 3). The pH in the unbuffered seawater in a sealed vial with *H. fusiforme* grown at ambient CO_2 level increased with the time of incubation to reach a compensation point of 9.66 in 6 h (Fig. 2).

The rates of photosynthetic oxygen evolution by the low, ambient, and high CO_2-grown *H. fusiforme* were determined in pH-buffered seawater containing 2.0 mM C_i with or without the addition of the inhibitors AZ and DIDS (Fig. 3). The lack of sensitivity of O_2 evolution to Tris buffer (Table 1) provided the actual photosynthetic rates of *H. fusiforme* measured here. Oxygen evolution rates were significantly ($P < 0.05$) reduced by the rise of pH from 8.2 to 9.0, irrespective of the CO_2 levels in culture. Net photosynthetic rates were higher with the low CO_2 but lower with the high CO_2 grown samples, compared with those of the ambient CO_2 grown samples at pH 8.2 as well as pH 9.0. AZ had pronounced inhibitory effects on the rates at both pH 8.2 and 9.0 despite the background CO_2 levels. The inhibition was highest in the low CO_2 grown and lowest in the high CO_2 grown samples. Addition of DIDS did not affect ($P > 0.1$) the photosynthetic rate irrespective of the CO_2 concentrations in culture (Fig. 3). Moreover, no effect by AZ or DIDS was found at pH 6.5 for all the samples grown at the varied CO_2 levels.

TABLE 2. Relative enzyme activities (REA) of extracellular and total CA in *Hizikia fusiforme* thalli grown at varied CO_2 concentrations.

CO_2 (ppmv) in culture	External CA (REA · g^{-1} FW)	Total CA (REA · g^{-1} FW)	External CA as a percentage of total CA
20	25.1 ± 9.0[a]	526.6 ± 51.5[a]	4.6
360	17.3 ± 3.3[a]	344.2 ± 95.9[b]	4.9
10,000	9.5 ± 2.1[b]	233.3 ± 88.0[b]	4.1

Values are means ± SD ($n = 6$).

[a,b] Different superscripts in the same row are significantly different ($P < 0.05$).

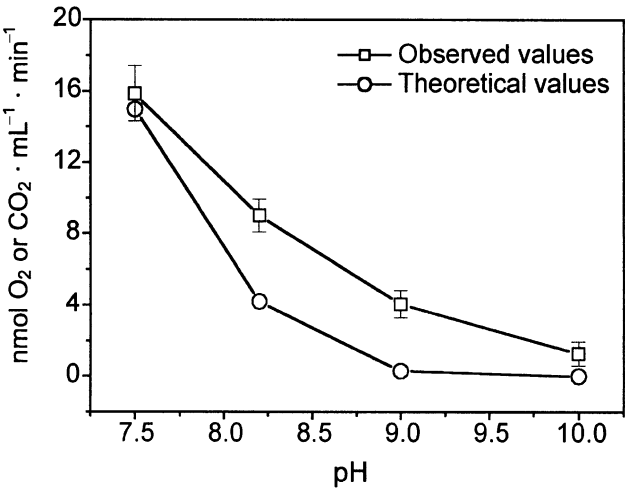

FIG. 1. Observed rates of photosynthetic oxygen evolution by *Hizikia fusiforme* and the theoretical maximum rates of CO_2 supply derived from uncatalyzed dehydration of HCO_3^- as a function of pH in the seawater containing 1.0 mM C_i. Vertical bars represent ± SD ($n = 6$).

When the samples grown at the varied CO_2 levels were incubated in the seawater of varied C_i concentrations at pH 6.5, their photosynthetic O_2 evolution appeared to be saturated at 1.1 mM C_i (Fig. 4). The apparent half-saturation constant was not significantly ($P > 0.05$) affected by the CO_2 levels in culture, but the C_i-saturated photosynthetic rate was markedly reduced in the samples grown at the high CO_2 (Table 4). When incubated at pH 8.2, a condition of lower CO_2 supply compared with that at pH 6.5, net photosynthesis appeared to be saturated at 4.4 mM C_i in the low CO_2 and at higher C_i concentrations in the ambient and high CO_2 grown samples (Fig. 4). Additionally, the high CO_2 grown samples showed lower O_2 evolution rates as well as lower apparent affinities for C_i compared with those grown at ambient and low CO_2 levels (Table 4).

