Photosynthetic carbon acquisition in the lichen photobionts *Coccomyxa* and *Trebouxia* (Chlorophyta)

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Processes involved in photosynthetic CO₂ acquisition were characterised for the isolated lichen photobiont *Trebouxia erici* (Chlorophyta, Trebouxiphyceae) and compared with *Coccomyxa* (Chlorophyta), a lichen photobiont without a photosynthetic CO₂-concentrating mechanism. Comparisons of ultrastructure and immuno-gold labelling of ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco; EC 4.1.1.39) showed that the chloroplast was larger in *T. erici* and that the majority of Rubisco was located in its centrally located pyrenoid. *Coccomyxa* had no pyrenoid and Rubisco was evenly distributed in its chloroplast. Both species preferred CO₂ rather than HCO₃[−] as an external substrate for photosynthesis, but *T. erici* was able to use CO₂ concentrations below 10–12 μM more efficiently than *Coccomyxa*. In *T. erici*, the lipid-insoluble carbonic anhydrase (CA; EC 4.2.1.1) inhibitor acetazolamide (AZA) inhibited photosynthesis at CO₂ concentrations below 1 μM, while the lipid-soluble CA inhibitor ethoxycarbonyl (EZA) inhibited CO₂-dependent O₂ evolution over the whole CO₂ range. EZA inhibited photosynthesis also in *Coccomyxa*, but to a much lesser extent below 10–12 μM CO₂. The internal CA activity of *Trebouxia*, per unit chlorophyll (Chl), was ca 10% of that of *Coccomyxa*. Internal CA activity was also detected in homogenates from *T. erici* and two *Trebouxia*-lichens (*Lasallia hispanica* and *Cladonia rangiferina*). In all three, the predominating CA had α-type characteristics and was significantly inhibited by low concentrations of AZA, having an I₅₀ below 10–20 nM. In *Coccomyxa* a β-type CA predominates, which is much less sensitive to AZA. Thus, the two photobionts differed in three major characteristics with respect to CO₂ acquisition, the subcellular location of Rubisco, the relative requirement of CA and the biochemical characteristics of their predominating internal CA. These differences may be linked to the ability of *Trebouxia* to accumulate dissolved inorganic carbon internally, enhancing their CO₂ use efficiency at and below air-equilibrium concentrations (10–12 μM CO₂) in comparison with *Coccomyxa*.

Key words – Carbonic anhydrase, *Coccomyxa*, CO₂-concentrating mechanism, green alga, immuno-gold labelling, lichen photobiont, photosynthesis, pyrenoid, Rubisco, *Trebouxia erici*.

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Introduction

Lichens are the symbiotic phenotype of nutritionally specialised fungi (mycobionts) that derive carbon nutrition from algal and/or cyanobacterial photobionts, located extracellularly within a matrix of fungal hyphae (Honegger 1991). The majority of lichens, about 90%,

have green algal photobionts, most of which belong to the class Trebouxiphyceae, a sister-taxon to Chlorophyceae within Chlorophyta (Friedl 1995). *Trebouxia* is the most frequent genus, being present in approximately 20% of all lichens, mainly within Lecanorales. Recent data, based on 18S rRNA analysis, suggest that *Coccomyxa* should also be included within Trebouxio-

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phyceae (T. Friedl, Plant Ecology and Systematics, University Bayreuth, Germany, personal communication), a genus which is less common as lichen photobionts, occurring in the fungal families Baeomycetaceae, Peltigeraceae and in lichenised Basidiomycetes (Friedl and Büdel 1996). *Trebouxia* is rarely found free-living in nature, whereas *Coccomyxa* also comprises non-symbiotic species (Honegger 1991). The physiological integration between mycobiont and photobiont is moreover closer in lichens with *Trebouxia* photobionts compared to lichens with *Coccomyxa* (Honegger 1991).

Lichens are metabolically active when their water content is above a critical threshold level, which varies between species. The water content optimal for photosynthesis also varies, which is caused by several factors, including the morphology of the thallus and the ability of the photobiont to acquire and fix CO₂ from an environment where CO₂ diffusion is impeded (Lange et al. 1993). Cyanobacterial photobionts and algae with a pyrenoid in the chloroplast, such as *Trebouxia* spp. and some species in the genus *Stichococcus*, possess a photosynthetic CO₂-concentrating mechanism (CCM) (Badger et al. 1993, Palmqvist 1993, Smith and Griffiths 1996a), which allows the cell to accumulate and maintain a higher concentration of dissolved inorganic carbon (DIC) inside the chloroplast in relation to the environment (Badger and Price 1994). However, a CCM in lichen photobionts has mainly been inferred from measurements of internal DIC pools, carboxylation efficiency of intact lichens and carbon isotope discrimination characteristics (Badger et al. 1993, Palmqvist 1993, Palmqvist et al. 1994b, Máguas et al. 1995, Smith and Griffiths 1996a) and the mechanistic details have not been resolved.

