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Evidence for a plasmalemma-based CO_2 concentrating mechanism in *Laminaria saccharina*

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Abstract

A kinetic analysis of the photosynthesis inhibition by buffers allowed quantification of some components of the carbon concentrating mechanism (CCM) of the brown macroalga *Laminaria saccharina*. The CCM was based on the presence of acid regions outside the plasma membrane that increased the CO_2 concentration available for photosynthesis by 10–20 times above that of the bulk medium at alkaline pH. Furthermore, the results suggested that the CCM is located mainly on the cell membrane and not in the chloroplast, as suggested for most macroalgae. The degree of dissipation of the acid regions by a buffer was related to the buffer anion concentration (B^-), estimated from the titration of the buffer from bulk medium pH to a pH endpoint value close to the first pK_a of the carbonic acid system. A kinetic model describing the relationship between inhibition of photosynthesis by a buffer and B^- was developed suggesting that buffers act as competitive inhibitors with IC_{50} (the concentration of the buffer anion which reduces the reaction velocity by half) of 5.0 mol m^{-3}. This model can be used to estimate the inhibitory effect of any buffer on the photosynthesis of *L. saccharina*. Nevertheless, some buffers tested showed a lower effect than that predicted from the hyperbolic model suggesting that their strength as inhibitors depended on: (1) the pK_a in relation to the first pK_a of the carbonic acid system and (2) its molecular weight (i.e. its mobility).

Introduction

The presence of carbon concentrating mechanisms (CCMs) that generate a high CO_2 concentration around Rubisco (higher than that explained by diffusion of inorganic carbon from the bulk medium) has been well documented in algae. In spite of their likely polyphyletic origin (Badger et al. 2002), it is usually assumed that all CCMs are composed of at least three functional elements (Badger 2003): (i) influx of CO_2 and/or HCO_3^-, (ii) capture of dissolved inorganic carbon inside the cell (usually as HCO_3^-) and (iii) production of CO_2 from the dissolved inorganic carbon (DIC) pool around Rubisco. The subcellular location and function of these components have been extensively studied in cyanobacteria and microalgae (see Colman et al. 2002 and Badger 2003, for recent reviews). In all cases, internal carbonic anhydrase (CA) seems to be an essential component of the CCMs.

Comparatively few investigations have dealt with marine macroalgae. Many macroalgae, especially sublittoral species, feature a photosynthetic physiology that suggests the presence of CCMs and/or mechanisms for HCO_3^- utilization (Cook et al. 1986; Surif and Raven 1989; Maberly 1990; Axelsson et al. 1991; Johnston et al. 1992; Mercado et al. 1998). For most of the studied species, carbonic anhydrase was involved in the HCO_3^- utilization presumably via its transformation into CO_2 (Smith and Bidwell 1987; Björk et al. 1992, 1993; Haglund et al. 1992a; Mercado et al. 1997). HCO_3^- uptake through the cell membrane via an anion exchanger has been demonstrated in many species of green macroalgae (reviewed by Beer 1994; Larsson and Axelsson 1999). Active transport of CO_2 has been suggested in a red alga species (Mercado et al. 1997), although no direct evidence was presented. Sensitivity to buffers, which indicates a HCO_3^- utilization relying on extracellular acidic compartments (Prins et al. 1980; Price and Badger 1985), has so far been presented only for a few green and brown macroalgae, although preliminary results indicate that this mechanism might be quite common among brown and red macroalgae (Axelsson and Beer 2001).

Support for the presence of an internal component of the CCM in macroalgae is largely based on the inhibition of the internal CA, which is often linked to a remarkable decrease of the photosynthesis rate at alkaline pH, higher than that obtained by inhibition of the external CA (Mercado et al. 1998). Further evidence comes from the parallel de-activation of internal CA and CCM during growth under increased CO_2 concentrations, although this has been demonstrated only in a few species (García-Sánchez et al. 1994; Mercado et al. 1997). Most of the CA activity in macroalgae appears to be internal (Giordano and Maberly 1989; Haglund et al. 1992b). Given that the presence of CA in the cytosol could increase the CO_2 leakage, it has been postulated that CA is located mainly in the chloroplast. However, the sub-cellular location of the CA has only been determined in a few macroalgal species. Thus, Haglund et al. (1992b), Mercado et al. (1999) and Andria et al. (2000) reported CA activity in thylakoid membranes, indicating the presence of chloroplast-associated CA in four species of green and red algae with CCM.

