Planta (1992) 187:152–156

Planta

Inorganic-carbon assimilation in the green seaweed *Ulva rigida* C.Ag. (Chlorophyta)

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Received 20 July; accepted 15 October 1991

Abstract. Mechanisms of carbon assimilation were investigated in thalli and protoplasts of *Ulva rigida* by measuring HCO_3^--dependent O_2 evolution at pH 6.5 and 8.6. In thalli, dextran-bound azetazolamide (DBAZ), a specific inhibitor of extracellular carbonic anhydrase (CA), inhibited the rate of O_2 evolution at pH 8.6 when HCO_3^- was the only available form of inorganic carbon (C_i) in the medium. At pH 6.5 when CO_2 is accessible, DBAZ did not affect photosynthetic O_2 evolution. Inhibition of total CA activity (extracellular and intracellular) by ethoxyzolamide (EZ) inhibited photosynthesis at pH 6.5 and 8.6. During illumination of thalli the medium was alkalized at a rate which increased with increasing light. This alkalization decreased during inhibition of extracellular CA by DBAZ. Protoplasts at pH 6.5 exhibited a higher rate of O_2 evolution than in pH 8.6. Addition of CA to protoplasts at pH 8.6 increased the rate of O_2 evolution, whereas EZ was inhibitory at both pH 6.5 and 8.6, and DBAZ did not affect photosynthesis at either pH. We suggest that both extracellular and intracellular CA are present and that *Ulva rigida* assimilates HCO_3^- by an indirect mechanism. A theoretical scheme for carbon utilization is suggested.

Key words: Carbon-assimilation – Carbonic anhydrase – Photosynthesis (*Ulva*) – Protoplast (photosynthesis) – *Ulva*

Introduction

Marine macroalgae have been shown to utilize both CO_2 and HCO_3^- from the surrounding seawater (Smith and Bidwell 1989; Raven 1990); CO_2 can enter through membranes by diffusion, or by active transport as in microalgae (Sültemeyer et al. 1988). Carbon dioxide is the most accessible form of inorganic carbon (C_i), but considering the greater availability of HCO_3^- in seawater, this latter carbon source could well be more important in natural systems (Smith and Bidwell 1987, 1989). The mechanisms for uptake of HCO_3^- are still not clear. It has been proposed that HCO_3^- is transported through the plasmalemma by an HCO_3^-/OH^- antiporter or HCO_3^-/H^+ symporter mechanism (Lucas 1983) which may involve ATPases (Raven and Lucas 1985). Bicarbonate could also be assimilated by an indirect mechanism, i.e. after dehydration to CO_2 in the cell wall (Smith and Bidwell 1987). This mechanism would require an extracellular carbonic anhydrase (CA) (Smith and Bidwell 1987, 1989). Carbonic anhydrase catalyzes the reversible hydration and dehydration of CO_2 and HCO_3^-, and has been shown to be involved in active C_i transporting and accumulating mechanisms in seaweeds (Smith and Bidwell 1987, 1989; Beer and Israel 1990). Another possibility is the spontaneous low-pH-dependent dehydration of HCO_3^- in "acidic regions" of the cell wall of aquatic plants (Lucas 1983). It has been suggested that extracellular CA can increase the rate of conversion of HCO_3^- to CO_2 in the extracellular space; however, CA analyses on several seaweeds have shown no indications of extracellular CA activity (Cook et al. 1986, 1988).

In the present work we demonstrate that the green seaweed *Ulva rigida* can assimilate both CO_2 and HCO_3^-, but that protoplasts isolated from this plant take up only CO_2. We suggest that *U. rigida* assimilates HCO_3^- by an indirect mechanism, that extracellular CA is involved in C_i uptake, and the evidence for the presence of extracellular and intracellular CA is presented.

