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Role of carbonic anhydrase in photosynthesis and inorganic-carbon assimilation in the red alga *Gracilaria tenuistipitata*

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Abstract. The mechanism of inorganic-carbon (C_i) accumulation in the red seaweed *Gracilaria tenuistipitata* Zhang et Xia has been investigated. Extracellular and intracellular carbonic-anhydrase (CA) activities have been detected. Photosynthetic O_2 evolution in thalli and protoplasts of *G. tenuistipitata* were higher at pH 6.5 than at pH 8.6, where HCO_3^- is the predominant form of C_i. Dextran-bound sulfonamide (DBS), a specific inhibitor of extracellular CA, reduced photosynthetic O_2 evolution at pH 8.6 and did not have any effect at pH 6.5. After inhibition with DBS, O_2 evolution was similar to the rate that could be supported by CO_2 from spontaneous dehydration of HCO_3^-. The rate of photosynthetic alkalization of the surrounding medium by the algal thallus was dependent on the concentration of C_i and inhibited by DBS. We suggest that the general form of C_i that enters through the plasma membrane of *G. tenuistipitata* is CO_2. Bicarbonate is utilized mainly by an indirect mechanism after dehydration to CO_2, and this mechanism involves extracellular CA.

Key words: Carbon assimilation – Carbonic anhydrase – Carbon dioxide uptake – *Gracilaria* – Photosynthesis

Introduction

Seawater at normal pH (8.0–8.2) contains 2 mM HCO_3^- and only 10 μM CO_2 (Skirrow 1975) for which reason marine red seaweeds have been proposed to take up both these forms of inorganic carbon (C_i) (Sand-Jensen and Gordon 1984; Bidwell and McLachlan 1985; Beer and Israel 1990; Maberly 1990). Carbon dioxide can enter through lipid membranes by a diffusion process (Gutknecht et al. 1977) and, in addition to this uncatalyzed diffusion of CO_2, several other models for C_i acquisition from the medium into the cell in aquatic plants have been proposed: (i) ATPase-dependent HCO_3^- transport (Raven and Lucas 1985); (ii) H^+/HCO_3^- cotransport or OH^-/HCO_3^- antiport systems (Lucas 1983); (iii) HCO_3^- dehydration in the cell wall dependent on acidic regions created by H^+ extrusion (Lucas 1983); (iv) an "indirect mechanism" of HCO_3^- transport after dehydration to CO_2, i.e. a mechanism requiring extracellular carbonic anhydrase (CA) (Smith and Bidwell 1989a); and (v) active CO_2 transport (Sültemeyer et al. 1989). A specific protein for the transport of HCO_3^- has not yet been identified. Also, the mechanism by which an ATPase is involved in C_i transport is still unknown.

Alkalization of the medium resulting from uptake of C_i has been suggested as being caused by OH^- efflux simultaneous with active influx of HCO_3^- (Lucas 1983; Cook et al. 1988), H^+/HCO_3^- symport (Lucas 1983) or simultaneous uptake of CO_2 and H^+ (Axelsson 1988). The processes involving HCO_3^- transport imply that the dehydration reaction of HCO_3^- to CO_2, which is the substrate for the carbon-fixing enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco), is intracellular. In the reaction, OH^- is produced and intracellular pH is maintained by efflux of OH^- or influx of H^+. However, for many seaweeds, it has not been determined if the mechanism of alkalization is extra- or intracellular. The extracellular conversion of HCO_3^- to CO_2 and OH^-, and the following uptake of CO_2 would result in direct alkalization of the surrounding medium.

In *Chondrus crispus*, extracellular CA was found to be involved in C_i uptake (Smith and Bidwell 1989a). Involvement of CA was greatest at high pH and low CO_2 concentration, and no evidence of any active or facilitated mechanism for the transport of HCO_3^- in *C. crispus* was detected. However, it has been demonstrated for a wide variety of seaweeds that no extracellular CA activity

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Abbreviations: C_i = inorganic carbon ($CO_2 + HCO_3^-$); CA = carbonic anhydrase; DIC = dissolved inorganic carbon (total); DBS = dextran-bound sulfonamide; EZ = ethoxyzolamide; NSW = natural seawater; PPFD = photosynthetic photon flux density; REA = relative enzyme activity; Rubisco = ribulose-1,5-bisphosphate carboxylase/oxygenase

is present (Cook et al. 1988; Giordano and Maberly 1989).