For some macroalgae, with obvious capacity to use HCO_3^-, direct measurements of internal CA have resulted in surprisingly low levels (Giordano and Maberly 1989; Surif and Raven 1989), especially within members of the family Laminariaceae. These results suggest that the function of the internal CA in the accumulation and mobilization of the hypothetical internal DIC pool could be replaced by another mechanism. As an alternative, these species might use external CA to accumulate DIC in the internal intracellular spaces. Thus, the place for DIC accumulation could be (at least partly) outside the cells as suggested by Schmid and Dring (1996). Some evidence supporting the latter suggestion has been presented by Axelsson et al. (2000) for *Laminaria saccharina*, suggesting that the use of HCO_3^- in *L. saccharina* is based on the simultaneous operation of two components; proton pumps, which contribute to the creation of acid zones in the cell wall region just outside the cell membrane, and periplasmic CA activity, which speeds up the dehydration of HCO_3^- to CO_2 within these zones. Based on the low levels (or likely lack) of internal CA activity (Surif and Raven 1989), Axelsson et al. (2000) postulated that the CCM in *L. saccharina* is located at the plasmalemma, in contrast to the majority of macroalgae. Figure 1a shows a model of this putative CCM. This model depicts the two functional elements mentioned above: (1) a local H^+ extrusion (which must be counterbalanced by OH^- extrusion in other areas) which creates a low pH (pH_a) area where CO_2 concentration ($[CO_2]_a$) is increased above its concentration in the bulk medium ($[CO_2]_o$); and, (2) an external CA activity (reaction (i) in Figure 1a) that speeds up HCO_3^- dehydration to CO_2 within these zones. In the present study, new data and kinetic analysis supporting this model are presented.

Materials and methods

Laminaria saccharina (L.) Lamour (Phaeophyta) and *Ulva lactuca* L. (Chlorophyta) were collected at 0.5–1.0 m depth outside Kristineberg Marine Research Station, on the Swedish west coast. The plants were either used immediately for the experiments, or kept at low irradiance (ca. 200 μmol photons m^{-2} s^{-1}) in a flux of natural seawater (NSW) from 35 m depth (salinity 28–30 PSU;

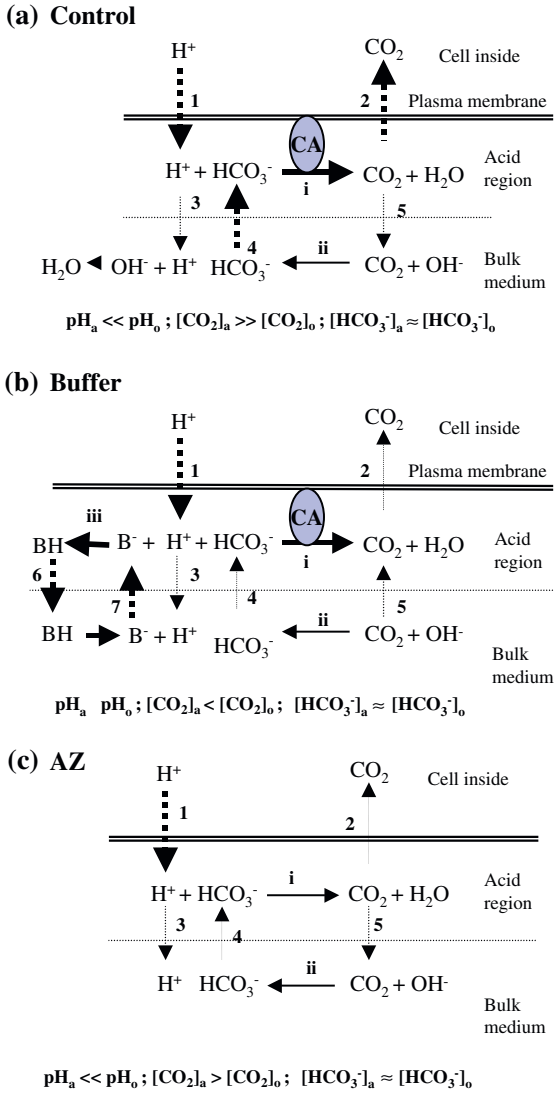

Figure 1. Functional model of the inorganic carbon acquisition mechanism in *Laminaria saccharina*. (a) Control (photosynthesis in natural seawater at pH 8.1); (b) after buffer (Buffer) addition; (c) after acetazolamide (AZ) addition. The main components of the model are: 1. An extracellular acidic compartment with a pH (pH_a) different from that of the bulk medium (pH_o). 2. External carbonic anhydrase (CA) that speeds-up the HCO_3^- dehydration (reaction (i)). The vertical dotted arrows denote flux reactions of different chemical species.