Coccomyxa and other species without a pyrenoid in the chloroplast, such as *Trentepohlia* and some other species of *Stichococcus* lack a CCM (Palmqvist 1993, Palmqvist et al. 1994a, Smith and Griffiths 1996a). Among these, *Coccomyxa* has been studied in more detail. This alga has a relatively more efficient Rubisco, compared to *Chlamydomonas reinhardtii*, an alga with a well developed CCM (Palmqvist et al. 1995). Moreover, *Coccomyxa* possesses high activity of an intracellular carbonic anhydrase (CA) of β -type (Hiltonen et al. 1995), not found in *C. reinhardtii*, which, similar to other β -type CAs has a relatively low sensitivity to CA inhibitors (Hewett-Emmett and Tashian 1996). It is not clear, however, to what extent this CA is involved in photosynthetic CO₂ acquisition in *Coccomyxa* (Palmqvist et al. 1995).

In contrast to this, numerous studies have established an important role of different CA isozymes in the CO₂ acquisition process of algae with a CCM (Badger and Price 1994). Among these, a low CO₂-induced periplasmic CA has been particularly well characterised (Coleman et al. 1984, Fukuzawa et al. 1990), even though internal CAs are also involved in the CCM (Karlsson et al. 1995, Eriksson et al. 1996). Attempts have been made to

detect and quantify the activity of both internal and periplasmic CA also in *Trebouxia* and *Trebouxia*-lichens (Badger et al. 1993, Palmqvist et al. 1994b). So far, however, it was only the extremely sensitive mass spectrometer method, measuring ¹⁸O exchange in vivo, that succeeded in detecting any CA activity in these. However, due to the complexity of in vivo systems the latter results were difficult to interpret (Palmqvist and Badger 1996).

This study aimed at characterising mechanisms involved in photosynthetic CO₂ acquisition, including CA, in an isolated strain of *Trebouxia*, to avoid the complexity of the intact lichen system. Some measurements were, however, performed also with intact *Trebouxia*-lichens to see whether the results obtained from the isolated alga were valid for lichenised cells. The results are compared with new and previous data from both isolated and lichenised *Coccomyxa* with the aim of characterising differences and similarities in CO₂ acquisition between the two photobionts and to see the extent of different components affecting their photosynthetic physiology.

Abbreviations – AZA, acetazolamide (5-acetamido-1,3-thiadiazole-2-sulfonamide); BBM, Bold's basal medium; BTP, 1,3-bis[tris(hydroxymethyl)methylamino]propane; CA, carbonic anhydrase (EC 4.2.1.1); CCM, CO₂-concentrating mechanism; CO₂(aq), CO₂ dissolved in aqueous solution; Chl, chlorophyll; EPPS, N-(2-hydroxyethyl)piperazine-N'-(3-propanesulfonic acid); EZA, ethoxzolamide (6-ethoxy-2-benzothiazole-2-sulfonamide); DIC, dissolved inorganic carbon (CO₂ + HCO₃⁻); I₅₀, concentration required to obtain 50% inhibition of an enzyme activity; Rubisco, ribulose-1,5-bisphosphate carboxylase-oxygenase (EC 4.1.1.39).

Materials and methods

Collection of lichen material and growth of algae

Cladina rangiferina (L.) Wigg. was collected near Umeå University, Umeå, Sweden in October and November 1995 and used for enzyme preparations immediately after collection. *Lasallia hispanica* (Frey) Sancho & Crespo was collected in October 1995 in Sierra de Guadarrama, Madrid, Spain in their dry state. These samples were stored dry in darkness during transport and kept dry and dark in a cold room (4°C) in the Umeå laboratory prior to reactivation and experimental use. *Peltigera aphthosa* (L.) Willd. was collected outside Botsmark, Västerbotten, Sweden in August 1995, dried, stored and reactivated as specified in Palmqvist (1993).

