Abstract. The capacity for HCO$_3^-$ use by Porphyra leucosticta Thur. in Le Jolis grown at different concentrations of inorganic carbon (C$_i$) was investigated. The use of HCO$_3^-$ at alkaline pH by P. leucosticta was demonstrated by comparing the O$_2$ evolution rates measured with the O$_2$ evolution rates theoretically supported by the CO$_2$ spontaneously formed from HCO$_3^-$). Both external and internal carbonic anhydrase (CA; EC 4.2.1.1) were implied in HCO$_3^-$ use during photosynthesis because O$_2$ evolution rates and the increasing pH during photosynthesis were inhibited in the presence of azetazolamide and ethoxyzolamide (inhibitors for external and total CA respectively). Both external and internal CA were regulated by the C$_i$ level at which the algae were grown. A high C$_i$ level produced a reduction in total CA activity and a low C$_i$ level produced an increase in total CA activity. In contrast, external CA was increased at low C$_i$ although it was not affected at high C$_i$. Parallel to the reduction in total CA activity at high C$_i$ is a reduction in the affinity for C$_i$, as estimated from photosynthesis versus C$_i$ curves, was found. However, there was no evident relationship between external CA activity and the capacity for HCO$_3^-$ use because the presence of external CA became redundant when P. leucosticta was cultivated at high C$_i$. Our results suggest that the system for HCO$_3^-$ use in P. leucosticta is composed of different elements that can be activated or inactivated separately. Two complementary hypotheses are postulated: (i) internal CA is an absolute requirement for a functioning C$_i$-accumulation mechanism; (ii) there is a CO$_2$ transporter that works in association with external CA.

Key words: Carbonic anhydrase – Inorganic carbon – Macroalga – pH – Photosynthesis – Porphyra

Introduction

The total concentration of inorganic carbon (C$_i$) in natural seawater is approx. 2.2 mM. At equilibrium with air, CO$_2$ represents 8% and HCO$_3^-$ 92% of the total C$_i$ in seawater. The CO$_2$ diffusion rate in seawater is low and about 10,000 times lower than in air. Since the diffusion rate is so low, the occurrence of purely diffusive entry of CO$_2$ into the cell in aquatic plants imposes constraints on the in-vivo rate of photosynthesis as a function of external CO$_2$ concentration, taking into account the quantity and kinetics of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). In fact, it has been shown that the photosynthetic rates of various macroalgae are not saturated at the C$_i$ concentration of seawater (Surif and Raven 1989). However, many another macroalgae, including the red macroalgae, show low rates of photorepiration and very high affinity for C$_i$, probably because of the presence of a carbon-concentrating mechanism (CCM; Johnston and Raven 1987). It has been suggested that macroalgal CCMs can be biochemical, as in terrestrial C$_4$ plants (Reiskind and Bowes 1991) and/or biophysical (by means of active transport of C$_i$).

Carbon dioxide is the only source of C$_i$ for some macroalgae (Holbrook et al. 1988; Surif and Raven 1989; Maberly 1990). However, the O$_2$ evolution rates of many other macroalgae in seawater are higher than the maximal rates that theoretically could be supported by the CO$_2$ formed in the medium by uncatalyzed spontaneous dehydration of HCO$_3^-$). The use of HCO$_3^-$ by these macroalgae has been suggested. Several mechanisms of HCO$_3^-$ incorporation have been proposed: CO$_2$ uptake dependent on a dehydration of HCO$_3^-$ catalyzed by an external carbonic anhydrase (CA; EC 4.2.1.1), ATPase-dependent HCO$_3^-$ transport, and H$^+$/HCO$_3^-$ co-transport and OH$^-$/HCO$_3^-$ antiport systems (Lucas 1983).