temperature ca. 10 °C). The thalli of *U. lactuca* were cultivated in Perspex cylinders, 0.6 m long and 0.2 m in diameter, supplied with a continuous flux (1 l min^{-1}) of temperature controlled natural seawater (NSW; 30 PSU, 18 °C). The water in the cylinders was bubbled with air (1 l min^{-1}) to gently move the thalli continuously in a vertical loop, and supplied with additional NO_3^- and PO_4^{3-} to yield a steady state concentration in the medium above 10 mmol m^{-3} NO_3^- and 2 mmol m^{-3} PO_4^{3-}. A 16 h/8 h light/dark cycle was provided by a metal halogen lamp (Power Star HQI – T 400 W/D; Osram, www.osram.ge) at a distance of 0.15 m, yielding an irradiance of ca. 1700 μmol photons m^{-2} s^{-1} at the side of the cylinder facing the lamp (as measured with a LI 1000 datalogger, equipped with a cosine corrected quantum sensor; LI-COR, www.env.licor.com). Due to the movement of the algal thalli in the cylinder, they were exposed to a strongly fluctuating light field.

Oxygen electrode set-up

Photosynthetic O_2 evolution was measured under light saturating conditions (ca. 1000 μmol photons m^{-2} s^{-1}) in temperature-controlled O_2-electrode chambers (15–16 °C) with a volume of 3.0 ml. Thallus discs were punched out with a 12-mm diameter cork borer. One to three discs (ca. 18 mg fresh weight per disc) were washed in NSW before being enclosed in the electrode chambers. Oxygen electrode tracings were recorded on a strip chart recorder (Seconic flat bed recorder, Seconic Inc, Japan). Once steady photosynthesis was reached, the inhibitory effects of several buffers and of the CA inhibitors, alone and in different combinations, were tested.

The CA inhibitors, acetazolamide (AZ), dextran-bound sulfonamide (DBS) and ethoxyzolamide (EZ), were prepared as stock solutions of 20 mol m^{-3} dissolved in 20 ml m^{-3} NaOH. They were added to a final concentration of 0.1 mol m^{-3} (referring to the concentration of the active ligand for DBS). The buffers used were Tris [(hydroxymethyl)-aminomethane], Hepes [*N*-(2-Hydroxyethyl)piperazine-*N*-(2-ethanesulfonic acid)], Ampso [*N*-(1,1-Dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid] and Mes [2-Morpholinoethanesulfonic acid] (Biological buffers, Sigma). Stock solutions of 1 or 2 kmol m^{-3} were prepared by adding either HCl or NaOH (DIC free) solution. The buffers were added to a final concentration of 50 mol m^{-3}, except for the Mes buffer, where a final concentration of 25 mol m^{-3} was used.

The inhibitory effect of Tris and AZ was tested at different pH values (7.0, 7.5, 8.1, 8.7, 9.0 and 9.5). Before these measurements, a seawater

medium was prepared from natural seawater by adding small amounts of NaOH or HCl solutions. After steady-state photosynthesis was reached, the inhibitors were added from stock solutions, prepared to maintain the pH of the seawater medium. Since changes in pH affected the free-CO_2 concentration, O_2 evolution rates vs. free-CO_2 concentration curves were constructed by calculating the CO_2 concentration at each pH assayed. The concentration of different inorganic carbon species was calculated according to Millero (1979).

Titration of seawater and pH measurement

The buffering range of the medium was analysed by titration of 20 ml samples, collected from the O_2-electrode chambers directly after an experiment. The titration was performed at 20 °C, adding small aliquots of 100 mol m^{-3} HCl and measuring the pH after each addition. As a measure of the H^+ capturing capacity of the buffer anion, the acid volume added to a chosen endpoint (usually pH 5) was calculated. The pH was measured with a probe equipped with a glass electrode and a double junction Ag/AgCl reference electrode calibrated according to the NBS scale.

