**Communication**

Carbonic Anhydrase-Dependent Inorganic Carbon Uptake by the Red Macroalga, *Chondrus crispus*¹,²

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**ABSTRACT**

The rate of photosynthetic carbon uptake of *Chondrus crispus* Stackhouse plants, at various CO₂ concentrations and pretreated with carbonic anhydrase (CA) inhibitors, was determined using an air-suspension, differential infra-red gas analyzer technique. It was found that the CA inhibitors, acetazolamide, dextran-bound acetazolamide (DBI, which does not permeate cell membranes), and subtilisin (a protease that attacks the cell surface) inhibit photosynthetic carbon uptake in *C. crispus*. Inhibition was greatest at low CO₂ concentrations, and decreased at CO₂ saturation. Acetazolamide inhibited carbon uptake to a greater extent than DBI. The data support the conclusion that *C. crispus* plants utilize HCO₃⁻ for photosynthesis, and that both cell-surface and internal CA are involved in the photosynthetic uptake of inorganic carbon.

The growth of the red macroalga, *Chondrus crispus*, may be limited by Cᵢ supply in tank culture conditions (5) as well as in tidal pools and dense seaweed beds. An understanding of the mechanism of Cᵢ uptake is therefore important for the growth and cultivation of this economically important species.

Seawater contains approximately 2 mM HCO₃⁻ and 10 μM CO₂, and it is not surprising that marine macroalgae have been reported to utilize HCO₃⁻, the more abundant molecular species, for photosynthesis (4, 6, 7, 11, 12, 23). Freshwater phytoplankton species have also been reported to utilize HCO₃⁻ for photosynthesis (e.g. 3, 8, 18, 20). It has been suggested that a CA-dependent Cᵢ uptake mechanism is induced in some species of phytoplankton when they are adapted to low CO₂ conditions (3, 10). To our knowledge, CA-dependent Cᵢ uptake mechanisms have not been reported in marine macroalgae, although they have been reported to contain CA (e.g. 9).

In this paper we present data that further support the conclusion that *C. crispus* utilizes HCO₃⁻ as a source of photosynthetic carbon, and suggest the involvement of CA in the uptake and utilization of Cᵢ.

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³ Abbreviations: Cᵢ, inorganic carbon; IRGA, infra-red gas analyzer; CA, carbonic anhydrase (EC 4.2.1.1.); DBI, dextran-bound acetazolamide; REA, relative enzyme activity.

**MATERIALS AND METHODS**

**Plant Material.** *Chondrus crispus* Stackhouse plants were collected from the intertidal zone at Herring Cove, Nova Scotia, Canada. The plants were held in a flow-through seawater tank (Aquatron) prior to experimental use for 10 d or less. The photosynthetic characteristics of the plants (light, temperature, and CO₂ responses) did not change over the 10-d period. Only healthy looking plants free of visible epibions were used in the experiments.

**Inorganic Carbon Uptake.** The photosynthetic rates of plants were determined using the air-suspension, differential IRGA technique described by Bidwell and McLachlan (4). Plants were placed wet, but in air, on an inert support in a thermostatted photosynthesis chamber. Gas was humidified, passed through the photosynthesis chamber, dehumidified, and its CO₂ content was compared with that of the incoming gas using the differential mode of an IRGA (Analytical Development Company, Hoddesdon, England, Mark II). The CO₂ in the gas stream dissolves in the seawater film surrounding the alga and further undergoes hydration and dissociation reactions according to its equilibrium constants, so both CO₂ and HCO₃⁻ are available to the plants. Gas containing various concentrations of CO₂ was supplied at a rate of 1.0 L·min⁻¹ from compressed gas cylinders (Analysed Gas, Liquid Carbonic Ltd., Toronto, Canada) using a gas mixing manifold (4). Concentrations of CO₂ ranged from 150 to 3000 μL·L⁻¹ CO₂, balance N₂. A second IRGA monitored the absolute concentration of CO₂ in the gas stream.

The temperature of the incubation chamber was kept at 18°C by a surrounding thermostatted circulating water bath. Light was supplied by a bank of power-groove fluorescent lamps (CGE Ltd., Toronto, Canada) and was set at saturating irradiances of 350 or 400 μE·m⁻²·s⁻¹.

Prior to the air-suspension incubation, the plants were weighed and pretreated in either seawater (control) or one of the following inhibitors in seawater. Acetazolamidde (Sigma) was used as a CA inhibitor that is reported to diffuse across cell membranes (15). DBI (mol wt about 5500) was used as a CA inhibitor that cannot diffuse across cell membranes (16, 24). Subtilisin (Sigma) was used as a protease that reportedly attacks CA (and other proteins) at the cell surface (1). The control, acetazolamide, and DBI treatments lasted 10 min in an ice bath and the subtilisin treatment lasted for 30 min at 0, 12, or 20°C.