The Rhodophyta cover a large range in their ability to use HCO$_3^-$ from seawater. A small fraction of the marine red macroalgae examined by Johnston et al. (1992) and Maberly (1990) appeared unable to use HCO$_3^-$; most of
these algae, which apparently use only CO\textsubscript{2}, are subtidal. Cook et al. (1986) found that HCO\textsubscript{3} entry is necessary to explain the rate of photosynthesis by 15 species of marine red algae, based on photosynthesis at a faster rate than uncatalysed conversion of HCO\textsubscript{3} to CO\textsubscript{2} in the medium and the absence of external CA; however, these results do not conflict for any species with those of Johnston et al. (1992) and Maberly (1990). The presence of an external CA involved in C\textsubscript{i} uptake has been described in other algae of this group (Giordano and Maberly 1989; Surif and Raven 1989; Haglund et al. 1992a; Süttemeyer et al. 1993), including Porphyra sp., the apparently most efficient HCO\textsubscript{3} -user among Rodophyceae (Axelsson et al. 1991).

The aim of this paper is to describe the role of CA in carbon assimilation by the red alga Porphyra leucosticta. The affinity for HCO\textsubscript{3} and effect of the inhibitors of CA when the plants are exposed to low and high C\textsubscript{i} concentrations are described.

Materials and methods

Plant material. Porphyra leucosticta Thur. in Le Jolis was collected in the supralittoral zone near Lagos (Málaga, Southern Spain) during winter 1995. It was maintained in natural, nutrient-poor seawater aerated vigorously (about 3 L air min\textsuperscript{-1}) at 15 °C under white fluorescent lamps (F20W; CW Osram, Munich, Germany), at 12 h light per day and a photon fluence rate of 60 \textmu mol m\textsuperscript{-2} s\textsuperscript{-1} (Figueroa et al. 1995). The photon fluence rate was determined by means of a quantum spherical PAR sensor (193SB, Li-Cor, Lincoln, Neb., USA) connected to a radiometer (Li-1000; Licor).

Experimental design. P. leucosticta was cultivated at high, normal and low C\textsubscript{i} concentrations for 7–8 d. Discs (9 mm in diameter) taken from the thallus were cultured in Plexiglas cylinders containing 3 L of seawater enriched with Provasoli medium (Starr and Zeikus 1987) and buffered with Tris 50 mM. For each culture 1.2–1.5 g of alga was used. The different C\textsubscript{i} concentrations in the cultures were obtained by bubbling air with different CO\textsubscript{2} concentrations. In the control culture (normal C\textsubscript{i}), seawater was aerated with air from outside (0.035% CO\textsubscript{2}). In the high-C\textsubscript{i} treatment, seawater was bubbled with air enriched with 1% CO\textsubscript{2}. In the low-C\textsubscript{i} treatment, air was passed through a 5 N KOH solution (final CO\textsubscript{2} concentration in air less than 0.0001%). Light and temperature conditions were as described above. Duplicate experiments gave similar results.

Oxygen evolution and pH change. Oxygen evolution was measured in small (8–9 ml) temperature-controlled (15–16 °C) seawater chambers containing oxygen electrodes, at an irradiance of 250 \textmu mol photons m\textsuperscript{-2} s\textsuperscript{-1}. Chambers were equipped with an oxygen probe (522; Yellow Springs, Ohio, USA). The suitable agitation of the medium in the chamber was obtained by a magnetic stirrer. Ten P. leucosticta discs were transferred to the oxygen-evolution chamber containing C\textsubscript{i}-free synthetic seawater medium buffered to a pH of 5.6, 8.7 or 9.2 with 50 mM buffered (final concentration). Biological buffers 2-(N-morpholino)ethanesulfonic acid (Mes) and Tris, respectively, were used. After a zero net O\textsubscript{2} exchange rate, different amounts of 200 mM NaHCO\textsubscript{3} solution were injected into the chambers in order to create different concentrations of C\textsubscript{i}. Evolution of O\textsubscript{2} was recorded for 10–15 min. Values for I\textsubscript{m} and V\textsubscript{max} were estimated from the fit to the Michaelis-Menten equation using the KaleidaGraph program (Abelbeck Software, USA). The goodness of fit was tested by using least-squares regression analysis.