Trebouxia erici (IB 364) was obtained from the algal collection in Innsbruck, Austria. Liquid cultures were grown in 0.5-l glass bottles at 20°C in Bold's basal medium (BBM) (Nichols and Bold 1965) and continuously irradiated (80 $\mu\text{mol m}^{-2} \text{s}^{-1}$) with a bank of fluorescent tubes (TL 20W/55, Philips, Eindhoven, The Netherlands). The cultures were bubbled with ambient air (35 Pa CO₂), stirred with a magnetic stirrer to obtain a homogenous distribution of cells (5–10 $\mu\text{g Chl ml}^{-1}$) and diluted every week with fresh medium. Cells were also

grown in air enriched with 1–2% CO₂ (1 000–2 000 Pa). However, these cultures turned yellow and died after only a few days of high-CO₂ treatment and could not be used for experiments. *Coccomyxa* was grown as described in Palmqvist et al. (1994a).

Electron microscopy and immunolabelling

For conventional observation of ultrastructures, samples of lichen or algal cells were fixed in 3% (v/v) glutaraldehyde in 0.1 M sodium phosphate (pH 7.1). Samples were dehydrated and embedded in Spurr's resin as in Ascaso et al. (1988). For immunocytochemical studies the samples were fixed in 2.5% (v/v) glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4), dehydrated and embedded in LR-White as in Ascaso et al. (1995). Algal cells were blocked in 2% (w/v) agar after fixation. Immunolabelling was carried out with anti-Rubisco antiserum of *Euglena gracilis*, provided by Dr Joaquin Moreno (Biochemistry Dept, Valencia Univ., Spain) as detailed in Ascaso et al. (1995). Ultrathin sections were post-stained with uranyl acetate followed by lead citrate (Reynolds 1963) and observed in a Philips 300 transmission electron microscope.

O₂ evolution measurements

Photosynthetic O₂ evolution of *T. erici* was measured in an oxygen (O₂) electrode (Hansatech Ltd, King's Lynn, UK) where DIC and CO₂ response curves were obtained as described in Palmqvist et al. (1994a). Prior to measurements the cells were harvested from their growth medium by a light centrifugation (700 g, 5 min) and washed twice in CO₂-free assay medium; BBM buffered with 25 mM 1,3-bis[tris(hydroxymethyl) methylamino]propane (BBM-BTP). The final algal pellet was suspended in the assay medium to 1–2 µg Chl ml⁻¹, bubbled with CO₂-free air and kept in low light (5 µmol m⁻² s⁻¹) at room temperature for a maximum of 2 h before experimental use. When CA inhibitors were used, the sample was incubated with the inhibitor for 20 min in darkness before measurements. On these occasions the controls were treated in a similar way, but without inhibitor. All experiments were done at light saturation (150 µmol m⁻² s⁻¹), measured at the site of the O₂ electrode chamber with a quantum sensor (LI-189, Li-Cor Inc., Lincoln, NE, USA). Light was provided by a slide projector lamp (Philips 250W, 24V).

CO₂ gas exchange measurements

Steady state gas exchange and CO₂ pool sizes were measured on individual lichen thalli or in *T. erici* cells transferred to a glass-fiber filter (1 µm pore-size; Micron Separations Inc., Westboro, MA, USA) using an open system (Compact Minicuvette System 400 with gas-mixing unit GMA1; Walz, Effeltrich, Germany) as in Palmqvist et al. (1994b). The air-stream passing the cuvette had a

dew-point temperature of 1°C below the cuvette temperature in order to minimise desiccation of the sample. Lichens were measured with a thallus water content optimal for photosynthesis at 15°C and illuminated with 400 µmol m⁻² s⁻¹ provided from a slide projector (specified above). Algae were measured at 25°C and 200 µmol m⁻² s⁻¹. Lichen thalli used for CA inhibition experiments were treated as described in Palmqvist and Badger (1996). Algal cells were incubated with the CA inhibitors in their assay medium (BBM-BTP) (specified above) prior to filtration. DIC pool sizes were calculated from the initial DIC uptake pool in dark-light-dark transient experiments as described in Palmqvist et al. (1994b).

Chlorophyll determinations

Chlorophyll was quantitatively determined for all assayed lichen and algal samples after experiments by extraction in MgCO₃-saturated dimethyl sulfoxide (DMSO) (Ronen and Galun 1984).

Measurements of carbonic anhydrase activity in algae and lichens

The CA activity was electrochemically determined by measuring the time (t) for the pH to decrease from 8.0 to 7.2, at 2°C, in a 4-ml sample upon addition of 2 ml of ice-cold CO₂-saturated distilled water. One WAU (Wilbur-Anderson unit) of activity was defined as: WAU = (t₀–t)/t, where t₀ was the time for the pH to change in pure buffer (20 mM Veronal, pH 8.3, buffer A) and t was the time when CA-containing samples were added.