In this work we demonstrate the presence of extracellular and intracellular CA in the red seaweed *Gracilaria tenuistipitata*. The role of CA in carbon assimilation is discussed and we propose a model in which seawater HCO_3^- at alkaline pH is converted by external CA to CO_2. The rate of alkalization of the surrounding medium is inhibited by dextran-bound sulfonamide (DBS), which specifically inhibits extracellular CA. We suggest that the alkalization of the medium by *G. tenuistipitata* is an extracellular mechanism.

Material and methods

Cultivation. Gracilaria tenuistipitata var. *liui* Zhang et Xia was collected in Hainan Island, south China and has since then been kept in unialgal culture in the laboratory. Plant material was brought to the Marine Plant Biotechnology Laboratory (MPBL), Gran Canaria, where the experiments reported in this paper have been carried out. *Gracilaria tenuistipitata* was cultivated in Plexiglas cylinders as described by Lignell et al. (1987). The photon fluence rate followed natural daily irradiances and was at midday maximum 1200–1350 µmol · m^{-2} · s^{-1}. The seawater salinity was 38‰ and temperature varied between 20 and 30° C during the day. pH and growth rates were measured daily and algal biomass was harvested down to initial level. Nutrients were applied according to Provasoli (1968) with the following exceptions: nitrogen was added as NH_4NO_3 at 410 µM, phosphorus was added as NaH_2PO_4 at 30 µM and vitamins were excluded. Daily additions were made according to the produced biomass in order to restore the nitrogen and phosphorus concentrations. Fresh weight was determined after mild centrifugation to remove excess water.

Extraction and fractionation of algal material. Algal thallus (10 g) was carefully ground in liquid nitrogen and extracted with a buffer containing 50 mM Tris (pH 8.5), 25 mM dithiothreitol (DTT), 25 mM isoascorbic acid and 5 mM EDTA. Buffer was added to a total volume of 60 ml. Fractionation of the obtained homogenate was then carried out according to Palmqvist et al. (1990b) with some modifications. The procedure is outlined in Fig. 1. The homogenate was first centrifuged at 100 · *g* for 15 min. Pelleted material contained cell debris including cell-wall material and was not used for assay in this study. The 100 · *g* supernatant was centrifuged at 100 000 · *g* for 40 min and the supernatant from this centrifugation was saved for assay of CA. The pellet from this centrifugation contained chloroplast and plasma membranes and was bright green. This pellet was resuspended in 15 ml extraction buffer and spun down at 10 000 · *g* for 10 min. The new pellet was resuspended in 10 ml extraction buffer and contained chloroplast membranes while plasma membranes could be found in the supernatant. Both these fractions were saved for determination of CA activity.

Preparation of thallus fragments. Algal thallus was chopped to 1-mm pieces with a razor blade and rinsed several times in seawater to get rid of soluble protein that may have leaked out from the cut surfaces. Fragments were then transferred to seawater medium and kept with continuous air bubbling and 100 µmol · m^{-2} · s^{-1} light for at least 6 h before use.

Protoplast isolation. Protoplasts were isolated following the procedure of Björk et al. (1990) with some modifications. Cellulysin 2% (Calbiochem AG, Switzerland) and Agarase 0.01% (Sigma, St. Louis, Mo., USA) were dissolved in seawater at a salinity of 38‰ with addition of 0.2 M mannitol and 20 mM bis[2-hydroxyethyl]imino-tris[hydroxymethyl]-methane (Bis-Tris), final pH-6.0. The algal thallus was chopped into 1-mm pieces using a razor blade. The fragmented thallus was then rinsed five times in washing buffer

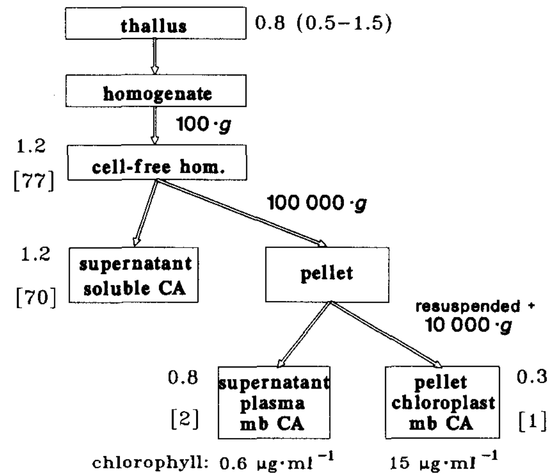

Fig. 1. Flow scheme showing the procedure for fractionation of homogenate from *G. tenuistipitata*. The enzyme activities are denoted beside the boxes of the different fractions and are expressed as REA units · mg^{-1} protein and also as total REA units in each fraction [in *square brackets*]. For thallus, CA activity is expressed per g FW (range indicated in parentheses, $n = 9$) and should not be directly compared with CA activities in the fractions. The fractionation experiment was repeated three times with similar results. The supernatant from the 100 000 · *g* centrifugation had a strong violet colour from phycobiliproteins and the washed pellet was bright green. Chlorophyll concentrations in the two membrane-containing fractions are denoted at the bottom of the figure. *mb*, membrane-bound