DISCUSSION

Comparison of the observed photosynthetic rates with the theoretical rates of CO_2 supply, together with the pH-drift pattern, indicated that *H. fusiforme* could use HCO_3^- as a source of C_i for photosynthesis, as reported in many other marine macroalgae (Axelsson and Uusitalo 1988, Surif and Raven 1989, Maberly 1990, Haglund et al. 1992a,b, Johnston et al. 1992). It has been found that direct HCO_3^- uptake could be facilitated by a mechanism with similar properties to the red blood cells anion exchanger (AE1; Drechsler et al. 1993, 1994), such as being inhibited by DIDS. A DIDS-sensitive mechanism had been reported in *Ulva* spp. (Drechsler et al. 1993, 1994, Axelsson et al. 1995,

TABLE 3. Ratios of the measured rates of photosynthetic O_2 evolution (nmol $O_2 \cdot mL^{-1} \cdot min^{-1}$) to the theoretical rates of CO_2 supply derived form uncatalyzed dehydration of HCO_3^- at pH 8.2 and 9.0 in the seawater containing 2.0 mM Ci for *Hizikia fusiforme* grown with 20, 360, and 10,000 ppmv CO_2.

	pH 8.2		pH 9.0	
CO_2 (ppmv) in culture	Control	+AZ	Control	+AZ
20	2.35 ± 0.11^a	0.65 ± 0.11^a	16.01 ± 0.70^a	2.51 ± 0.39^a
360	2.01 ± 0.18^b	0.61 ± 0.04^a	12.14 ± 1.11^b	2.61 ± 0.08^a
10,000	1.21 ± 0.24^c	0.63 ± 0.12^a	7.30 ± 1.57^c	2.00 ± 0.54^a

The theoretical values were calculated by assuming that the whole volume of the bathing medium was available for uncatalyzed conversion of HCO_3^-. The concentration of AZ was 200 μM. Values are means ±SD ($n = 6$).
[a,b,c] Different superscripts in the same column are significantly different ($P < 0.05$).

1999) and *Enteromorpha intestinalis* (Larsson et al. 1997). Andría et al. (1999) first reported the presence of a direct HCO_3^- transport via a DIDS-sensitive mechanism in a red macroalga, *Gracilaria gaditana* nom. prov. However, in the present study, *H. fusiforme* appeared to lack such a DIDS-sensitive mechanism, because no photosynthetic inhibition by DIDS at all the pH levels tested was recognized. Additionally, a decrease in photosynthetic O_2 evolution with an increase of pH in this species was also an indication for a lack of direct HCO_3^- uptake, as suggested by Axelsson et al. (1995), Mercado et al. (1998), and Mercado and Niell (1999).

Both external and internal CA activities in *H. fusiforme* could be detected by the potentiometric method. The external CA activity in *H. fusiforme* represented about 5% of the total CA, a value comparable with that found in the red macroalgae *Chondrus cripus* (Smith and Bidwell 1989), *Himanthalia elongata*, and *Ceraminnm rubrum* (Giordano and Maberly 1989) and in the brown algae *Fucus serratus* and *F. vesiculosus* (Surif and Raven 1989). The extracellular CA inhibitor, AZ, inhibited net photosynthesis of *H. fusiforme* at alkaline pH (8.2 and 9.0) but not at acidic pH (6.5). Moreover, the effect of pH became more obvious when AZ was added. Those results implied that the external CA played an important role in HCO_3^- utilization at alkaline pH, because it acts on the dehydration of HCO_3^- to CO_2 before its transport across the plasmalemma. However, a higher net photosynthesis at a lower pH at a limiting C_i level indicated that *H. fusiforme* had a higher affinity for CO_2 than for HCO_3^- ions.

CO_2 concentrations in culture played an important role in regulating the photosynthetic characteristics of *H. fusiforme*. When CO_2 level was raised in culture, CO_2 affinity for photosynthesis was greatly lowered. At the same time, the external CA activity was considerably reduced when *H. fusiforme* was grown at the higher CO_2 level. Decreased activity of the external CA in the higher CO_2 culture resulted in the insensitiveness of