T. erici was harvested from the growth medium by centrifugation (700 g, 5 min), washed twice and resuspended in buffer A and then broken twice in a pre-cooled French pressure cell (160 MPa). The homogenate was subsequently centrifuged at 100 000 g for 1 h, using a fixed angle rotor (Beckman Ti70), whereafter the majority of the CA activity was recovered in the pellet. Some CA was present in the soluble fraction, but this concentration was too low to allow biochemical characterisations. The pellet was resuspended in a buffer consisting of 200 mM KCl, 20 mM Veronal (pH 8.3) and 1 µM ZnSO₄ to solubilise the CA. The suspension was gently stirred on ice for 15 min and centrifuged at 100 000 g for 1 h. After this procedure most of the CA was localised in the supernatant which was used to determine the AZA inhibition characteristics of the enzyme.

L. hispanica and *C. rangiferina* were rinsed with distilled water, pulverised with liquid nitrogen in a mortar and homogenised in buffer A (5 ml g⁻¹ dry weight) with an Ultra-Turrax (Janke & Kunkel, TP 18/2N). The crude lichen homogenate was centrifuged at 1 000 g for 5 min and the supernatant was kept at 4°C before further measurements, while the pellet was resuspended in buffer A (3 ml g⁻¹ dry weight), gently stirred on ice for 1 h and again centrifuged at 1 000 g for 5 min. This pellet, now

Fig. 1. Electron micrographs with the ultrastructure of free-living *T. erici* (A), *Coccomyxa* in *Peltigera aphthosa* (B) and free-living *Coccomyxa* (C). Plates D and E show free-living *T. erici* (D) and *Coccomyxa* (E) labelled with anti-Rubisco. d, Dictyosome; p, pyrenoid; s, starch; m, mitochondria; n, nuclei; ch, chloroplast; mc, multivesicular complex; pg, plastoglobuli. Bar = 0.5 μm.

containing washed disrupted fungal hyphae and some intact algal cells was discarded, while the supernatant was pooled with the first supernatant. The CA activity was measured and related to the Chl content of the pooled homogenate. Thereafter, the homogenate was centrifuged at 17 000 g for 15 min. Again, most of the activity was recovered in the pellet although a small amount of CA was present in the soluble fraction, as in *T. erici* (see above). The pellet was treated as *T. erici* to solubilise the CA, but the ultracentrifugation was replaced by a lower speed centrifugation at 12 500 g for 15 min. The final supernatant was used to determine the AZA inhibition characteristics.

Results

Ultrastructure and distribution of Rubisco in *Trebouxia* and *Coccomyxa*

T. erici was characterised by a large and highly lobed chloroplast, with thylakoid lamellae, mainly consisting of grana stacks extending throughout the stroma. A pyrenoid with numerous and large pyrenoglobuli was present in the centre of the chloroplast. These were associated with short, single thylakoids extending from grana stacks in the stroma (Fig. 1A).

The free-living cells of *Coccomyxa* were characterised by a parietal, single, chloroplast without any signs of a pyrenoid (Fig. 1C). The chloroplast of *Coccomyxa* was moreover proportionally smaller in relation to the

protoplast, compared to *T. erici*. The thylakoids were either single or piled up to form grana stacks similar to the observations of Peveling and Galun (1976). In lichenised *Coccomyxa*, the chloroplast was cup-shaped and more developed, compared to the free-living cells, occupying a proportionally larger volume of the protoplast and with more starch and plastoglobuli between its thylakoids (Fig. 1B). Multivesicular complexes were present in both free-living and lichenised *Coccomyxa* (Fig. 1B,C). In the lichenised *Coccomyxa*, these areas could contain both vesicles and storage bodies (Fig. 1B), while in the free-living cells, the areas of multivesicular complexes only contained black storage bodies (Fig. 1C).

When the location of Rubisco was compared between *T. erici* and *Coccomyxa* there were clear differences between the two species. In *Coccomyxa*, Rubisco was evenly distributed throughout the stromal part of the chloroplast (Fig. 1E). In *T. erici*, the highest concentration of Rubisco was found in the pyrenoid even though some gold particles were also present in the stroma (Fig. 1D), similar to previous observations of lichenised *Trebouxia* (Ascaso et al. 1995, Balaguer et al. 1996).