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Photosynthetic conductance (g\textsubscript{p}) based on the concentration of C\textsubscript{i} was calculated as the initial slope of C\textsubscript{i} saturation curves (Johnston et al. 1992). According to Figueroa et al. (1995), a value of 5.91 for the fresh weight per unit surface area relationship (mg FW per cm\textsuperscript{2}) was used in calculating the O\textsubscript{2} evolution rate per surface unit. Chlorophyll \textalpha was extracted in N,N-dimethylformamide (Inskeep and Bloom 1985).

pH change during photosynthesis was recorded in the oxygen-evolution chamber by fitting a pH electrode (Ingold micropH 2001; Crison Instruments S.A., Barcelona, Spain) equipped with pH-meter (micropH 2001; Crison Instruments S.A.). Oxygen evolution and pH change were measured simultaneously. The photosynthetic quotient (PQ) was estimated according to Axelsson (1988).

In order to obtain the pH compensation point, pH-drift experiments were conducted in the photosynthesis chambers: pH was measured until there was not further increase (after about 6 h).

The maximum rate of dehydration of HCO\textsubscript{3} to CO\textsubscript{2} was calculated for a given salinity and temperature and pH according to Johnson (1982). The apparent dissociation constants of carbonic acid in seawater were taken from Riley and Chester (1977); K\textsubscript{1} and K\textsubscript{2} at 15 °C were 9.12·10\textsuperscript{-7} and 6.17·10\textsuperscript{-10} respectively. Theoretical O\textsubscript{2} production was calculated assuming that algae consumed CO\textsubscript{2} at a rate causing the CO\textsubscript{2} concentration to approach zero. Values for PQ estimated from the simultaneous measurements of change of pH and O\textsubscript{2} evolution were used.

Preparation of inhibitors and assay of CA (EC 4.2.1.1) in discs. 6-Ethoxyazolamide (EZ) and azetazolamide (AZ) were used for these experiments (Sigma-Aldrich Química S.A., Madrid, Spain). It is generally assumed that AZ cannot penetrate into the cell and only inhibits the extracellular CA. The EZ penetrates into the cell and inhibits external and internal CA. Carbon dioxide-free stock solutions of the inhibitors were prepared in 0.05 N NaOH to a final concentration of 50 mM.

Carbonic anhydrase activity was measured potentiometrically at 0–2 °C by determining the time taken for a linear drop of 1.0 unit of pH range 8.5–7.5, according to Haglund et al. (1992a). A cuvette containing 3 ml of buffer (50 mM Tris, 25 mM (oxalo)acetic acid, 25 mM mercaptoethanol, 5 mM EDTA) was used. External CA activity was measured using seven to ten algal discs (60–90 mg) transferred directly from the culture to the cuvette. In order to estimate total CA activity, 10–20 mg of sample was extracted in the buffer described above and assayed immediately. The reaction was started by introducing 1 ml of ice-cold CO\textsubscript{2} distilled water. One unit of relative enzyme activity (REA) was defined as (t\textsubscript{e} / t\textsubscript{c}) – 1, where t\textsubscript{e} and t\textsubscript{c} were the time for pH change of the nonenzymatic (buffer) and enzymatic reactions, respectively.

Extraction and determination of the amount of Rubisco (EC 4.1.1.39). Samples of 0.2 g FW were powdered in liquid nitrogen and 1.2 ml of extraction buffer (0.1 M Na\textsubscript{2}EDTA and 2 mM phenylmethylsulfonyl fluoride (PMSF), pH 6.5) were added. The extract was centrifuged at 20 000·g for 15 min at 4 °C. The supernatants were subjected to SDS-polyacrylamide gel electrophoresis (10% separating gel; 5% stacking gel) using the discontinuous buffer system of Laemmli (1970). All the reagents and molecular-weight markers were from Sigma-Aldrich Química S.A. Purified Rubisco of spinach was used as a standard. Gel staining and destaining were carried out according to Rintamaki et al. (1988).