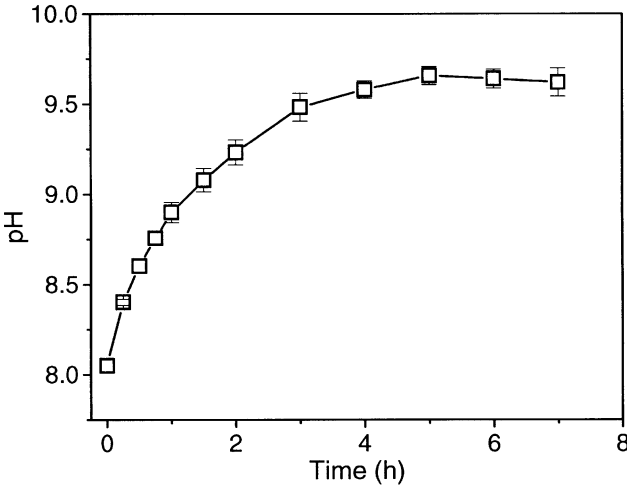

FIG. 2. The pH-drift curve for *Hizikia fusiforme* in natural seawater. Vertical bars represent ±SD ($n = 6$).

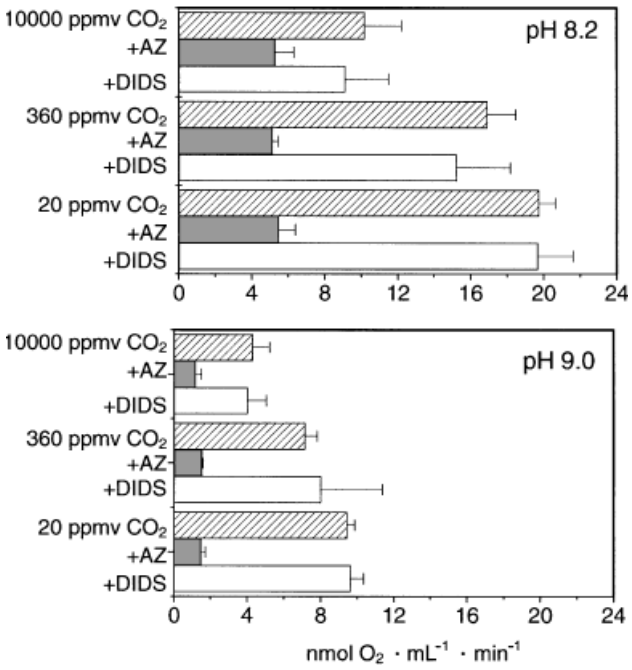

FIG. 3. Rates of photosynthetic oxygen evolution at pH 8.2 and 9.0 in the seawater of 2.0 mM C_i with addition of AZ or DIDS in *Hizikia fusiforme* grown with 20, 360, and 10,000 ppmv CO_2 in aeration. Horizontal bars represent ±SD ($n = 6$).

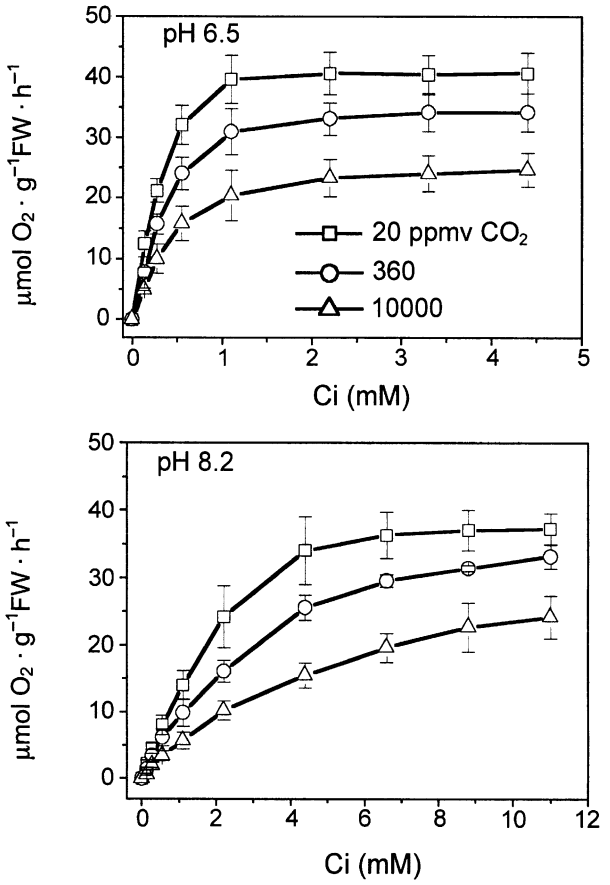

FIG. 4. Rates of photosynthetic oxygen evolution as a function of C_i concentrations in *Hizikia fusiforme* thalli grown with (□) 20, (○) 360, and (△) 10,000 ppmv CO_2. The seawater was buffered at pH 6.5 and 8.2. Vertical bars represent ±SD ($n = 6$).