Experiments reported in this paper were carried out in December 1985 and June 1986. Data are consistent within each experiment, and show parallel trends. However, there were marked differences in absolute rates of Cᵢ uptake and inhibition-values between experiments (cf. Fig. 2 versus Fig. 3B and Table 1). These
differences are probably due to known seasonal variation in the behavior of *Chondrus* plants (14).

CA Activity. The CA activity of crude extracts of *C. crispus* plants was determined by bubbling N₂ gas at a rate of 500 ml min⁻¹ through 150 ml of seawater buffered with 50 mm Tris (pH 7.5), containing 1 ml of octanol (a defoaming agent) and either crude extract or purified CA (Sigma, C7500). The bubbled gas was passed through a condenser and an IRGA (Analytical Development Company, model LCA2). The amount of CO₂ released from the seawater buffer was integrated over a 10-min period starting after 2 min. The REA of the extract, defined as (E-U)·U⁻¹ (where E is the amount of CO₂ released in the presence of CA and U is the amount of CO₂ released in the absence of CA) was compared with the REA of purified CA. The REA of acetazolamide-treated crude extracts and purified CA solutions was zero, as would be expected. The crude extract of *C. crispus* was prepared by chopping the plants very fine with razor blades, mixing with buffer, centrifuging (10,000g, 15 min) and decanting the extract. Generally, 10 ml of buffer were used for 0.5 to 1.0 g of alga. The buffer was composed of 50 mm Tris (pH 7.5), 5 mm EDTA, and 0.25% (v/v) mercaptoethanol in distilled H₂O.

**RESULTS**

**Inorganic Carbon Uptake.** *C. crispus* plants are saturated with respect to C, at 2000 μl·L⁻¹ CO₂, attaining carbon uptake rates of 10.6 μl CO₂·g⁻¹·min⁻¹ (Fig. 1). Acetazolamide, free or dextran-bound, inhibited photosynthetic carbon uptake at subsaturating and saturating CO₂ concentrations (Fig. 2). At both CO₂ levels, inhibition approached a maximum level at about the equivalent of 2 μM acetazolamide. However, inhibition was greatest at low CO₂ concentrations and decreased as CO₂ became saturating. Inhibition at the lowest CO₂ concentration used was 52 and 45%, respectively, for 2 μM acetazolamide and DBI. Inhibition decreased to 33 and 19%, respectively, at saturating CO₂. A further analysis of the CO₂-concentration effect on acetazolamide inhibition (Fig. 3) revealed that the degree of inhibition was inversely proportional to CO₂ concentration in the range from 150 to 3000 μl·L⁻¹ CO₂.

To determine if the higher inhibition by acetazolamide than DBI was due to the fact that both surface and internal CA was involved in carbon uptake, plants were treated first with seawater, then DBI, then acetazolamide. CO₂ uptake was measured following each treatment. Parallel samples were treated in the same way but with the order of inhibitor treatments reversed, and a sample was treated with both inhibitors together (Table I). Ace-

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**Fig. 1.** Rate of photosynthetic carbon uptake by *C. crispus* plants (in N₂; 400 μE·m⁻²·s⁻¹; 18°C) as affected by CO₂ concentration. Data points and vertical bars represent the means of 10 plants and their respective 95% confidence intervals.

**Fig. 2.** Rate of photosynthetic carbon uptake by *C. crispus* plants (in N₂; 400 μE·m⁻²·s⁻¹; 18°C) following a 10-min treatment with various concentrations of acetazolamide (Δ) or DBI (○) in seawater. CO₂ upper curve (high CO₂) 2500 μl·L⁻¹ for acetazolamide, 3000 μl·L⁻¹ for DBI; lower curve (low CO₂) 470 μl·L⁻¹ for acetazolamide, 320 μl·L⁻¹ for DBI. Each data point is the mean for 10 plants, bars show 95% confidence intervals.

**Fig. 3.** Rate of photosynthetic carbon uptake by *C. crispus* plants (in N₂; 400 μE·m⁻²·s⁻¹; 18°C) as affected by CO₂ concentration and inhibitors. A, Upper curve (○): plants previously held in seawater. Lower curve (●): plants were treated with 2 μM acetazolamide for 10 min prior to measurement. B, Percentage inhibition of carbon uptake caused by acetazolamide, calculated from data in A. Regression line for these data is y = -0.005x + 28.86, n = 70, r = -0.38 (significant at the 95% confidence level).

Acetazolamide inhibited carbon uptake more than DBI, either before or after DBI treatment. Addition of DBI to acetazolamide-inhibited plants did not increase inhibition. This shows that both surface and internal CA was being inhibited by acetazolamide.