Material and methods

Algal material and culture. Healthy *Ulva rigida* C.Ag. plants were collected from Taliarte, Gran Canaria, Spain and cleaned thoroughly in sterile seawater. Plants were then kept in 20-l tanks with running seawater for a maximum time of 12 h.

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Abbreviations: CA = carbonic anhydrase; C_i = inorganic carbon; DIC = dissolved inorganic carbon; DBAZ = dextran-bound acetazolamide; EZ = 6-ethoxyzolamide

Protoplast isolation. Cellulysin (Calbiochem AG, Lucerne, Switzerland) and Abalone Acetone Powder (Sigma, St. Louis, Mo., USA) were dissolved in seawater at a salinity of 38 ‰ with addition of 0.4 M mannitol and 20 mM 2,2-bis(hydroxymethyl)-2,2′,2″-nitrilo triethanol (Bis)-Tris. The enzyme solution was stabilized by stirring on ice for 30 min, centrifuged at 1000 · *g* for 10 min, the pH adjusted to 6.0, and then filtered through a 0.8-µm sterile filter. The enzyme solution was frozen in 10-ml portions and kept at −20° C prior to use. The tissue was chopped into 1- to 2-mm² pieces using a razor blade. The fragmented thallus was then rinsed five times in wash buffer (0.2 M mannitol and 20 mM 4-(2-hydroxyethyl)-1 piperazine-ethanesulfonic acid (Hepes), in seawater, pH 7), and incubated for 30 min in seawater with addition of 20 mM Hepes and 0.8 M mannitol, final pH 7.0. One gram of fragmented thallus was immersed in 10 ml enzyme solution and incubated at 20° C under constant shaking (60 rpm). After digestion the protoplast suspension was filtered through a 100-µm nylon mesh to remove cell-wall debris and non-digested material, rinsed with wash buffer and centrifuged in a swing-out rotor at 100 · *g* for 5 min. The pellet was resuspended in test buffer and centrifuged again, the resulting pellet of protoplasts was resuspended in a test buffer for photosynthesis experiments.

Preparation of inhibitor and CA solutions. Two inhibitors of CA were used, 6-ethoxyzolamide (EZ) which penetrates through the cell wall and membranes, and dextran-bound acetazolamide (DBAZ). When bound to dextran, DBAZ cannot penetrate into the cell and inhibits only the extracellular CA (Moroney et al. 1985; Palmqvist et al. 1990). Stock solutions were prepared as follows: EZ was dissolved in 0.05 N NaOH to a concentration of 10 mM; DBAZ, kindly provided by Prof. Göran Samuelsson (Umeå, Sweden), was dissolved in 0.05 N NaOH to a concentration of 0.1 $g \cdot ml^{-1}$. Bovine CA (Sigma) was dissolved in ultra-pure water (milliQ) to a concentration of 0.1 $mg \cdot ml^{-1}$.

Measurement of photosynthetic O_2 evolution. When thalli were used they were chopped with a razor blade to approx. 10 mm² pieces and rinsed several times in seawater. The fragments were transferred to seawater medium and maintained with continuous air bubbling and 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light for at least 6 h before use. Protoplasts were suspended in test buffer and transferred to the measuring chamber. Photosynthesis was measured in 1-ml samples with an oxygen electrode (Hansatech, Kings Lynn, Norfolk, UK). Two test buffers were used, consisting of seawater, 100 mM 3-(N-morpholino)ethanesulfonic acid (Mops) (Sigma) or 2-(N-cyclohexylamino)ethane-sulfonic acid (Ches) (Sigma) adjusted to pH 6.5 and 8.6 respectively. When protoplasts were measured, an addition of 0.2 M mannitol was used to prevent lysis. Buffers of low dissolved-inorganic-carbon content (low-DIC buffer) were obtained by acidifying natural seawater to pH 3, bubbling for 12 h with CO_2-free air to remove DIC and then adjustment of pH with carbonate-free NaOH. Before HCO_3^- was added, samples were allowed to consume the remaining C_i of the buffer and the intracellular pool of C_i until no net O_2 evolution was observed. Final inhibitor concentrations used here were 100 µM EZ and 100 µM DBAZ. The final CA concentration used was 1 $mg \cdot l^{-1}$. The experiment was repeated four times with material from the same batch of cut thalli or protoplasts, then repeated up to six times with new material.