photosynthesis to the addition of AZ at alkaline pH. The involvement of external CA activity in HCO_3^- utilization during photosynthesis has been shown for a great number of macroalgae (Mercado et al. 1997, 1998, Raven 1997) and is also confirmed here for the commercially important *H. fusiforme*. Regulation of CA activity with a clear connection with HCO_3^- utilization capacity has so far only been demonstrated in a few species, including *Fucus serratus* (Johnston and Raven 1990), *Ulva* spp. (Björk et al. 1993), and *Gracilaria tenuistipitata* (Garcia-Sanchez et al. 1994). Mercado et al. (1997) reported no reduction in external CA activity in the red alga *Porphyra leucosticta* when cultured with elevated C_i, though its activity was increased at a lowered C_i level. No evident relationship was observed between external CA activity and the capacity of HCO_3^- utilization in this alga, which was interpreted as the occurrence of a CO_2 transporter that worked in association with the external CA activity. In the present work, active CO_2-transport mechanism (CO_2-pump) in *H. fusiforme* was unlikely present, because the HCO_3^- utilization was closely associated with the external CA activity. If photosynthesis depended on both the CO_2 gradient created by the external CA activity and the CO_2 diffusive entries, then the energy cost could be lower than if only an active CO_2 pump was operating (Mercado et al. 1997). In the present study, net photosynthesis of *H. fusiforme* was not enhanced at the acidic pH when it was not C_i limited, which is suggestive of the absence of active CO_2-transport mechanism. Therefore, it appeared to depend on CO_2 diffusive entry driven by the external CA-mediated CO_2 gradient, as suggested in *Chondrus crispus* (Smith and Bidwell 1989), *Fucus serratus* (Haglund et al. 1992b), and *Bostrychia scorpioides* (Mercado and Niell 1999).

It seems that the ability of macroalgae to use HCO_3^- differ according to their zonations (Axelsson and Uusitalo 1988, Maberly 1990, Raven and Osmond 1992, Mercado et al. 1998). Surif and Raven (1989) reported that photosynthesis of eulittoral *Fucus* spp. tested was essentially saturated at the C_i level of seawater, whereas the normally submersed *Halidrys siliquosa*, *Alaria esculenta*, and *Laminaria spp*. were only about half C_i saturated in the seawater. *Hizikia fusiforme* is distributed at a lower intertidal zone; its photosynthesis was demonstrated in the present work to be C_i limited, with low C_i affinity (the half-saturating C_i level

TABLE 4. C_i-saturated rates of photosynthetic O_2 evolution (V_{max}) and apparent half-saturating C_i concentration ($K_{1/2}$) at pH 6.5 and 8.2 in *Hizikia fusiforme* grown at 20, 360, and 10,000 ppmv CO_2.

	CO_2 (ppmv) in culture		
	20	360	10,000
pH 6.5			
V_{max}(μmol · O_2 g^{-1} FW · h^{-1})	50.1 ± 3.7[a]	44.2 ± 2.0[a]	30.0 ± 2.1[b]
$K_{1/2}(C_i)$ (mM)	0.41 ± 0.07[a]	0.55 ± 0.12[a]	0.59 ± 0.15[a]
$K_{1/2}(CO_2)$ (μM)	93.9 ± 16.0[a]	126.0 ± 27.5[a]	135.1 ± 34.4[a]
pH 8.2			
V_{max}(μmol · O_2 g^{-1} FW · h^{-1})	58.5 ± 4.1[a]	69.3 ± 16.2[a]	26.4 ± 5.0[b]
$K_{1/2}(C_i)$ (mM)	1.62 ± 0.26[a]	2.28 ± 0.26[b]	3.06 ± 0.24[c]
$K_{1/2}(CO_2)$ (μM)	8.4 ± 1.4[a]	11.9 ± 1.4[b]	15.9 ± 1.2[c]

Values are means ±SD ($n = 6$).
[a,b,c] Different superscripts in the same row are significantly different ($P < 0.05$).

exceeded that of seawater), albeit it was capable of using HCO_3^- in seawater.

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