Results

The effect of EZ on photosynthetic oxygen evolution in *L. saccharina* was measured at pH 7.5 (Figure 2). EZ can easily penetrate into the cells and inhibits both external and internal CA activity. Therefore, to test the relevance of the internal CA activity for photosynthesis, the effect by EZ was tested in the presence of an inhibitor of the extracellular CA (AZ or DBS). At the assayed pH, the inhibition of the external CA by either AZ or DBS resulted in a 30% reduction of the net photosynthesis in *L. saccharina*. Inhibition to a similar degree was obtained for the green macroalga *U. lactuca* treated with AZ (Figure 2). The O_2 evolution rates achieved under the external CA inhibition indicate that the spontaneous HCO_3^- dehydration at pH 7.5 was fast enough (and the equilibrium CO_2 concentration high enough) to support a fairly high rate of net photosynthesis. Comparing results from EZ + EZ with AZ + EZ and DBS + EZ suggested an extra decrease in photosynthesis due to internal CA inhibition of

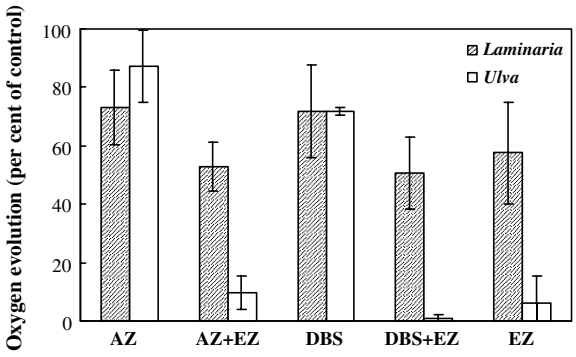

Figure 2. Inhibition of the net photosynthesis for *L. saccharina* (closed bars) and *U. lactuca* (open bars) following the addition of CA inhibitors (AZ and DBS). DBS does not penetrate the plasma membrane, while EZ readily penetrates cell membranes, and thus inhibits both external and internal CA. Each inhibitor was added to a final concentration of 0.1 mol m^{-3} (referring to the active ligand for DBS). The results are the mean values of four independent measurements; bars indicate standard deviations.

7.8 ± 5.3% (referring to the control in Figure 2; mean ± SD, $n = 8$), although the photosynthesis rates obtained in the presence of EZ did not differ significantly from the rates obtained in the presence of AZ or DBS (ANOVA, $p = 0.26$). In contrast, EZ inhibited almost completely (to more than 90%) the photosynthesis in *U. lactuca* (which has a CCM located to the chloroplast; Axelsson et al. 1999). If it is assumed that plasmalemma permeability to EZ was similar in *L. saccharina* and *U. lactuca*, it can be concluded that photosynthesis in *L. saccharina* did not rely on internal CA activity, in contrast to the situation proposed for *U. lactuca* and for most of the macroalgae previously tested (Mercado et al. 1998).

The CCM model for *L. saccharina* in Figure 1a assumes that DIC enters the cell through the cell membrane as free-CO_2 (flux 2). Proton pumps create acid regions close to the cell membrane and external CA (probably attached to the membrane outside) speeds up the reversible inter-conversion between HCO_3^- and CO_2 within these acid regions (reaction (i)). Thus, the low pH within the acid zones (pH_a) can maintain a high concentration of free-CO_2 as long as the HCO_3^- pool is refilled from the bulk medium (flux 4). To check this model, the effects of buffer and AZ additions on photosynthesis were measured at different pH values. The source of DIC (HCO_3^- or CO_2) was modified by changing the pH of the bulk medium (pH_o). Thus, an increase of pH_o from 7.0 to 9.6 results in a

decrease in the relative free-CO_2 to DIC concentration from 0.1 to less than 0.0001. The HCO_3^- concentration is less affected since it varies from 1.9 to 0.7 mol m^{-3} for the same pH range. Figure 3 shows the oxygen evolution rates as a function of the free-CO_2 concentration in the bulk phase ($[CO_2]_o$) calculated for each pH assayed. Four treatments were tested following this approach:

Control

Under control conditions (i.e., without AZ and/or buffer addition; Figure 1a), the changes in photosynthetic rate more or less coincide with the changes in HCO_3^- concentration, as should be expected for this HCO_3^- utilizing mechanism (Axelsson et al. 2000). However, between pH 9.0 and 9.5, the drop in net photosynthetic rate (87%) is much higher than the drop in HCO_3^- concentration (44%) suggesting that the loss of H^+ and CO_2 (fluxes 3 and 5) is considerable. The hyperbolic shape of the net photosynthesis vs. $[CO_2]_o$ curve is thus a consequence of the adjustment of the fluxes 3, 4 and 5 (Figure 1).