Statistics. The results were expressed as the mean value ± standard deviation (SD). Statistical significances of means were tested with a model 1 one-way analysis of variance (ANOVA) followed by a multirange test using Fisher’s protected least significant difference (Sokal and Rohlf 1981).
Results

The PQ′ of normal-Ci-grown discs, estimated from measurements of O2 evolution and change of pH, was 1.04. This value was used to calculate theoretical O2 evolution rates supported by CO2 spontaneously formed from HCO3−. The maximum theoretical rate of CO2 formation in 9 ml of seawater at 15 °C at pH 8.7 was 98.37 nmol O2·min−1, and at pH 9.2 it was 46.68 nmol O2·min−1. O2 evolution rates of discs cultivated under normal and low Ci were five times higher than the theoretical CO2-dependent O2 evolution rates at pH 8.7. At pH 9.2, measured O2 evolution exceeded the theoretical CO2-dependent O2 evolution by six and eight times in discs grown at normal and low Ci, respectively. In discs cultivated under high Ci, the ratio was approx. 1 at pH 8.7 and lower than 1 at pH 9.2.

Figure 1 shows the increase in pH in the medium during photosynthesis by discs of P. leucosticta cultivated at normal Ci. The increase in pH ceased in the dark and after the addition of AZ, an inhibitor of external CA. Ethoxyzolamide, an inhibitor of total CA, had a similar effect (data not shown). Similar patterns were found for discs grown at low and high Ci.

Table 1 contains the values for external and total CA activity and pH compensation point. The results show the presence of extracellular and intracellular CA in P. leucosticta. The external CA activity was significantly higher (P < 0.001) in the discs cultivated at low Ci. External CA activities in normal and high Ci did not differ significantly (P > 0.01). At high Ci, total CA activity decreased by 50%. In contrast, at low Ci, total CA activity increased by 90%. The pH compensation point obtained after 6 h was modified by the concentration of Ci. The lowest pH compensation point was obtained for discs grown at high Ci. An appreciable increase in this parameter was obtained at low Ci.

The effect of AZ on oxygen evolution rates at high pH values is shown in Fig. 2. The photosynthetic rates were expressed on chlorophyll a (Chl a) basis. The lowest Chl a content was obtained for discs grown at high Ci (0.75 ± 0.20 mg Chl a·(gFW)−1). The Chl a contents for discs grown at normal and low Ci were 1.40 ± 0.05 and 1.09 ± 0.05 mg Chl a·(gFW)−1, respectively. The change in pH from 8.7 to 9.2 did not affect photosynthesis by discs grown at low and normal Ci. However, O2 evolution rates by high-Ci-grown discs were reduced at pH 9.2. Oxygen evolution rates by high-Ci-grown discs were lower than O2 evolution rates by normal and low-Ci-grown discs (P < 0.01) at pH 8.7 as well as at pH 9.2. The inhibition of photosynthesis at pH 8.7 by AZ was nearly 80% in normal and low Ci, and almost 50% in high Ci. A similar effect was found at pH 9.2. Ethoxyzolamide completely inhibited photosynthesis in all the treatments. No effect by AZ or EZ was found at pH 5.6 (data not shown).

At pH 8.7, the HCO3− concentration is 99% of total inorganic carbon (TIC) and the CO2 concentration is 10 times the HCO3− concentration. The curves of Ci saturation at pH 8.7 and 5.6 are shown in Fig. 3. The Km(TIC) values for Ci (Table 2) at pH 8.7 were not significantly different (P > 0.01) in discs grown at the different Ci levels. At pH 5.6, the Km(TIC) obtained for high-Ci-grown discs was lower than that obtained for normal- and low-Ci-grown-discs. The value for Vmax was higher for low- and normal-Ci-grown discs than for cells grown at high Ci (P < 0.01) at pH 8.7 as well as at pH 5.6. The photosynthetic conductance (gph) based on the concentration of Ci estimated from the initial slope of Ci-O2 evolution curves at pH 8.7 was increased at low Ci and decreased at high Ci (P < 0.01). The gph values estimated from saturating Ci curves at pH 5.6 were not significantly different (P > 0.01).