Photosynthetic alkalization in whole thalli. Photosynthetic alkalization was measured with a pH meter (CKC UC–23 with UC–502E electrode; Central Kagaku Co., Tokyo, Japan). An Erlenmeyer flask was filled to 55 ml with medium and 0.5 g seaweed thallus (fresh weight) was placed in the flask together with a bar magnet. The medium consisted of natural seawater (pH 8.12). A closed system was obtained with the electrode tip immersed in the medium in a 50 ml Erlenmeyer flask by sealing the electrode and flask together with Parafilm and covering the seal with paraffin to prevent CO_2 exchange with the atmosphere. The pH change was recorded at photon irradiances of 75 and 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and at 23 ± 1° C. The experiment was repeated three times with material from the same batch of cut thalli or protoplasts, then repeated up to six times with new material.

Extraction of algal material. A 2-g sample of algal thallus was homogenized carefully in liquid nitrogen and extracted with a buffer containing 50 mM Tris (pH 8.5), 15 mM dithiothreitol (DTT), 25 mM isoascorbic acid and 5 mM EDTA. Buffer was added to a total volume of 20 ml.

Assay of carbonic anhydrase. Carbonic-anhydrase activity was measured potentiometrically at 0–2° C by determining the time taken for a linear drop of 0.4 pH units in the pH range 8.1 to 7.1 in the reaction mixture, which consisted of 2 ml sample and 2 ml substrate. Samples used were intact thalli, protoplast suspensions, and algal extracts centrifuged at 12000 · *g* for 15 min. The sample buffer used was the same as the extraction buffer. The reaction was started by rapidly introducing 2 ml of the substrate, ice-cold CO_2-saturated distilled H_2O. The method was modified after Ramazanov and Semenenko (1988). One unit of relative enzyme activity (REA) was defined as (t_0/t_c)-1 where t_o and t_c are the times for pH change of the nonenzymatic (sample buffer) and the enzymatic reactions, respectively. When measuring CA activity in thallus fragments, 0.16–0.29 g fresh weight was transferred directly from seawater to 2 ml of sample buffer. Prior to the measurements with protoplasts, they were lysed in the hypo-osmotic test buffer. The fragments were maintained in suspension by magnetic stirring. Results shown are means of three parallel determinations in two independent repetitions of the experiment.

Measurement of chlorophyll. Chlorophyll was measured spectrophotometrically after ethanol extraction according to Wintermans and de Mots (1965).

Results

Photosynthesis in thalli. The photosynthesis of *U. rigida* thalli fragments in seawater of pH 8.6 is shown in Fig. 1. The rate of photosynthetic O_2 evolution was inhibited by both DBAZ and EZ; however, the inhibition by EZ was 40% higher than that of DBAZ. After subsequent addition of CO_2, the rate of O_2 evolution recovered and under these conditions the inhibition by EZ was lower than in the presence of only HCO_3^- (Fig. 1). In the presence of CO_2 in the medium, DBAZ had no effect on the rate of O_2 evolution (data not shown). The photosynthesis of thalli fragments in seawater at pH 6.5 is also shown in Fig. 1. At this pH the photosynthetic rate was about three times higher than at pH 8.6 at the same concentration of C_i, and EZ strongly inhibited the rate of O_2 evolution whereas DBAZ had no effect.

The rate of pH change in natural seawater caused by thalli of *U. rigida* is shown in Fig. 2. The highest alkalization rate was obtained at the highest irradiance. Addition of DBAZ to the medium caused a decrease in the alkalization rate. When DBAZ was removed by washing the thallus in fresh medium the rate of alkalization increased again. No attempts have been made to correlate the rate of photosynthetic O_2 evolution with the rate of photosynthetic alkalization.