(0.2 M mannitol and 20 mM N-2-hydroxyethyl piperazine-N′-2-ethanesulphonic acid (Hepes) in seawater, pH 7), then incubated for 30 min in seawater with additions 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 incubation in enzyme solution was performed at 25° C under constant shaking (60 · min^{-1}). After digestion the protoplast suspension was filtered through a 50-µm nylon mesh filter to remove cell-wall debris and non-digested material, and rinsed with washing buffer. The suspension was layered on top of a centrifugation medium consisting of equal parts of Percoll (Pharmacia, Uppsala, Sweden) and 1.2 M mannitol in seawater buffered with 20 mM Bis-Tris (pH 7). This was then centrifuged in a swingout rotor at 100 · *g*, 15 min. The band containing *G. tenuistipitata* protoplasts was resuspended in buffered seawater of pH 6.5 or 8.6 with addition of 0.2 M mannitol to prevent lysis of the protoplasts and centrifuged again at 100 · *g* for 15 min. Yield of protoplasts ranged from 10^6 to 2 · 10^6 protoplasts per g FW. The resulting pellet of protoplasts was resuspended in the same buffered seawater of pH 6.5 or 8.6 for photosynthesis experiments, as described below.

Preparation of inhibitors. Two inhibitors specific for CA have been used in this study. Stock solutions were prepared as follows: EZ (ethoxyzolamide, Sigma) was dissolved in 0.05 N NaOH to a concentration of 10 mM. Dextran-bound sulfonamide (synthesized according to Tinker et al. (1981), kindly provided by Professor Göran Samuelsson, Department of Plant Physiol., University of Umeå, Sweden) was dissolved in 0.05 N NaOH to a concentration of 0.1 g · ml^{-1} corresponding to 80 mM active inhibitor. Dextran-bound sulfonamide does not penetrate cell membranes and specifically inhibits extracellular CA (Moroney et al. 1985).

Assay of CA in extracts and in thallus fragments. 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 a cuvette containing 2 ml of sample and 2 ml of substrate. The sample was either algal extract or finely chopped

thallus suspended in sample buffer. The sample buffer used was the same as the extraction buffer. The reaction was started by rapidly introducing 2 ml of ice-cold CO_2-saturated distilled H_2O (substrate). 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_0 and t_c are the times for pH change of the nonenzymatic (buffer) and the enzymatic reactions, respectively.

For measurement of CA activity in thallus fragments, 0.16–0.29 g FW was transferred directly from seawater to 2 ml of sample buffer. The same method was used as above with the addition of a magnetic stirrer to keep the fragments in suspension. Final inhibitor concentrations used in CA assay were 50–100 µM EZ and $1 g \cdot l^{-1}$ DBS (800 µM inhibitor).

Photosynthetic O_2 evolution in thallus fragments and protoplasts. Photosynthetic O_2 evolution was measured with a Clark-type electrode (Hansatech Instruments Ltd., Kings Lynn, Norfolk, UK) with the measuring-chamber thermostat set to 25° C. Thallus fragments (0.2–0.3 g FW) or protoplasts ($7–8 \cdot 10^5 ml^{-1}$) were transferred to the measuring chamber, which contained buffered seawater with a low content of dissolved inorganic carbon (low-DIC) of pH 6.5 (100 mM 3-[N-morpholino]propanesulphonic acid (Mops)) or pH 8.6 (100 mM 2[N-cyclohexylamino]ethanesulphonic acid (Ches)), or fresh natural seawater (NSW). Low-DIC seawater was obtained by acidifying NSW to pH 3 and bubbling for 12 h with CO_2-free air to remove DIC, and then adjusting to the desired pH with carbon-free NaOH. Samples were allowed to consume any remaining C_i and $NaHCO_3$ was then added to desired concentration. The volume was adjusted to 1 ml or 0.5 ml when using protoplasts. Oxygen evolution at a photosynthetic photon flux density (PPFD) of 500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ was followed on a flatbed recorder. In sequential experiments with thalli the same thallus fragments could be used while changing the solutions.