Figure 4 shows an electrophoretic analysis of soluble proteins from homogenates of P. leucosticta cells grown

Table 1. Carbonic anhydrase activity of P. leucosticta discs grown at different levels of Ci. Measurements were performed on either intact cells (to obtain activity of external CA) or on homogenate of cells (to obtain the total CA activity). Data are mean values (± SD) for n = 8 from two independent experimental series. The pH compensation point was obtained by incubation of discs in the photosynthesis chambers in natural unbuffered seawater for 6 h. REA = relative enzyme activity

<table>
<thead>
<tr>
<th>Culture</th>
<th>External CA activity (REA·(gFW)−1)</th>
<th>Total CA activity (REA·(gFW)−1)</th>
<th>pH compensation point</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low Ci</td>
<td>14.41 ± 3.66</td>
<td>76.52 ± 11.57</td>
<td>9.45</td>
</tr>
<tr>
<td>Normal Ci</td>
<td>6.05 ± 1.57</td>
<td>40.09 ± 5.64</td>
<td>9.25</td>
</tr>
<tr>
<td>High Ci</td>
<td>4.93 ± 0.77</td>
<td>20.41 ± 4.10</td>
<td>8.88</td>
</tr>
</tbody>
</table>
at low, normal and high $C_i$ concentrations. Each homogenate was obtained from 200 mg of plant material. The soluble protein concentration varied among the treatments (data not shown) with a similar pattern to that of Rubisco. It should be noted that the intensity of the Rubisco band decreased considerably at high $C_i$ and was similar for normal and low $C_i$. These results indicate that the Rubisco concentration is reduced at high $C_i$.

**Discussion**

The use of $HCO_3^-$ by *P. leucosticta*. Implication of external CA in photosynthesis at alkaline pH. From our results the use of $HCO_3^-$ by *P. leucosticta* can be inferred. The value of the pH compensation point obtained with air-grown discs of *P. leucosticta* also supports this conclusion. Based on data obtained using the pH-drift method, the rate of photosynthesis is inhibited by AZ at high $C_i$ concentrations.

<table>
<thead>
<tr>
<th>$C_i$ Level</th>
<th>$K_m$ (TIC) ($\mu$M)</th>
<th>$K_m$ (CO2) ($\mu$M)</th>
<th>$V_{\text{max}}$ ($\mu$mol O$_2$·mg Chl a$^{-1}$·h$^{-1}$)</th>
<th>$g_p$ ($10^{-6}$·m$^{-2}$·s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 8.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low $C_i$</td>
<td>240 ± 50</td>
<td>1.21 ± 0.13</td>
<td>125.75 ± 15.85</td>
<td>8.60</td>
</tr>
<tr>
<td>Normal $C_i$</td>
<td>610 ± 90</td>
<td>3.28 ± 0.05</td>
<td>165.50 ± 9.70</td>
<td>4.45</td>
</tr>
<tr>
<td>High $C_i$</td>
<td>277 ± 40</td>
<td>1.50 ± 0.22</td>
<td>35.89 ± 1.45</td>
<td>2.12</td>
</tr>
<tr>
<td>pH 5.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low $C_i$</td>
<td>24.31 ± 4.37</td>
<td>21.18 ± 3.10</td>
<td>152.63 ± 20.67</td>
<td>147.86</td>
</tr>
<tr>
<td>Normal $C_i$</td>
<td>26.87 ± 4.83</td>
<td>23.41 ± 3.43</td>
<td>242.02 ± 24.24</td>
<td>103.08</td>
</tr>
<tr>
<td>High $C_i$</td>
<td>5.51 ± 0.58</td>
<td>4.80 ± 0.50</td>
<td>29.29 ± 0.46</td>
<td>87.30</td>
</tr>
</tbody>
</table>