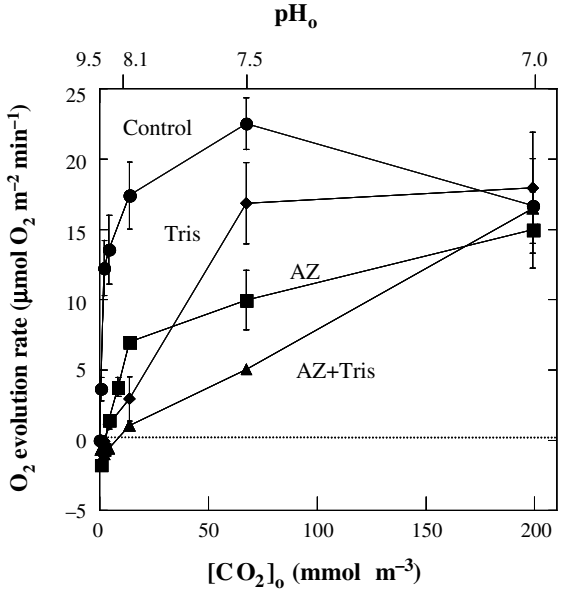

Figure 3. Net photosynthesis rate of *L. saccharina* in natural seawater as a function of the free-CO_2 concentration at different pH. The pH of the seawater was adjusted to pH values ranging from 7.0 to 9.5 by aliquots of HCl or NaOH. Following the achievement of a steady-state photosynthesis, Tris buffer (final concentration 50 mol m^{-3}) and/or AZ (concentration 0.1 mol m^{-3}) prepared to maintain the actual pH were added as indicated. The results are the mean values of six independent measurements; bars indicate standard deviations.

Buffer

The addition of Tris buffer resulted in a significant decrease in the photosynthetic rates at pH values above 7.5 (i.e. at $[CO_2]_o$ below 60 µM; Figure 3). The buffer effect at alkaline pH_o strongly suggests that the acid regions were disrupted under this treatment. Figure 1b shows the mechanism responsible for that reaction (fluxes 6 and 7). Thus, the buffer is assumed to act as a proton dissipating system facilitating the H^+ diffusion out of the acid zone (as proposed by Price and Badger 1985 for *Chara corallina*) and equilibrating, consequently, pH_o and pH_a. Under such conditions, HCO_3^- use should depend exclusively on the external CA activity, which is not able to increase the CO_2 concentration above the chemical equilibrium (i.e. that at the pH of the bulk medium). Therefore, flux 2 will be limited by the CO_2-equilibrium concentration just outside the cell membrane. As a consequence of the high HCO_3^- concentration and the CA activity located at the cell membrane, this CO_2 concentration will be about the same as that of the medium. The initial slope of the curve (1.0 µmol O_2 m^{-2} min^{-1} [mmol m^{-3} of CO_2]$^{-1}$) was much reduced as compared to that of the control curve (21 µmol O_2 m^{-2} min^{-1} [mmol m^{-3} of CO_2]$^{-1}$). If we assume that the driving force for flux 2 is the CO_2 concentration just outside the plasma membrane it suggests that CO_2 concentration in the acid zones could be up to 20 times higher in the control samples than in the samples with Tris buffer.

AZ

Following the addition of AZ, photosynthesis vs. $[CO_2]_o$ curve described a bi-linear kinetic (Figure 3). The only part of the HCO_3^- use system activated under these conditions was the noncatalysed HCO_3^- dehydration in the acid zones. The change in the slope was observed at CO_2 concentration of 14 mmol m^{-3} (i.e. at pH_o 8.1). The initial slope (i.e. below 14 mmol m^{-3} CO_2) was twofold higher than that recorded for the buffer treatment indicating that $[CO_2]_a$ was higher than that expected from the chemical equilibrium at pH_o. Therefore, the acid regions must remain

operative under this treatment (i.e. pH_a must be lower than pH_o). However, the initial slope was also significantly lower than that of the Control, indicating that $[CO_2]_a$ was lower than the corresponding value of the control. This, in turn, indicates that the noncatalysed HCO_3^- dehydration rate (i.e. the reaction (i) when CA is inhibited, Figure 1c) was slower than the CA-catalysed HCO_3^- dehydration. Consequently, flux 2 was lower than in the control samples.