Measurement of pH change in the medium caused by whole thalli. Alkalization of the medium was measured with a pH meter (CKC UC–23 with UC–502 E electrode; Central Kagaku Co., Tokyo, Japan). A closed system was obtained with the electrode tip immersed in the medium in a 50-ml Ehrlenmeyer flask by sealing the electrode and flask together with Nescofilm and covering the seal with paraffin to prevent CO_2 exchange with the atmosphere.

Before sealing the Ehrlenmeyer flask it was filled to 55 ml with medium and 0.5 g FW *G. tenuistipitata* was placed in the flask together with a bar magnet. The medium consisted of natural seawater (pH 8.12) or low-DIC seawater (pH 8.12) obtained as described above. The change of pH was recorded after turning on the light. The PPFD used was 440 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and the temperature was 22–24° C.

Measurement of chlorophyll and protein. Chlorophyll was measured after extraction in absolute ethanol according to Wintermans and De Mots (1965), and protein concentration was measured using Serva blue reagent (Serva, Heidelberg, FRG) as described by Peterson (1983). Both chlorophyll and protein were measured in triplicate.

Calculations of spontaneous dehydration of HCO_3^-. The rate of uncatalyzed formation of CO_2 from HCO_3^- in seawater was calculated according to Johnson (1982) using the values of C_i ratios in seawater from Skirrow (1975). The assumption was made that in alkaline pH conditions the CO_2 present in the seawater at the start, as well as the CO_2 formed by dehydration, was consumed almost instantly in photosynthesis and that the CO_2 concentration thus tends to zero. The photosynthetic quotient, i.e. the ratio between O_2 evolution and CO_2 uptake, was not calculated for *G. tenuistipitata*, but the value of 1.17 reported for the O_2/CO_2 ratio of several red, green and brown macroalgae (Axelsson 1988) has been used here.

Results

Carbonic-anhydrase activity. In Fig. 1 we indicate total REA and $REA \cdot mg^{-1}$ protein in thallus fragments and different fractions of homogenized thalli after centrifugation. The results show the presence of extracellular and intracellular CA in *G. tenuistipitata*. Most of the activity was found in the soluble fraction of the cell-free homogenate, although some activity was also found in the green fraction containing chloroplast and plasma membranes. Carbonic-anhydrase activity in fractions of homogenate from *G. tenuistipitata* was inhibited by EZ (DBS not tested); CA activity in *G. tenuistipitata* thallus was inhibited by EZ and DBS

Photosynthetic O_2 evolution in thallus and effect of specific CA inhibitors. The recorded rate of O_2 evolution of *G. tenuistipitata* thallus in unbuffered NSW (pH 8.1, DIC 2 mM) was 400 nmol $O_2 \cdot g\ FW^{-1} \cdot min^{-1}$ (Fig. 2). In buffered seawater the rate of photosynthetic O_2 evolution was highest at pH 6.5 where $NaHCO_3$ had been added to a DIC concentration of 0.5 mM. At pH 8.6 with a DIC concentration of 2 mM, O_2 evolution was one-third of the rate at pH 6.5. When the thallus was transferred from pH 8.6 (2 mM DIC) to pH 6.5 (0.5 mM DIC), photosynthetic O_2 evolution increased to the level recorded first at pH 6.5 (data not shown). The rates of photosynthetic O_2 evolution recorded at pH 8.1 (NSW, 2 mM DIC) and at pH 8.6 (buffered seawater, 2 mM DIC) (Fig. 2) are both higher (1.7- to 3-fold) than the theoretical rate that can be supported by the CO_2 derived