**Table 2.** Rates of oxygen evolution at $C_i$ saturation ($V_{\text{max}}$) and half-saturated rate of photosynthesis for total inorganic carbon ($K_m$ (TIC)) and CO$_2$ ($K_m$ (CO2)) at pH 8.7 and 5.6. Conductance for $C_i$ ($g_p$) was obtained as the initial slope of photosynthesis-$C_i$ curves with units of mol·m$^{-2}$·s$^{-1}$ and mol·m$^{-3}$, respectively.
Fig. 4. Protein analysis of homogenates of *P. leucosticta* grown at normal low and high C<sub>i</sub>. The extracts were subjected to SDS-polyacrylamide gel electrophoresis (10% separating gel; 5% stacking gel) using the discontinuous buffer system of Laemmli (1970) and gels were stained with Coomassie blue. *Lane 1*, homogenate from high-C<sub>i</sub>-grown discs; *lane 2*, homogenate from low-C<sub>i</sub>-grown discs; *lane 3*, homogenate from normal-C<sub>i</sub>-grown discs; *lane 4*, purified Rubisco of spinach that was used as a standard; *Lane 5*, molecular-weight markers. The arrowhead indicates the Rubisco band.

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The role external CA in C<sub>i</sub> use. Two functions of external CA can be presumed in macroalgae: (i) the conversion of HCO<sub>3</sub><sup>-</sup> to CO<sub>2</sub> that is taken up by the macroalga (Haglund et al. 1992b); (ii) participation in active transport of HCO<sub>3</sub><sup>-</sup> across the plasma membrane by conversion of CO<sub>2</sub> to HCO<sub>3</sub><sup>-</sup>. A way of determining the true role of the CA-system in acquisition of C<sub>i</sub> by algae is to check the effect of C<sub>i</sub> level on both CA activity and capacity for HCO<sub>3</sub><sup>-</sup> use. A regulation of both external and internal CA by the level of external CO<sub>2</sub> has been reported in microalgae (Nelson et al. 1969; Muñoz and Merrett 1988; Ramazanov and Cardenas 1992; Sütlemeyer et al. 1993). Similar results have been described for macroalgae (Johnston and Raven 1990; Björk et al. 1993) although equivalent data for red macroalgae are relatively sparse (García-Sánchez et al. 1994). In general, a correlation between external CA activity and HCO<sub>3</sub><sup>-</sup> use was found by these authors. In our report it is shown that external CA activity *P. leucosticta* was increased at low C<sub>i</sub> but that there was no reduction in external CA activity under high C<sub>i</sub>. The mechanism for using HCO<sub>3</sub><sup>-</sup> in *P. leucosticta* is activated at low and normal C<sub>i</sub> levels but, in contrast, is partially inactivated at high C<sub>i</sub>. Photosynthetic conductance based on the concentration of C<sub>i</sub> the pH compensation point and photosynthetic rates at pH 9.2 were dramatically reduced at high C<sub>i</sub> and these were increased at low C<sub>i</sub>. It might look paradoxical that the *K<sub>m(TIC)</sub>* at pH 8.7 was not reduced at high C<sub>i</sub>. However, it must be noted that the suitability of the half-saturation constant as a measurement of the ability to extract C<sub>i</sub> from seawater can be questioned. According to Raven and Johnston (1991), the C<sub>i</sub> kinetic can be viewed as two limiting steps: (i) the maximum velocity limited by the rate of electron transport which controls the rate of resupply of Rubisco for carboxylation and (ii) the initial slope controlled by the activity and CO<sub>2</sub> affinity of Rubisco and the supply of C<sub>i</sub>. A decreasing Rubisco concentration can produce a change in the *K<sub>m(TIC)</sub>* for TIIC without a change in the affinity for C<sub>i</sub>. In that sense, we have found that the different treatments produced changes in Rubisco concentration. The decreasing Rubisco concentration at high C<sub>i</sub> can explain the low values of *V<sub>max</sub>* and *K<sub>m(TIC)</sub>* obtained. The inherent problems in interpreting the *K<sub>m(TIC)</sub>* values can be overcome using the initial slope of photosynthesis-C<sub>i</sub> curves (*g<sub>p</sub>*)