Photosynthesis in protoplasts. The rate of O_2 evolution in protoplasts of *U. rigida* was also dependent on the pH in the medium (Fig. 3), and was higher at pH 6.5 than at pH 8.6. At pH 6.5, photosynthesis was saturated at a

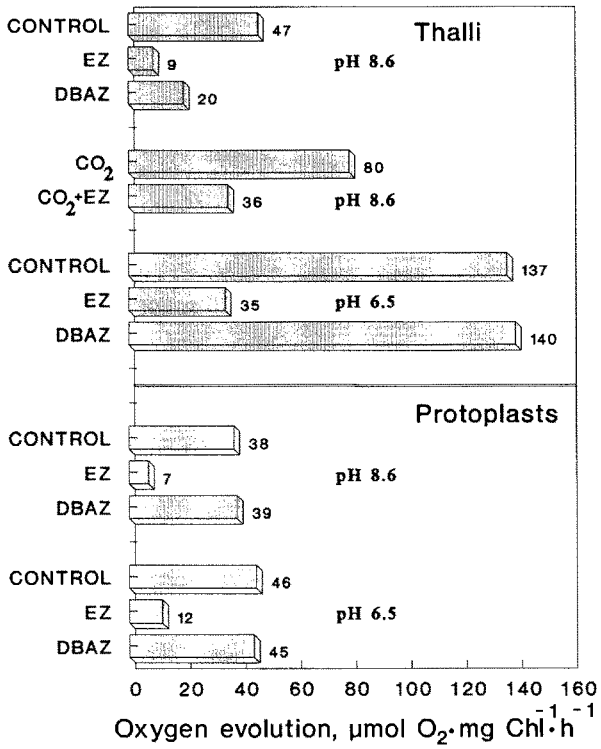

Fig. 1. Photosynthesis of *Ulva rigida* thalli and protoplasts at pH 6.5 and 8.6 In low-DIC medium composed of natural seawater depleted of C_i, with addition of HCO_3^- to a final concentration of 100 μM (for thalli at both pH values, and protoplasts at pH 6.5) or 600 μM (for protoplasts at pH 8.6). Fresh weight of thalli was 0.5 g/50 ml medium, light irradiance was 400 μmol photons · m^{-2} · s^{-1}. *EZ*, 6-ethoxyzolamide (100 μM); *DBAZ*, dextran-bound acetazolamide (100 μM); *CO_2*, addition of 330 μM CO_2. Mean values ($n=4$); SD not exceeding 7%.

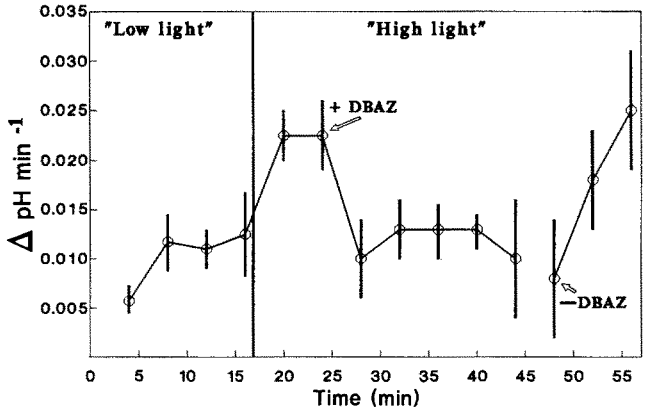

Fig. 2. Effect of DBAZ on the rate of light-dependent alkalization of the medium by *Ulva rigida* thalli. *Arrows* indicates the addition of DBAZ to a final concentration of 100 μM (*+DBAZ*) and the removal of DBAZ by washing with new medium (*−DBAZ*). *Low light*, 75 μmol photons · m^{-2} · s^{-1}; *High light*, 200 μmol photons · m^{-2} · s^{-1}. Values are means ± SD ($n=3$)