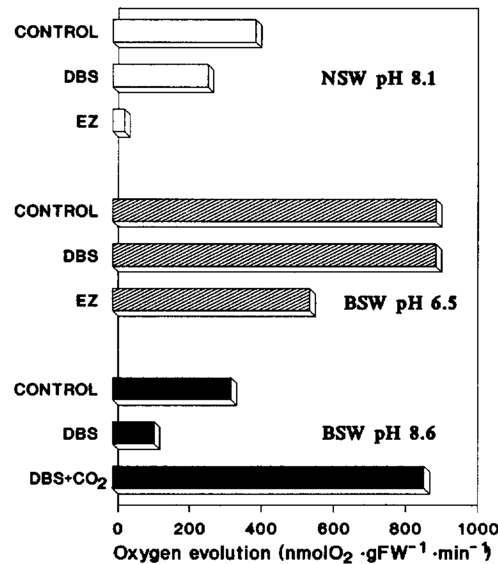

Fig. 2. Photosynthetic O_2 evolution in small thallus pieces of *G. tenuistipitata* and the effect of specific CA inhibitors. Oxygen evolution was measured in three different media, in NSW (pH 8.1, DIC 2 mM), buffered seawater of pH 6.5 (DIC 0.5 mM) and in buffered seawater of pH 8.6 (DIC 2 mM). Inhibitor concentrations: EZ = 100 µM; DBS = $1 g \cdot l^{-1}$ (800 µM inhibitor). The PPFD was 500 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Values are means of ten measurements and variability for the datum points never exceeded 10% of the mean results. *NSW*, natural seawater; *BSW*, buffered seawater

Table 1. Comparison of the observed rates of photosynthetic O_2 evolution at alkaline pH in *G. tenuistipitata* with the O_2 evolution that could, in the present experimental setup, theoretically be supported by the CO_2 derived from uncatalyzed, spontaneous dehydration of HCO_3^- (according to Johnson 1982), assuming a photosynthetic quotient of 1.17 (Axelsson 1988). pH and HCO_3^--concentration both determine the theoretical dehydration rate. The observed photosynthesis rates were calculated from the values shown in Fig. 2. *NSW*, natural seawater; *BSW*, buffered seawater

Treatment	O_2 evolution (nmol · ml^{-1} · min^{-1})			
	NSW pH 8.1, 2 mM DIC		BSW pH 8.6, 2 mM DIC	
	Observed	CO_2-supported	Observed	CO_2-supported
Control	100	56	83	27
DBS 800 μM	67	56	29	27

from uncatalyzed, spontaneous dehydration of HCO_3^- (see Table 1).

In natural seawater (pH 8.1, DIC 2 mM) EZ inhibited O_2 evolution by 90% while the inhibition with DBS was approx. 30%. At pH 8.6 (2 mM DIC) in buffered seawater, DBS reduced O_2 evolution by 65%. The rates of O_2 evolution after inhibition by DBS were, at both pHs, similar to the theoretical rates that could be supported by spontaneous dehydration of HCO_3^- (Table 1). Photosynthetic O_2 evolution at pH 6.5 (0.5 mM DIC) was not affected by DBS but was reduced by 40% by EZ (Fig. 2). At pH 8.6 (2 mM DIC) upon addition of 10 μl CO_2-saturated water (340 μM CO_2) the rate of O_2 evolution increased to the same level as in pH 6.5 conditions.

Photosynthetic O_2 evolution in protoplasts. In Fig. 3 we show photosynthetic O_2 evolution of *G. tenuistipitata* protoplasts at pH 6.5 and pH 8.6 in the presence of various concentrations of HCO_3^-. At pH 6.5, O_2 evolution increased strongly upon addition of 100 μM HCO_3^- and remained fairly constant at higher concentrations. However, at pH 8.6, O_2 evolution increased slowly and at a more linear rate with increasing HCO_3^- concentration. The O_2 evolution rates at both pH values are lower than can be supported by spontaneous dehydration of HCO_3^-, indicating that the measuring conditions induce some stress to the protoplasts. Oxygen evolution is expressed per 10^6 protoplasts which is the average yield from 1 g FW thallus and also the number of protoplasts present in the measuring chamber (1 ml). Assuming a density of the protoplasts of 1.1 g · cm^{-3} and an average diameter of 11 μm, 1.3 · 10^9 protoplasts would have the biomass of 1 g FW.

Effect of DBS on the rate of pH change. During cultivation the growth rate of *G. tenuistipitata* was 18–37% · d^{-1} and the pH increased from 8.2 to 9.2 in 7 d. The alkalization only took place during the light period of cultivation; in darkness the medium was slowly acidified.