DIC and CO₂ responses of *Trebouxia* and *Coccomyxa*

Figure 2A,B shows the photosynthetic CO₂ and HCO₃⁻ responses of *T. erici* measured at two pH values, 6.2 and 7.9, respectively. At the lower pH, photosynthesis was

Fig. 2. Net rate of photosynthetic O₂ evolution as a function of HCO₃⁻ (lower x-axis) or CO₂(aq) (upper x-axis) in cultured cells of *T. erici*. The measurements were carried out at 25°C and light saturation (200 μmol m⁻² s⁻¹) in BBM-BTP medium at pH 6.2 (A) (●) or pH 7.9 (B) (○) with controlled additions of HCO₃⁻ to DIC-depleted medium. The CO₂ and HCO₃⁻ concentrations were calculated as in Palmqvist (1993). Data represent the average ± SE of at least three independent experimental series. In C, (+) and solid line shows data obtained in a similar way for cultured cells of *Coccomyxa* (Palmqvist 1993, Palmqvist et al. 1994a) compared with the *T. erici* data obtained at the two pH values.

saturated when both CO_2 and HCO_3^- was about $100\ \mu\text{M}$, respectively, with a significant decline above $200\ \mu\text{M}$ CO_2 and/or HCO_3^- (Fig. 2A). The reason for this decline is not clear, but a similar response has previously been observed also for higher plant leaves, at even lower concentrations of CO_2 (Ögren and Evans 1993). Interestingly though, as $200\ \mu\text{M}$ $\text{CO}_2(\text{aq})$, corresponds to ca $500\ \text{Pa}$ $\text{CO}_2(\text{g})$, this decline in photosynthetic capacity at suprasaturating DIC concentrations might explain the failure to grow *T. erici* with CO_2 -enriched ($1\ 000$ – $2\ 000\ \text{Pa}$) air (see Materials and methods).

At the higher pH, photosynthesis was saturated at a somewhat lower concentration of CO_2 ($40\ \mu\text{M}$), but at a considerably higher concentration of HCO_3^- ($1\ 500\ \mu\text{M}$) (Fig. 2B). Thus, since the affinity to low concentrations of CO_2 was similar irrespective of pH (Fig. 2C), while the affinity to HCO_3^- was strikingly different (Fig. 2A, B), it may hence be concluded that *T. erici* appears to prefer CO_2 over HCO_3^- as an external substrate for photosynthesis. However, since photosynthesis was saturated at a somewhat lower CO_2 concentration at the higher pH, this also suggests that *T. erici* may have the ability to use some HCO_3^- as an external carbon source. The affinity to CO_2 was moreover higher for *T. erici* as compared to *Coccomyxa* (Fig. 2C).

Incubation of *T. erici* with the lipid-insoluble CA inhibitor acetazolamide (AZA) resulted in more than 50% inhibition of net photosynthesis below $1\ \mu\text{M}$ CO_2 (Fig.

3), indicating the participation of a periplasmic CA in this alga, a possibility which will be discussed below. In the presence of the lipid-soluble CA inhibitor ethoxymethylolamide ($350\ \mu\text{M}$ EZA), photosynthesis was even more affected, with a 100% inhibition below $1\ \mu\text{M}$ CO_2 . This inhibition gradually declined to about 50% at air concentrations of CO_2 (Fig. 3). In *Coccomyxa*, the effect of EZA was less severe, particularly at the lowest CO_2 concentrations (Fig. 3), even though a higher concentration of the inhibitor was used ($500\ \mu\text{M}$).

Photosynthetic CO_2 fixation in relation to external CO_2 was also measured in intact thalli of the *Trebouxia*-lichen *Lasallia hispanica* (Fig. 4). Similar to *T. erici* there was a decrease in photosynthesis at high CO_2 concentrations also in the lichen, as well as inhibition of photosynthesis in the presence of EZA. This inhibition decreased with increasing CO_2 , similar to what has previously been shown for another *Trebouxia*-lichen, *Hypogymnia physodes* (Badger et al. 1993).