concentration of 400–500 μM HCO_3^-, while at pH 8.6 no saturation was observed. Addition of CA to the medium at pH 8.6 increased the rate of O_2 evolution. The effect of CA-inhibitors, EZ and DBAZ, on the rate of O_2 evolution by protoplasts at pH 6.5 and pH 8.6 is shown in Fig. 1. The effects of the inhibitors were similar in both pH conditions. The rate of photosynthetic O_2 evolution was not affected by DBAZ, which does not enter into the protoplast as it cannot penetrate the membrane, whereas EZ decreased the rate.

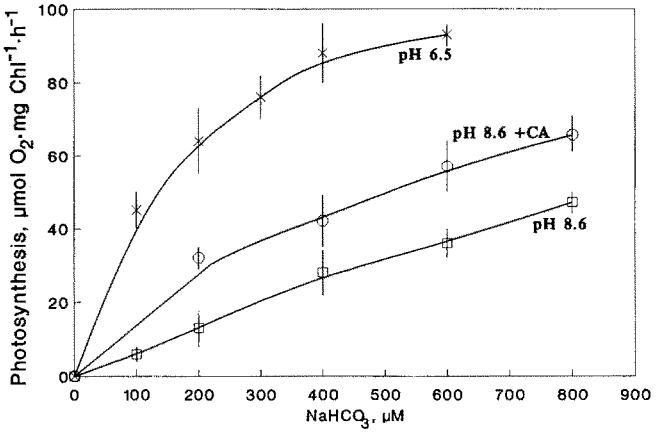

Fig. 3. Photosynthesis of *Ulva rigida* protoplasts at pH 6.5 and 8.6 in low-DIC medium composed of natural seawater depleted of C_i, which was then added as HCO_3^-. Light irradiance was 400 μmol photons · m^{-2} · s^{-1}. *+CA*, experiment performed with medium containing bovine CA at a final concentration of 1 μg · ml^{-1}. Values are means ± SD ($n = 4$)

Table 1. Relative enzyme activity (REA) of CA in an extract of 1 g of *Ulva rigida* thallus tissue. Activity was measured in the homogenate before centrifugation and in the supernatant and the pellet fractions after centrifugation. Protoplasts were lysed in buffer prior to measurement. Whole thallus was submersed in buffer and measured directly. Data are from two separate experiments, each with three parallel sample measurements. The protoplast fraction was measured only once. Values are means ± SD

	REA in 1 g thallus	
Homogenate	5 ± 0.5	($n=6$)
Supernatant	2 ± 0.7	($n=6$)
Pellet	1 ± 0.6	($n=5$)
Protoplast (lysed)	4	($n=1$)
Living thalli	4 ± 1.1	($n=6$)

Carbonic-anhydrase activity. Measurements of the activity of CA in thallus extract showed a higher activity in the soluble fraction than in the insoluble pellet (Table 1), although a comparison with the total CA activity in the homogenate shows that some activity is lost during fractionation. Lysed protoplasts and living intact thalli also posessed CA activity, but these values cannot be directly compared with those of the extracts because of the methodological differences involved in obtaining the measurements.

Discussion

The results in this study demonstrate that, for the green alga *Ulva rigida*, the predominant form of C_i entering

into the cell through the plasmalemma is CO_2. Bicarbonate is dehydrated to CO_2 in the cell wall, an event most likely catalysed by an external CA.