The pH change caused by photosynthetic alkalization of natural seawater by *G. tenuistipitata* was recorded with and without addition of DBS. The results are shown in

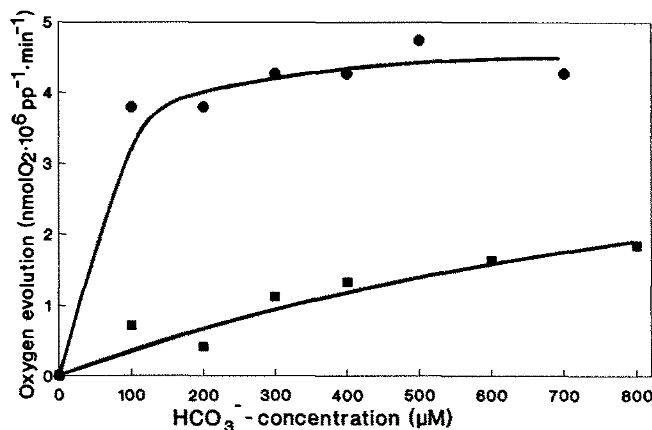

Fig. 3. Photosynthetic O_2 evolution by protoplasts isolated from *G. tenuistipitata* in buffered seawater of pH 6.5 (●) and pH 8.5 (■) with increasing concentration of HCO_3^-. Values on the x-axis show added concentrations of HCO_3^-. 1 g FW thallus yields approx. 10^6 protoplasts, i.e. the amount used per ml in the experiments. The PPFD was 500 μmol · m^{-2} · s^{-1}. Datum points represent one experiment. The experiment was repeated twice with similar results. *pp*, protoplasts

Fig. 4A where pH is plotted against time after turning on the light. Initially, we observed a slight acidification and, after 4–5 min, a linear alkalization. The alkalization rate in the presence of DBS was less than half the rate of the control. The alkalization rate was steady for longer periods than indicated in the figure.

In seawater with low DIC (see *Material and methods*) a slow acidification continued for 3 min and the following alkalization was slow and not linear (Fig. 4B). Upon addition of 1 mM HCO_3^-, alkalization increased to a rate similar to the control experiment in Fig. 4A. This demonstrates that the alkalization rate is dependent on the concentration of DIC. Subsequent addition of DBS strongly inhibited this alkalization. After rinsing the algal thallus to wash away DBS, the alkalization rate was restored to the rate before addition of DBS (data not shown).

Discussion

To our knowledge this is the first report on the presence of CA in *Gracilaria*. The presence of CA in marine macroalgae has previously been demonstrated by several authors, both in homogenates (Bowes 1969; Graham and Smillie 1976; Smith and Bidwell 1987; Beer and Israel 1990) and extracellularly (Giordano and Maberly 1989; Smith and Bidwell 1989a). The presence of CA in microalgae, including the red microalga *Porphyridium cruentum*, both intra- and extracellularly, is also well documented (e.g. Yagawa et al. 1987; Burns and Beardall 1988). Its role in carbon assimilation and photosynthesis is best characterized in the green algal genus *Chlamydomonas*. Studies of C_i accumulation in *C. reinhardtii* (Moroney et al. 1985; Palmqvist et al. 1990a) have shown that external CA at alkaline pH catalyzes HCO_3^- to CO_2 which then crosses the plasmalemma.