The relationship between photosynthetic capacity and DIC pool sizes in *Trebouxia* and *Trebouxia*-lichens

Trebouxia-lichens accumulate an internal pool of DIC, which has been taken as evidence for the presence of a CCM in this genus (Badger et al. 1993, Palmqvist et al. 1994b, Smith and Griffiths 1996a). EZA increases the size of this pool, suggesting the involvement of a CA

Fig. 3. Effects of the CA inhibitors AZA ($\times = 5\ \mu\text{M}$; $\Delta = 30\ \mu\text{M}$) and EZA ($\blacktriangle = 350\ \mu\text{M}$) on photosynthetic net O_2 evolution as a function of CO_2 in *T. erici*. The measurements were carried out at 25°C and light saturation ($200\ \mu\text{mol m}^{-2}\text{ s}^{-1}$) in BBM-BTP medium (pH 7.9) with controlled additions of HCO_3^- to DIC-depleted medium. The CO_2 concentrations were calculated as in Palmqvist (1993). Data represent the average of two independent experimental series with each inhibitor concentration and compared with un-inhibited samples. The solid line shows data obtained for cultured cells of *Coccomyxa* using $500\ \mu\text{M}$ EZA (Palmqvist et al. 1994a).

Fig. 4. Gross rate of CO_2 assimilation in relation to external $\text{CO}_2(\text{g})$ in the *Trebouxia*-lichen *Lasallia hispanica*. Measurements were carried out at 15°C and light saturation ($400\ \mu\text{mol m}^{-2}\text{ s}^{-1}$). Samples were incubated in $20\ \text{mM}$ EPPS buffer, pH 8.0 (control, $\square$), or in $20\ \text{mM}$ EPPS buffer, pH 8.0 with $500\ \mu\text{M}$ EZA ($\blacksquare$) prior to measurements as detailed in Palmqvist and Badger (1996). Error bars ($\pm$ SD) are indicated when they exceed the symbol size; $n \geq 2$ for each series.

Fig. 5. Initial DIC pool size in relation to external CO₂(g) in *T. erici* filtered onto a glass fiber filter and in intact thalli of *L. hispanica*. Samples were incubated in BBM growth medium (*T. erici*, ○) or in 20 mM EPPS buffer, pH 8.0 (*L. hispanica*, □), or in the same medium/buffer with 500 μM EZA prior to measurements: *T. erici* (●), *L. hispanica* (■). Lichens were measured at 15°C and 400 μmol m⁻² s⁻¹ and algae at 25°C and 200 μmol m⁻² s⁻¹. Error bars (± SD) are indicated when they exceed the symbol size; n≥2 for each series.

somewhere between the DIC pool and CO₂ fixation by Rubisco (Badger et al. 1993, Palmqvist et al. 1994b). These characteristics were also found in *T. erici* and *L. hispanica*, where the size of the DIC pool was increased both with increasing CO₂ and in the presence of EZA (Fig. 5).

Overall, however, the internal DIC pool of *Trebouxia* and *Trebouxia*-lichens, is relatively small when compared to free-living cyanobacteria (reviewed in Badger and Price 1994), cyanobacterial *Nostoc*-lichens (Badger et al. 1993, Sundberg et al. 1996) and *C. reinhardtii* (Badger et al. 1980). This includes both the absolute size of the pool and its size in relation to photosynthetic rate. The latter relation may be quantified by dividing the size

of the pool with the steady-state CO₂ fixation rate, measured under the same experimental conditions. This gives an estimate of the duration (in seconds) for which the pool would theoretically support photosynthetic CO₂ fixation with substrate (Tab. 1). In a *Nostoc*-lichen, the pool would theoretically support photosynthesis for about 35 s, while it would last for about 3–4 s in *T. erici* and *Trebouxia*-lichens. In the presence of EZA, the pool would last for about 8 s in *Trebouxia* and for about 17 s in *C. reinhardtii* (Tab. 1). *Coccomyxa* also appears to accumulate a small pool upon illumination (Tab. 1). However, using the same assumptions and calculations as in Badger et al. (1993), this pool corresponds to passive accumulation of DIC related to alkalisation of the chloroplast stroma upon illumination.

Evidence for the presence of internal α-CA in *Trebouxia* and *Trebouxia*-lichens

The data presented in Fig. 3 indicate that both intracellular and periplasmic CA participate in photosynthetic CO₂ acquisition in *Trebouxia*. Despite this, it was not possible to detect any CA activity in washed and concentrated intact *T. erici* cells, suggesting that the effect of AZA (Fig. 3) may be caused by penetration of the inhibitor to internal CA. Indeed, internal CA activity could be found and quantified in crude homogenates of *T. erici*, *L. hispanica* and *C. rangiferina*, another *Trebouxia*-lichen (Tab. 2). In all three, the CA activity was somewhat higher, expressed per unit Chl, compared to the internal activity of *C. reinhardtii*, but significantly lower than the total CA activity, including periplasmic CA, of low-CO₂ grown *C. reinhardtii* (Tab. 2). The CA activity of *Trebouxia* was though considerably lower than that of both free-living and lichenised *Coccomyxa* (Tab. 2).