Tris plus AZ

When both inhibitors were added simultaneously, a linear relationship between O_2 evolution and $[CO_2]_o$ was observed (Figure 3; $r^2 = 0.99$, $p < 0.001$). Interestingly, the slope of this line (0.3 μmol O_2 m^{-2} min^{-1} [mmol m^{-3} CO_2]$^{-1}$) was similar to that obtained from O_2 evolution vs. DIC concentration curves performed in synthetic seawater at pH 5.6 (where 72% of DIC is in the CO_2 form). The linear shape of the curve indicates that the CO_2 diffusion from the bulk medium towards the cell proximity limited photosynthesis. Also, this treatment suggests that the involvement from any direct HCO_3^- uptake mechanism to the DIC-supply for photosynthesis (such as the DIDS sensitive anion exchange mechanism of green macroalgae, Beer 1994, Axelsson et al. 1995, Larsson and Axelsson 1999) is unlikely since photosynthesis rates at alkaline pH were low despite the rather high HCO_3^- concentration. The very low photosynthesis rates obtained at alkaline pH_o in this treatment thus indicate that photosynthesis of *L. saccharina* control samples was based mainly on the use of HCO_3^- via external CA and acid zones.

The pH_a values and the CO_2 concentration gradients driven by the H^+ pump when CCM of *L. saccharina* is fully functioning can be roughly estimated from the results showed in Figure 3. *A priori*, the photosynthetic CO_2 flux (flux 2 in Figure 1) should be driven mainly by the CO_2 gradient between the chloroplast and the outside of the cell membrane, where the CA and the acid regions are located. This should be the case for all the treatments, provided photosynthesis is limited by this CO_2 concentration. Thus, for a specific rate of photosynthesis, this CO_2 gradient (and also $[CO_2]_a$, i.e. the CO_2 concentration outside the cell membrane) should be the same in all the curves of Figure 3. When the acid zones are dissipated (i.e. after buffer addition), $[CO_2]_a$ should be very close to $[CO_2]_o$, i.e. the CO_2 concentration of the bulk medium. The conditions for such approximation are a high HCO_3^- concentration (resulting in a similar HCO_3^- concentration in the medium and the acid zones) and the presence of CA in the acid zones. Thus, the Tris curve can be used as a key graph to estimate the value for $[CO_2]_a$ of the other curves. As an example, at pH 7.5 photosynthesis is about 60 μmol O_2 m^{-2} min^{-1} and the CO_2 concentration (both $[CO_2]_a$ and $[CO_2]_o$) is about 70 mmol m^{-3}. For the control, a similar rate of photosynthesis occurs at pH 8.1, where $[CO_2]_o$ is below 20 mmol m^{-3}, suggesting that a pH close to 7.5 in the acid zones of the control have caused an increase in the CO_2 concentration by ca. 50 mmol m^{-3}.

According to the model described in Figure 1b, buffer anion (B^-) competes with the HCO_3^- for H^+. Therefore, the degree of disruption of the external acidic band by the buffer should primarily depend on the B^- concentration. This concentration depends on the buffer pK_a relative to pH_o and pH_a (Price et al. 1985). The titration of the buffer from pH_o to pH_a could be an estimation of the active B^- concentration. Figure 4 shows the inhibition by different Tris concentrations as a function of the active B^- concentration calculated by choosing a pH_a of 6.5. As expected for a competitive inhibition, data fitted to a hyperbola ($r^2 = 0.98$, $p < 0.001$), with a maximum inhibition of 94.5%. From this function, a B_{50}^- value (the concentration of the buffer anion which reduces the photosynthesis rate by half) of 5.0 mol m^{-3} was obtained. The hyperbolic function also predicts acceptably the inhibition obtained using other buffers (Mes, Ampso and Hepes, Figure 5). However, some significant deviations were obtained, particularly the inhibitory effects calculated for Mes and Hepes were overestimated.

Interestingly, this hyperbolic model explains adequately the Tris inhibitory effect at the different pH_o's used in Figure 3 (as calculated taking into account the B^- concentration estimated for each given pH_o, c.f. Figure 4). Thus, a partial inhibition of approx. 50, 75 and 90% at 50 mol m^{-3} Tris concentration and pH_o of 7.5, 8.1 and 8.7, respectively, should be expected. These inhibition percentages fit well those shown in Figure 3.