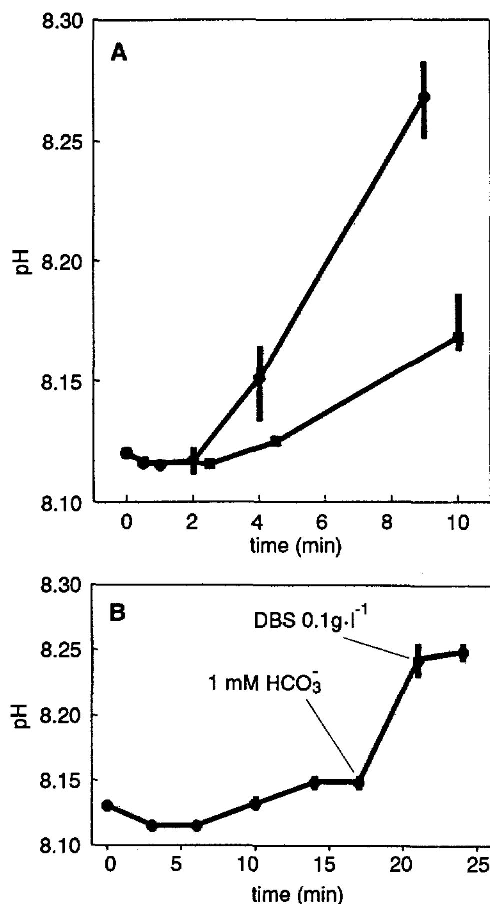

Fig. 4. A Influence of the CA inhibitor DBS on the rate of alkalization by whole thallus of *G. tenuistipitata* in natural seawater. Light (PPFD = 440 µmol · m^{-2} · s^{-1}) was turned on at time 0. Datum points are obtained from calculating the average slope of *n* separate pH-change curves (●, control, $n = 7$; ■, DBS 0.1 g · l^{-1} (80 µM inhibitor), $n = 4$). **B** Rate of alkalization by whole thallus of *G. tenuistipitata* in low-DIC seawater and the influence of HCO_3^- and DBS. Additions of HCO_3^- (1 mM) and DBS (0.1 g · l^{-1}, 80 µM inhibitor) are indicated by *arrows*. Light (PPFD = 440 µmol · m^{-2} · s^{-1}) was turned on at time 0. Datum points are obtained from calculating the average slope of 2 separate pH-change curves. *Vertical bars* indicate range. When bars are absent the range is smaller than the size of the symbols

The present report of CA activity in chloroplast membranes in *G. tenuistipitata* is the first for red macroalgae. It is in accordance with the presence of CA in the chloroplasts of microalgae reported by many authors (e.g. Sültemeyer et al. 1990; Coleman et al. 1991). Also, Palmqvist et al. (1990b) demonstrated the presence of CA activity in thylakoid and plasma membranes of four different green algae. The chloroplast of microalgae has been suggested to be the site for a C_i-concentrating mechanism (Beardall 1981; Moroney et al. 1985, 1987). Carbonic-anhydrase activity has also been found in the chloroplasts of the green macroalgae *Codium* and *Udotea* (Reiskind et al. 1988). The presence of CA in different fractions of homogenized *G. tenuistipitata* could involve the presence of different isoenzymes of CA.

Several results in this work indicate that *G. tenuistipitata* possesses extracellular CA and that this enzyme is involved in photosynthesis. Both DBS, which does not penetrate membranes, and EZ, which penetrates easily (Moroney et al. 1985), inhibit photosynthetic O_2 evolution in thallus fragments. However, EZ has a stronger inhibitory effect than DBS since it inhibits both extra- and intracellular CA activity. Smith and Bidwell (1987) have reported similar results with *Chondrus crispus*. Photosynthetic C_i uptake in this alga was inhibited by both acetazolamide and DBS. Inhibition by acetazolamide was greater and the effect was greatest at low CO_2 concentrations.

Since DIC in natural seawater of pH 8.0–8.2 predominantly consists of HCO_3^-, it seems likely that marine algae would be able to utilize this form of C_i. Carbon-acquisition strategies vary among marine algae, as well as the form of C_i utilized for growth and photosynthesis. Marine algae have been shown to use only CO_2 (Tseng and Sweeney 1946; Jolliffe and Tregunna 1970; Maberly 1990), or to have also the ability to utilize HCO_3^- (Sand-Jensen and Gordon 1984; Bidwell and McLachlan 1985; Smith and Bidwell 1987, Lignell and Pedersén 1989; Beer and Israel 1990; Maberly 1990). Results are sometimes conflicting. Thus *Chondrus crispus* has been shown to utilize HCO_3^- (Bréchignac et al. 1986; Smith and Bidwell 1987; Maberly 1990) but Smith and Bidwell (1989a) reported the absence of an active HCO_3^--transporting mechanism in this species. Instead they found CA at the cell surface and proposed a model whereby CA converts HCO_3^- to CO_2 which then passively diffuses into the cell. The same mechanism has been suggested by Surif and Raven (1989) for brown algae of the Fucales and the Laminariales, although they state that diffusive entry of CO_2 into the cell is not enough to supply the Rubisco of these algae and therefore imply that a CO_2-concentrating mechanism operates even when extracellular CA is present.

By contrast, the absence of extracellular CA has been reported in several (including red) macroalgae (Cook et al. 1986, 1988; Maberly 1990). Giordano and Maberly (1989) reported CA to be absent in *Chondrus crispus*, both externally and internally, contrary to the findings of Smith and Bidwell (1989a). In several algae reported to lack extracellular CA, photosynthesis rates exceeding the rate that could be supported by uncatalyzed conversion of HCO_3^- to CO_2 have been found, indicating an uptake of HCO_3^- by these algae (Cook et al. 1986, 1988; Maberly 1990).