From our results, there is no evident relationship between external CA activity and the capacity for using HCO<sub>3</sub><sup>-</sup> in *P. leucosticta*, although this enzyme is necessary for HCO<sub>3</sub><sup>-</sup> use. A similar result was found by Palmqvist et al. (1990) for cells of the microalga *Chlamydomonas reinhardtii* which showed a higher C<sub>i</sub>-affinity when grown at low C<sub>i</sub> than cells grown at normal C<sub>i</sub> although the external CA was equal in cells from the two growth conditions. The increased affinity was interpreted as resulting from the activation of a plasma-membrane-located HCO<sub>3</sub><sup>-</sup>-pump under conditions where external-CA-mediated diffusion of CO<sub>2</sub> was insufficient. In that sense, our results suggest that the system for HCO<sub>3</sub><sup>-</sup> concentrating in *P. leucosticta* is composed of different elements that could be activated or inactivated independently. Although HCO<sub>3</sub><sup>-</sup> use is linked to external CA in *P. leucosticta*, other components are essential requirements for a HCO<sub>3</sub><sup>-</sup>-concen-
trating system. We have no evidence for postulating a HCO₃⁻ transporter in P. leucosticta because photosynthesis by low-Cᵢ-grown discs was almost completely inhibited by AZ. Two complementary hypotheses can be postulated.

(i) Internal CA is an absolute requirement for a functioning Cᵢ-accumulation mechanism. The reduction in internal CA activity can explain the loss of capacity of HCO₃⁻ use at high Cᵢ. However, if internal CA was really important for photosynthesis, an inhibitory effect of EZ would be expected even under acidic conditions.

(ii) There is a CO₂ transporter which works in association with external CA. This transporter could be inactivated at high Cᵢ. Our data indicated that external CA catalyzes the conversion HCO₃⁻ to CO₂ at the surface of the plasmalemma. Carbon dioxide could penetrate into cells both by diffusion (Gutknecht et al. 1977) and by an active CO₂-transport mechanism (Sülttemeyer et al. 1989). The presence of this enzyme could become redundant when the CO₂-pump is inactivated. The simultaneous presence of external CA and direct uptake of HCO₃⁻ and/or CO₂ has been proposed in microalgae (Badger and Price 1994; Palmqvist et al. 1994).

A way to investigate the presence of a CO₂-pump in P. leucosticta would be to determine the energy cost of the CO₂ accumulation at alkaline pH. If photosynthesis depends on both the CO₂ gradient created by external CA and the CO₂ entries by diffusion, then the energy cost could be lower than if an additional pump was activated. An indication of the energy cost in the mechanism for CO₂ accumulation at alkaline pH in P. leucosticta is that the Cᵢ-saturated photosynthesis by low- and normal-Cᵢ-grown discs at acidic pH was higher than at alkaline pH. This preference for CO₂ as a carbon source was also pointed out by Haglund et al. (1992a) for Gracilaria tenuistipitata.

In conclusion, we suggest that external CA catalyzes the external dehydration of HCO₃⁻ followed by CO₂ uptake. Uptake of CO₂ could be a transporter-mediated process that is regulated by Cᵢ level. The activation of both CA and a hypothetical CO₂-pump could explain the high affinity for Cᵢ in P. leucosticta grown at low Cᵢ. Further experiments are necessary in order to validate this hypothesis.

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