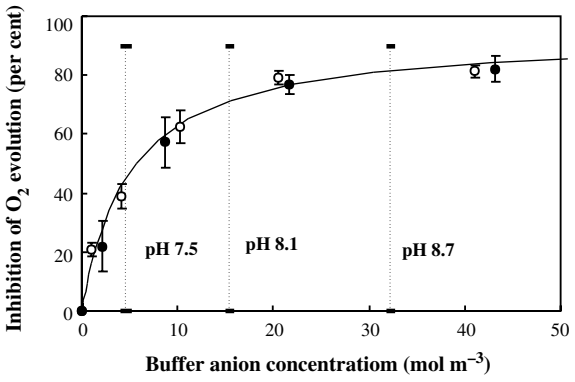

Figure 4. Inhibition of net photosynthesis (in percentage of initial oxygen evolution rate) of *L. saccharina* by different Tris concentrations plotted against the concentration of a putative active part of the buffer (anion). The concentration of the active part was calculated as the titre of the buffer from the pH of the medium (pH_o) to an endpoint at pH 6.5. The experiment was performed in natural seawater, salinity 30, DIC 2.2 mol m^{-3}. Open marks refer to experiments at pH 8.1, closed marks to experiments at pH 8.7. The curve is a hyperbola with a $K½$ (half way inhibition) of 5 mol m^{-3} and a maximum inhibition of 94.2%. The vertical lines show the titre of 50 mol m^{-3} Tris in seawater of different pH, as indicated. The results are the mean values of six independent measurements; bars indicate standard deviations.

Discussion

The data presented in this study agree with the model by Axelsson et al. (2000) describing the mechanism for HCO_3^--use in *L. saccharina*. In this model, HCO_3^- use is facilitated by the excretion of protons outside of the plasma membrane, which creates regions of low pH (acid zones), resulting in a higher-than-ambient CO_2 concentration. The CO_2 enters the cell by diffusion either through the cell membrane proper or via proteinaceous pores. Similar mechanism has been proposed for other macrophytes (Lucas 1975; Prins et al. 1980; Price and Badger 1985; Price et al. 1985; Hellbom et al. 2001). The involvement of plasma membrane associated P-type H^+-ATPase in the DIC uptake of *Laminaria* spp., as demonstrated recently by Klenell et al. (2004), is a strong support for this model. Theoretically, it might be expected that the cell membrane potential, generated from the H^+ ATPases in the cell membrane, could drive a HCO_3^- uptake via a H^+/HCO_3^- symport. However, the proton buffers would not inhibit such mechanism. Moreover, this mechanism is rather unlikely in marine plants due to a low HCO_3^- uptake efficiency (i.e. a poor HCO_3^-/H^+ yield, Raven 1997). In fact, direct uptake of HCO_3^- must be low in our study, as the addition of AZ and proton buffer separately produced a full inhibition of photosynthesis at alkaline pH.

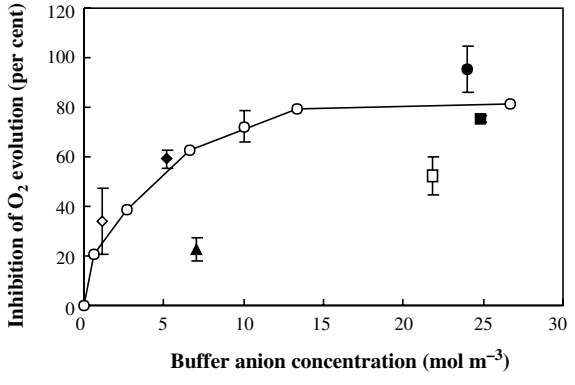

Figure 5. Inhibition of net photosynthesis (in percentage of initial oxygen evolution rate) of *L. saccharina* by different buffers plotted against the concentration of a putative active part of the buffer. The concentration of the active part was calculated as the titre of the buffer from the pH of the medium (pH_o) to pH 6.5. The curve represents data from consecutive addition of Tris (to a concentration of 50 mol m^{-3}). Tris, *circles*; Mes, *triangles*; Hepes, *squares*; Ampso, *diamonds*. Open marks refer to experiments at pH 8.1, closed marks to experiments at pH 8.7. The results are the mean values of 3–6 independent measurements; bars indicate standard deviations.

Klenell et al. (2004) demonstrated that AZ has also some buffering capacity in seawater, which could increase its inhibitory effect on the *Laminaria* photosynthesis. According to these authors, the addition of 0.2 mol m^{-3} AZ increases the buffering capacity of the seawater to the same extent as the addition of 0.2 mol m^{-3} Tris solution. According to our data in Figure 4, the photosynthetic inhibition due to 0.2 mol m^{-3} Tris is about 1%. Therefore, the pronounced inhibitory effect by 0.1 mol m^{-3} was mainly due to the inhibition of the external CA activity; consequently AZ and buffer acted on different components of the HCO_3^- utilization mechanism.