Changes in pH have been used as a measure of carbon uptake in macroalgae (Lindahl 1963; Haglund et al. 1987; Axelsson 1988). Moreover, the photosynthetic alkalization of the medium has been suggested as being caused by an energy-driven process involving OH^- efflux or H^+ influx coupled with active transport of HCO_3^- (Kerby and Raven 1985; Cook et al. 1988). In this case, the alkalization takes place inside the cell, and intracellular pH is maintained through efflux of OH^- or influx of H^+.

In *G. tenuistipitata*, we have found extracellular CA and have observed that specific inhibition of this CA considerably reduces photosynthesis, both measured as O_2 evolution and as pH change. We suggest that this alga

uses HCO_3^- and that extracellular CA catalyses the conversion to CO_2 at the surface of the plasmalemma. Carbon dioxide then enters into the cell where its further transport to the site of CO_2 fixation is enhanced by interconversion between CO_2 and HCO_3^- catalysed by intracellular CA (Enns 1967; Kerby and Raven 1985).

This hypothesis is supported by our observation that at alkaline pH photosynthetic O_2 evolution in *G. tenuistipitata* is higher than could be supported by spontaneous conversion of HCO_3^- to CO_2 (see Table 1 and Fig. 2). Also, DBS, a specific inhibitor of extracellular CA, reduces photosynthetic O_2 evolution both in natural seawater (pH 8.2) and in buffered seawater (pH 8.6) to levels similar to those that could be supported by spontaneous dehydration of HCO_3^- to CO_2. These findings imply the involvement of extracellular CA in the photosynthetic carbon uptake of this alga. Photosynthetic O_2 evolution in *G. tenuistipitata* was higher in buffered seawater of pH 6.5 (DIC 0.5 mM), when approx. 30% of the C_i exists as CO_2, than at pH 8.6 (DIC 2 mM), when the concentration of CO_2 is close to zero. This indicates a preference for CO_2 as a carbon source in this alga (Fig. 2). As a further indication of CO_2 preference, addition of CO_2 to *G. tenuistipitata* at pH 8.6 increased O_2 evolution to the same rate as at pH 6.5. This CO_2-addition took place in the presence of DBS, which shows that the inhibition of extracellular CA does not affect CO_2 uptake. Addition of EZ at pH 6.5, however, inhibited the O_2 evolution by 40%, showing the importance of the interconversion between CO_2 and HCO_3^- catalyzed by intracellular CA. Protoplasts of *G. tenuistipitata* exhibited a similar preference for CO_2 as carbon source when O_2 evolution was measured in response to an increased concentration of DIC. The rates of photosynthesis for protoplasts are lower than the rates recorded for thalli if the FW per protoplast is calculated according to our method, indicating that the protoplasts are subjected to stress in vitro. This is in accordance with the results reported by Smith and Bidwell (1989b) for protoplasts of *Chondrus crispus*. The CO_2-preference of *G. tenuistipitata* thalli is in agreement with a report by Sand-Jensen and Gordon (1984) that photosynthetic O_2 evolution declined at pH values above 8.0–8.4 for two red algae also able to use HCO_3^-. As early as 1946, Tseng and Sweeney showed that photosynthetic O_2 evolution in newly collected *Gelidium cartilagineum* was higher at pH 6.5 than at pH 8.5 in normal seawater.

A further indication that extracellular CA is involved in photosynthesis and C_i uptake is the observation that photosynthetic alkalization of the seawater medium by *Gracilaria tenuistipitata* is inhibited by DBS. Such an extracellular-CA-dependent alkalization has recently been reported for the chlorophyte *Ulva rigida* by Björk et al. (1992).

Conclusions. We assume that extracellular CA in *G. tenuistipitata* converts HCO_3^- in the surrounding medium to CO_2, which is the form of C_i entering through the plasma membrane. The high amount of soluble CA in *G. tenuistipitata* indicates that intracellular CA is involved in the subsequent transport and assimilation of C_i. The presence of CA in the fraction containing chloroplast membranes indicates the involvement of CA in interconversion and transport of CO_2 and HCO_3^- also in the chloroplast.

Furthermore, we suggest that the CA-catalyzed dehydration of HCO_3^- followed by CO_2 uptake causes alkalization of the surrounding medium, i.e. the photosynthetic alkalization is an extracellular process in *G. tenuistipitata*. From the presented data we cannot entirely exclude the presence of an additional HCO_3^--transporting system in this alga, but such a contribution would be minor. Investigations of the role of the chloroplast in C_i acquisition in *G. tenuistipitata* are needed and further experiments to clarify the C_i-uptake mechanisms close to the site of Rubisco are in progress.

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