Most of the CA was found in the insoluble fraction after centrifugation (see Materials and methods). This fraction was separated on a protein gel and tested against antibodies raised towards the intracellular β-CA of *Coccomyxa* (Hiltonen et al. 1995) and a chloroplast α-CA from *Chlamydomonas* (Karlsson et al. 1995). However, there was no immunological cross-reaction between any of these and the *Trebouxia* CA/CAs (results not shown).

Tab. 1. The ratio (s) of DIC pool sizes (nmol [mg Chl]⁻¹) to CO₂ assimilation rates (nmol [mg Chl]⁻¹ s⁻¹) for *T. erici* and *L. hispanica* compared with literature data on five *Trebouxia*-lichens, five *Coccomyxa*-lichens, six *Nostoc*-lichens and low-CO₂ grown *C. reinhardtii*. All rates were obtained at light saturation of photosynthesis. DIC pool sizes and EZA inhibition were obtained as detailed in the respective studies. Data represent the average ± SE for [n] number of comparable experiments at CO₂ concentrations below saturation of both DIC accumulation and photosynthesis. All data from lichens with the same photobiont genus were pooled; ¹ Badger et al. 1993, ² Palmqvist et al. 1994b, ³ Smith and Griffiths 1996a, ⁴ Sundberg et al. 1997. PD, pool decreases.

Species	Control (s)	[n]	EZA (s)	[n]	AZA (s)	[n]
<i>T. erici</i>	4.2 ± 0.5	[8]	8.7	[1]	4.1	[1]
<i>L. hispanica</i>	3.6 ± 1.1	[3]	12.8 ± 4.6	[3]	3.2 ± 2.1	[2]
<i>C. reinhardtii</i> ²	No pool		17.2	[1]		
<i>Coccomyxa</i> -lichens ^{2,3}	1.5 ± 0.6	[5]	0.8 ± 0.4	[4]	No data	
<i>Trebouxia</i> -lichens ^{1,2,3}	3.1 ± 0.2	[8]	8.3 ± 0.6	[7]	No data	
<i>Nostoc</i> -lichens ^{1,4}	34.5 ± 5.8	[6]	PD ¹		No data	

Tab. 2. Carbonic anhydrase activity, expressed as Wilbur-Anderson units [WAU = (t₀ - t)/t] measured and calculated as defined in Materials and methods for cell extracts of *T. erici*, *L. hispanica* and *C. rangiferina*. Data is compared with data obtained in the same experimental set-up for *Coccomyxa* and *C. reinhardtii*. Data represent average ± SE of [n] independent enzyme preparations from each species; ¹ Palmqvist et al. 1994b, ² Palmqvist et al. 1990.

Species	CA activity (WAU [mg Chl] ⁻¹)	[n]
<i>T. erici</i>	24.0 ± 2.3	[2]
<i>C. rangiferina</i>	8.0	[1]
<i>L. hispanica</i>	25.7 ± 6.7	[3]
<i>Coccomyxa</i> ¹	300	
<i>Coccomyxa</i> -lichens ¹	85–110	
<i>C. reinhardtii</i> ²	60 (tot)	
<i>C. reinhardtii</i> ²	3 (internal)	

The sulfonamide inhibition characteristics though indicate that an α -type CA predominates with an I₅₀ of about 10–20 nM AZA in *T. erici* and *C. rangiferina*. In *L. hispanica*, the CA was even more sensitive to AZA inhibition, with an I₅₀ below 3 nM (Fig. 6). The high sensitivity of the internal CA of *Trebouxia* gives further support to the idea that the AZA effect on photosynthesis (Fig. 3) could reflect penetration of this inhibitor into the cell.

Discussion

The two photobionts acquire CO₂ differently

Based on physiological measurements and experiments mainly conducted with intact lichens it has been proposed that photosynthetic CO₂ acquisition may be differently organised in the two lichen photobionts *Coccomyxa* and *Trebouxia* (Palmqvist 1993, Palmqvist et al. 1994b, Máguas et al. 1995, Smith and Griffiths 1996a).

Fig. 6. Acetazolamide inhibition of in vitro CA activity in *T. erici* (○), *C. rangiferina* (△) and in two independent extractions of *L. hispanica* (□, ×).