The higher photosynthetic rate of thalli at pH 6.5 compared with pH 8.6, at the same concentration of C_i (Fig. 1), demonstrates the uptake of CO_2. In a closed chamber, in seawater at pH 8.6, the only available form of C_i is HCO_3^-, while at pH 6.5 CO_2 is readily accessible to the plant. If *U. rigida* has the ability to assimilate HCO_3^- directly, it is difficult to explain the effect of DBAZ on the rate of photosynthesis at pH 8.6 since DBAZ specifically inhibits extracellular CA and, accordingly, the catalysed dehydration of HCO_3^- to CO_2 in the cell wall. At pH 6.5, when the relative CO_2 concentration is higher, inhibition of external CA does not affect photosynthetic O_2 evolution. That C_i is mainly assimilated into the cell in the form of CO_2 is also supported by the observation that addition of CO_2 to the medium at pH 8.6 increased photosynthesis and countered the inhibitory effect of EZ (Fig. 1).

However, the mechanism of C_i assimilation in *U. rigida* cannot be explained only by the presence of a extracellular CA. The inhibition by EZ at both pH values demonstrates that the C_i assimilation mechanism in *Ulva*, as in green unicellular algae (Moroney et al. 1985), requires both extra- and intracellular CA catalysis. Smith and Bidwell (1987) showed that inhibition of carbon uptake in the red seaweed *Chondrus crispus* by CA inhibitors was largest at air levels of CO_2, when HCO_3^- absorption predominates, and least when CO_2 was readily available. This supports our results that indicate the involvement of CA in HCO_3^- uptake in *U. rigida*.

The CA-activity assay shows the presence of active enzyme in *U. rigida*. The higher activity found in the supernatant compared with the pellet indicates that a high proportion of the enzyme might be in solution inside the cell, or that the enzyme can be easily removed, still intact, during extraction. The variability of these measurements is however high and, as shown by comparison with the activity of the homogenate, some activity is lost during fractionation, making it difficult to compare the values. The different extraction methods also make it pointless to compare the activities of the homogenized tissue with the intact thalli. Nevertheless, the detection of CA activity at the surface of living thalli is evidence for the existence of cell-wall-bound CA.

The inhibition of alkalization by DBAZ provides additional evidence that *Ulva* assimilates HCO_3^- by an indirect mechanism. Lucas (1983) and Axelsson and Uusitalo (1988) reported that aquatic plants assimilated HCO_3^- by HCO_3^-/OH^- antiport or HCO_3^-/H^+ symport mechanisms and that the alkalization of the medium resulted from the efflux of OH^-. Accordingly, DBAZ would have no effect on the rate of alkalization at pH 8.6. The DBAZ inhibition of light-dependent alkalization in this study indicates that alkalization of the medium by *U. rigida* involves a mechanism requiring extracellular CA. This mechanism is different from the one suggested by Axelsson and Uusitalo (1988) and Lucas (1983). However, a contribution of active ion transport in C_i uptake cannot be excluded from the results presented here.

The pH dependence of protoplast photosynthesis demonstrates that HCO_3^- does not pass through the protoplast plasmalemma. Similar results were obtained for protoplasts from the red seaweed *Chondrus crispus* (Smith and Bidwell 1989). This may reflect a stress effect on the protoplasts in in-vitro pH conditions. Such an explanation does not, however, explain the observation that photosynthetic O_2 evolution by protoplasts was increased by addition of CO_2 and CA. Adding CA to the medium at pH 8.6 did not restore the rate of O_2 evolution to levels matching those at pH 6.5. However, CA dissolved directly in the medium can not be expected to act as efficiently as a CA incorporated into the wall of a intact cell. As mentioned above, there is also a stress upon the protoplasts in vitro, when exposed to pH 8.6, that might effect O_2 evolution negatively. At both pH values, DBAZ had no effect on the rate of photosynthesis which demonstrates that intact protoplasts have no extracellular CA. However, extracellular CA activity would be removed during protoplast preparation.

It is important to note that CA activity was detected in solutions of lysed protoplasts, indicating an intracellular CA. Further investigations are needed to clarify the intracellular localization of CA in *Ulva* and other seaweeds. Decreased photosynthesis in protoplasts during inhibition by EZ at both pHs demonstrated that intracellular CA plays an important role in the C_i-uptake system of *U. rigida*.