The proton pump *per se* seems to play a minor role when external CA is completely inhibited, suggesting that the acid zones must feature external CA activity. On the other hand, extracellular CA would play an insignificant role when acting alone, i.e. when all protons had been dissipated. This is quite different from the situation in some seagrasses (notably *Zostera* sp.), which are affected by buffers (Hellblom et al. 2001; Hellblom and Axelsson 2003; Mercado et al. 2003). External CA activity in the studied seagrasses is not essential for

an efficient use of HCO_3^- based on the proton pumps. This suggested the possibility of a direct HCO_3^- uptake coupled to the pump activity, although external noncatalysed HCO_3^- dehydration could not be excluded (Hellblom and Axelsson 2003). Contrastingly, the tight (functional) coupling between proton pump and external CA appears to be a particular feature of *L. saccharina*. Our data suggest that CA speeds up the transformation of the HCO_3^- into CO_2 within these acid bands. Otherwise, photosynthesis could be limited by the spontaneous dehydration of CO_2 even at the hypothetically low pH of the acid zones (Hellblom and Axelsson 2003).

Buffers act by dissipating the acid zones and consequently the CO_2 gradient between the plasma membrane and the bulk medium (Price and Badger 1985), probably by competing with HCO_3^- for H^+. The fact that the buffer inhibitory effect depends on the buffering capacity of the medium (as modified by buffer addition) strongly supports this hypothesis. Furthermore, the effect of a particular buffer can be predicted from our data (at least to some extent), provided the titration from the pH of the medium (pH_o) to the assumed pH of the acid band (pH_a) is known (which depends on the pK_a of the buffer; Price and Badger 1985). If we assume a pH of the acid band (pH_a) of 6.5, the inhibition by Tris and the other buffers fits to a hyperbolic function (a similar function is also obtained if pH 7.5 is chosen, data not shown), which agrees with a competitive action between the buffer and HCO_3^-.

Two of the buffers tested (Mes and Hepes) exhibit a lower effect than what would be predicted by the hyperbolic model. These buffers have a buffering range that overlaps the buffering range of the CO_2/HCO_3^- system (in contrast to Tris and Ampso, which have a much higher pK_a). This might suggest that the predicted inhibition should probably be corrected for the shorter distance between the buffer pK_a and the first pK_a of the carbonic acid system. On the other hand, the results from Mes buffer showed a good agreement with the general hyperbola if pH 7.5 was chosen as the endpoint of the titration. This is also the pH value of the acid zones calculated from Figure 3. Another explanation for the lower inhibitory effect could be a lower mobility of the buffer molecule through the cell wall (the molecular weight of Hepes is about twice that of Tris and Ampso; Price and Badger 1985; Axelsson et al. 2000). Consequently, the buffer efficiency in disrupting the acid zones can be described as a function of its pK_a and mobility (Price and Badger 1985).

It might appear surprising that the ability of a buffer to compete with HCO_3^- for H^+ is relatively low, with an IC_{50} of 5 mol m^{-3} at an HCO_3^- concentration of 2.4 mol m^{-3}. Thus, it is necessary to use a relatively high buffer concentration to fully inhibit the photosynthesis. The reason for this is probably that the CO_2 formed is easily removed over a short diffusion distance into the chloroplast, while the buffer acid (HB) has a much longer distance to travel (and also diffuses at a slower rate). Such conditions would obviously be in good agreement with the mechanism suggested for the HCO_3^- utilization.

The acid zones, as described in this study, constitute *per se* a CCM, as they are able to considerably increase the CO_2 concentration available for photosynthesis. Apparently, an internal component (e.g. at the chloroplast level) is not necessary for this CCM to function, as the inhibition of internal CA appeared to have a negligible effect on photosynthesis. Such a situation would be in agreement with the comparatively high CO_2 compensation point of this species (Surif and Raven 1990) and differ from the CCM-models described in the literature, where an internal component (mainly chloroplastic CA) is involved (Badger 2003). Contrastingly, *L. saccharina* exhibits an HCO_3^- use efficiency similar to other macroalgae where an internal component of the CCM has been more or less assumed (Surif and Raven 1989, Axelsson et al. 1991, 2000). Accordingly, the values of photosynthesis rate at alkaline pH reported in this paper are comparable to those found for *Porphyra leucosticta* and *U. lactuca* (Mercado et al. 1998), the most efficient HCO_3^- users among the red and green macroalgae (Axelsson et al. 1991, 1999).

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