Subtilisin was also inhibitory (Table II). As with acetazolamide and DBI, subtilisin inhibition was greatest at lower CO₂ concentration, and least at higher CO₂ concentration. The high inhibition obtained at temperatures above 0°C may have been due to disruption of cell membranes by the protease.

**CA Activity.** A crude extract of *C. crispus* had a REA for CA of 0.47 unit·g⁻¹, which corresponds to approximately 9 Wilbur-Anderson units of purified CA.
PHOTOSYNTHETIC CARBON UPTAKE BY CHONDROS

Table I. Effect of Acetazolamide and DBI in Sequence or Together on the Rate of Photosynthetic Carbon Uptake by C. crispus Plants

Plants were transferred to air suspension (400 μE·m⁻²·s⁻¹; 18°C) and their carbon uptake rate was determined. The plants were then treated sequentially for 10 min each with the two inhibitors at 2 μM, or with both together. Following each treatment plants were again transferred to air suspension and carbon uptake rates were determined. The mean percentage inhibition values and their 95% confidence intervals are given (n = 10). Different plants were used for each set of treatments.

<table>
<thead>
<tr>
<th>[CO₂] (μl·L⁻¹)</th>
<th>Treatment:</th>
<th>DBI then acetazolamide</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Acetazolamide</td>
<td></td>
</tr>
<tr>
<td>320</td>
<td>DBI</td>
<td>16 ± 15</td>
<td>39 ± 9</td>
</tr>
<tr>
<td>1502</td>
<td>DBI</td>
<td>9 ± 7</td>
<td>28 ± 5</td>
</tr>
<tr>
<td>2942</td>
<td>DBI then acetazolamide</td>
<td>7 ± 4</td>
<td>16 ± 4</td>
</tr>
<tr>
<td></td>
<td>Acetazolamide, then DBI</td>
<td>27 ± 8</td>
<td>21 ± 7</td>
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<tr>
<td></td>
<td>Acetazolamide + DBI</td>
<td>26 ± 9</td>
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</table>

DISCUSSION

C. crispus plants incubated in air suspension are saturated with respect to carbon uptake at 2000 to 2500 μl·L⁻¹ CO₂ (Fig. 1). It has been shown that this level of CO₂ in air is necessary to maintain saturating levels of Ci at the surface of the thallus of plants held in air suspension (4, 14).

In this study we found that a C. crispus extract contained a level of CA activity that corresponded with 9 Wilbur-Anderson units·g⁻¹. This is within the range of CA activities reported by Graham and Smillie (9) for a variety of marine macroalgae. CA is also present in phytoplankton (e.g. 8, 18, 20, 22, 25), where it has been hypothesized to be involved in a CA-dependent C uptake mechanism (3, 10). Evidence that supports this view includes the increased CA activity and HCO₃⁻ utilization in phytoplankton species that are transferred from high CO₂ culture (where CO₂ is supplied in excess) to low CO₂. Acetazolamide inhibits the utilization of HCO₃⁻ in such microalgae when they are transferred from high to low CO₂ (usually air-level) cultures (2, 21, 22).

The reduction of carbon uptake in acetazolamide-, DBI-, and subtilisin-treated plants (Figs. 2 and 3; Tables I and II) is consistent with the operation of a CA-dependent HCO₃⁻ uptake system in C. crispus. It has been shown that C. crispus absorbs mainly HCO₃⁻ under normal circumstances (4, 6, 7). Presumably, as with microalgae, the proportion of CO₂ absorbed increases with CO₂ concentration. Data in Figure 3B and Tables I and II show that inhibition of carbon uptake by the CA inhibitors was greatest at air levels of CO₂, when HCO₃⁻ absorption predominates, and least at high CO₂ concentrations, when CO₂ absorption would be greatest. This indicates that CA is involved in HCO₃⁻ uptake.

Inhibition of carbon uptake was caused by both DBI and subtilisin (Tables I and II). This indicates that CA located at the cell surface is involved in HCO₃⁻ uptake. However, acetazolamide inhibition was greater than that caused by DBI, although there was no cumulative effect (Table I), which indicates that internal CA located inside cells or below the cell surface is also involved. This is unlike the situation reported for Dunaliella tertiolecta (1) and Chlamydomonas reinhardtii (13, 26), where essentially all of the CA was found to be located at the cell membrane surface. However, it is comparable with the situation in some chloroplasts, which contain roughly 50% of their CA in the chloroplast stroma (17, 19).

The data presented in this paper support the conclusion (4, 6) that C. crispus can utilize HCO₃⁻ for photosynthesis, and further suggest that C uptake in this red macroalga involves both membrane-associated and internal CA. Work to characterize the Ci uptake mechanism is currently in progress.

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