The fact that seaweeds have a low photorespiratory rate indicates that the chloroplastic CO_2/O_2 ratio may be high enough to suppress photorespiration (Bidwell and McLachlan 1985). Low photorespiratory levels are typical of green unicellular algae grown in low-C_i conditions which reportedly induce C_i-concentrating mechanisms (Moroney et al. 1985). *Chlamydomonas reinhardtii* grown in low-C_i conditions accumulates C_i to a concentration 40 times that of the environment (Badger et al. 1980). However, no such mechanism has been found in *Chondrus crispus* which does not accumulate C_i above the concentration of its incubation medium, and probably acquires C_i through a diffusive rather than an active mechanism (Smith and Bidwell 1987). The ability of *Ulva lactuca* to assimilate HCO_3^- is induced at high pH (Carlberg et al. 1990). The degree of HCO_3^- utilization is also stimulated in calm waters, where dissolved CO_2 is more limited (Larsson et al. 1990), indicating an induced increase of extracellular CA activity.

We propose an indirect mechanism for C_i uptake and transport in *U. rigida* (Fig. 4, I). This mechanism for C_i transport requires extracellular CA, which catalyzes the dehydration of HCO_3^- to CO_2 in or at the surface of the cell wall. Our model agrees with that previously proposed by Smith and Bidwell (1989). Figure 4(II) also shows a scheme for an ATP-ase dependent C_i-transport mechanism in aquatic plants, as suggested by Lucas (1983 review). In this scheme acid regions are produced in the cell wall by H^+ extrusion, and this local acidification may stimulate pH-dependent conversion of HCO_3^- to CO_2. In both model I and II, internal CA regulates the CO_2 concentration inside the plasmalemma, although the details of this regulation are not known.

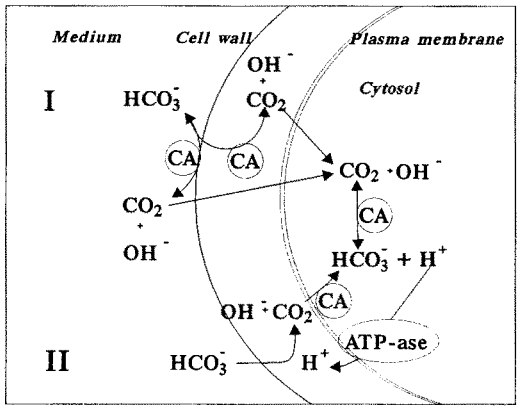

Fig. 4. Theoretical scheme for inorganic-carbon assimilation in *Ulva rigida*. *I*, Carbonic-anhydrase-requiring indirect mechanism of C_i assimilation. II, ATP-ase-dependent indirect mechanism of C_i assimilation

We suggest that, with the help of an extracellular CA, *Ulva rigida* has the ability to utilize the HCO_3^- pool present in seawater as a source of inorganic carbon for photosynthesis. Bicarbonate is dehydrated to CO_2 in the cell wall, and C_i crosses the plasmalemma in the form of CO_2. However, the possibility cannot be excluded that a ATP-ase dependent H^+-extrusion system as described above, co-exists with this system. The two systems might combine to enhance the conversion of HCO_3^- to CO_2.

The authors are very grateful to Juan-Luis Gómez-Pinchetti for valuable assistance in the laboratory, and to Erica Young for excellent help with the manuscript. This work has been supported by the Swedish Council for Forest and Agricultural Research (SJFR), Swedish Agency for Research Cooperation for Research with Developing Countries (SAREC) and CICYT-MAR91/1237 Spain. Z. Ramazanov is an invited professor from the Ministerio de Educación y Ciencia, Spain. The planning of the cooperation was facilitated by COST–48.

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