The data presented here give substantial support to this hypothesis, as judged from the different localisation of Rubisco in the two photobionts (Fig. 1, Ascaso et al. 1995, Balaguer et al. 1996), the differences in relative requirement and biochemical characteristics of their internal CA:s (Figs 3 and 7, Hiltonen et al. 1995, Palmqvist et al. 1995) and the larger internal DIC pool in *Trebouxia* (Tab. 1). Indeed, the pyrenoid location of Rubisco, the relatively high requirement of CA and the corresponding ability to actively accumulate DIC, support the hypothesis that photosynthetic CO₂ acquisition in *Trebouxia* is similar to that of other green algae with a CCM, such as *Chlamydomonas*, *Chlorella* and *Scenedesmus* (cf. Badger and Price 1994).

The CCM of *Trebouxia* in relation to the CCM of other green algae

However, there are some conspicuous differences between *Trebouxia* and the free-living algae with a CCM. First, the internal DIC pool appeared to be relatively smaller than in for instance *C. reinhardtii* (Tab. 1). The size of the DIC pool is determined by several factors, including the efficiency of the transport and accumulation system, the size of the chloroplast and/or cell, the rate of HCO₃⁻ dehydration as well as the efficiency of Rubisco to fix CO₂. Therefore, the smaller pool of *Trebouxia* could either be related to a less efficient accumulation system or to a somewhat more efficient Rubisco of this alga compared to *C. reinhardtii*. The latter hypothesis finds support from earlier findings of an inverse relationship between the size of the DIC pool and both the affinity and specificity for CO₂ of Rubisco (Badger and Andrews 1987, Palmqvist et al. 1995). Second, in the free-living algae, the external CO₂ concentration experienced during growth regulates both the relative strength and the induction/repression of the CCM and its components (Badger and Price 1994). However, since both photosynthesis (Fig. 2) and growth was inhibited in *Trebouxia* at high CO₂ it remains unclear whether the CCM can be repressed also in this genus. Third, the high activity of periplasmic CA that is induced by low CO₂, in for instance *C. reinhardtii*, *Chlorella* and *Scenedesmus* (Badger and Price 1994), could not be detected in free-living *T. erici*. Fourth, the subcellular location and the molecular details of internal CA in *Trebouxia* need to be further studied before we can compare the CAs of this alga with the other CCM algae, which are now known to possess more than one internal CA isozyme (Karlsson et al. 1995, Eriksson et al. 1996).

Why different strategies?

The absence of a CCM in *Coccomyxa* has previously been correlated with a relatively more efficient Rubisco of this alga, compared with that of *C. reinhardtii* (Palmqvist et al. 1995). Despite this, photosynthetic CO₂ acquisition was more efficient in *Trebouxia* compared with

Coccomyxa (Fig. 2C), so the reason(s) for the development of different CO₂ acquisition strategies among green algal lichen photobionts still needs to be resolved. Both *Coccomyxa* and *Trebouxia* belong to the same algal class, Trebouxiophyceae (Friedl 1995; T. Friedl, personal communication), a class which in recent studies appear to have a monophyletic origin (Friedl 1995). However, Trebouxiophyceae includes a broad range of species with a high degree of both morphological (cf. Friedl 1995) and physiological (Palmqvist et al. 1994b, Máguas et al. 1995, Smith and Griffiths 1996a) variation. Moreover, even though the hypothesis of a multiple origin of lichens was recently suggested also from DNA analysis (Gargas et al. 1995), it is not clear whether *Trebouxia* and *Coccomyxa* occurred as photobionts at different stages during lichen evolution. Neither do we know if the presence or absence of a CCM is related to the environmental conditions when the symbiosis first occurred or whether the CO₂ acquisition pathway has continued to evolve within the lichen. Still, it is tempting to suggest that *Trebouxia* with its pyrenoid and higher degree of lichenisation might be the older of the two genera as a lichen photobiont. If so, *Coccomyxa* may have lost the pyrenoid (Fig 1B,C), developed a Rubisco with a higher affinity and specificity to CO₂ (Palmqvist et al. 1995) and gained its β -type CA (Hiltonen et al. 1995) more recently during evolution. This hypothesis is supported by the findings of Smith and Griffiths (1996b), showing that those hornworts (Anthocerotae) that have retained a scattered pyrenoid also possess a CCM. Evidently, we need much more data and a multidisciplinary approach to resolve this problem, including phylogenetic relationships as well as physiological, biochemical (Rubisco) and immunocytological data for more species within Trebouxiophyceae, comparing a broader range of species both with and without a pyrenoid, respectively.

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