Investigations on the CO2 concentrating mechanism of two green algae
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Link to publication

Citation for published version (APA):

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Introduction

Photosynthetic organisms convert inorganic carbon to organic carbon compounds, while producing oxygen. On a geological time scale, this led to a decrease of the erstwhile high CO$_2$ and low oxygen level of the early atmosphere to the present day levels of 0.035% CO$_2$ and 21% oxygen. As a side effect, the drop in the CO$_2$ level of the atmosphere caused a diminished green house effect and a global temperature decrease.

The interest in these processes was until now focussed on the carbon fixation of higher terrestrial plants. Aquatic photosynthetic organisms were considered of no great importance for carbon fixation. Recent studies, however, showed that CO$_2$ fixation in the aquatic environment does contribute considerably to the global CO$_2$ fixation. In fact, algae are the dominant CO$_2$-fixating species, with an estimated annual carbon fixation of 43.5 Pg (10$^{11}$ tonnes; Behrenfeld and Falkowski 1997). Terrestrial photosynthetic organisms have an estimated annual carbon fixation of 60 Pg (Raven 1997). With more than 40% of the annual carbon fixation, the algae thus play an important role in the global carbon cycle. In this role, however, they are confronted with a number of adversities.

To begin with, in aquatic environments the amount of dissolved carbon is not constant over the time span of a year (Maberly et al 1996). Changes are due to, among others, the differences in temperature from summer to winter, the availability of light (this is especially true at higher latitudes in winter time), deposition and decomposition of organic material which changes over time and finally the activity of the aquatic photosynthetic organisms themselves. The problem, however, is that these organisms have to rely on a constant carbon flux for photosynthesis and therefore have to find means to adapt to changing conditions.

Secondly, the key enzyme of carbon fixation is Ribulose-bisphosphate-carboxylase/oxygenase, or Rubisco (EC 4.1.1.39). This enzyme binds CO$_2$ to ribulose-P$_2$ to produce two molecules of D-glycerate-3-P, which can be used as a building block for further organic components in the cell in the different carbon cycles. It is also able to use oxygen to produce one molecule of glycolate-2-P and one molecule of D-glycerate-3-P from ribulose-P$_2$. This reaction results in only one useful molecule for carbon metabolism. Thus photosynthetic organisms are at a disadvantage when the CO$_2$/O$_2$ ratio in the surrounding environment is low.

Furthermore, in order for Rubisco to be able to incorporate CO$_2$, it is necessary that the enzyme is activated (Pierce et al, 1982). For this activation the CO$_2$ concentration near the enzyme has to be higher than its K$_m$ for CO$_2$. The [CO$_2$] in
the surrounding aqueous environment is approximately 10 μM, which is much lower than the $K_m$ of Rubisco, which is about 200 μM in cyanobacteria (Moroney and Somanchi 1999) and between 15 μM and 100 μM in eukaryotic algae (Chapter 3; Falkowski 1997). Thus the rate of photosynthesis in aquatic photosynthetic organisms is often limited by the supply of CO$_2$.

A final problem is that at alkaline pH, most of the dissolved inorganic carbon (Ci) is in the form of HCO$_3^-$, while the substrate for Rubisco is CO$_2$, and the non-catalysed interconversion of CO$_2$ and HCO$_3^-$ is slow.

Fortunately, aquatic photosynthetic organisms have developed means to optimise their Ci uptake, resulting in intracellular CO$_2$ concentrations many times higher than the extracellular concentration (Coleman 1991; Rawat and Moroney 1995; Giordano et al. 1997; Amoroso et al. 1998; Sültemeyer et al 1998). This adaptation to low Ci levels is referred to as the Carbon Concentrating Mechanism (CCM). Different parts of the CCM have been well studied. In *Chlamydomonas* and many other algae this mechanism implies the synthesis of an extracellular carbonic anhydrase (EC 4.2.1.1), by which the interconversion between CO$_2$ and HCO$_3^-$ is accelerated (Moroney et al. 1985; Coleman 1991). This may avoid depletion of CO$_2$ and/or HCO$_3^-$ (depending on which carbon species is taken up by the cell) in the unstirred boundary layer surrounding the cells. The local concentration of CO$_2$ around Rubisco is increased by active transport of Ci into the cell. This may result in a CO$_2$ concentration around Rubisco that is much higher than in the surrounding aquatic environment, and thus in a large diffusion gradient. To overcome this potential problem, the local CO$_2$ concentration in each cellular compartment surrounding Rubisco is strictly controlled in order to limit diffusion of CO$_2$ out of the cells. For example, in cyanobacteria Rubisco is located in the carboxysomes (Fig. 1). Ci is pumped into the carboxysomes both as CO$_2$ and as HCO$_3^-$. A carboxysomal CA then may provide a rapid but regulated dehydration of accumulated HCO$_3^-$, and the CO$_2$ so generated can be effectively used by Rubisco (Fig. 2).

Photosynthetic eukaryotic aquatic organisms do not have a carboxysome, and the Rubisco is located on the stroma side of the thylakoids in the chloroplasts. There are studies which show that most of the Rubisco is located within a special structure called the pyrenoid (Moroney and Chen 1998; Morita et al. 1999). Since the stroma has an alkaline pH (pH 8), most of the Ci present there is in the form of HCO$_3^-$. This can not be used directly by Rubisco and this indicates the need for a CA near Rubisco (Fig 3). So far no direct evidence for the presence of such a CA exists.
Due to the specific location of Rubisco, Ci transport has to take place via both the plasma membrane and the chloroplast membrane. All components of the CCM, which are found at the plasma membrane (an active transport mechanism for both HCO$_3^-$ and CO$_2$ together with a CA), seem also to be present at the chloroplast membrane, suggesting that the chloroplast plays an important role in the transport of Ci (Amoroso et al 1998; Chapter 3). This makes an exact co-ordination between the different parts of the Ci transport system necessary for optimal functioning.

In the thylakoid membranes the photosystems are located. During photosynthetic electron transport a proton gradient is created over the thylakoid membranes by two processes: at the start of the electron transport oxygen is produced from water at the luminal side of the thylakoids by photosystem II.
Fig. 2: Detailed model of the various transport mechanisms for the different types of inorganic carbon in cyanobacteria according to Kaplan and Reinhold (1999)

(PSII), resulting in the production of protons in that compartment. Secondly, the electron transport via the cytochrome b6f complex is coupled by the import of protons into the lumen of the thylakoids. The proton gradient thus created can then be used by an ATP-ase to create ATP. Now we are confronted with a seemingly paradoxical situation: in the lumen a CA is located, while Rubisco is located in the stroma. The gene for this CA, CAH3, has been cloned from *C. reinhardtii* and studied (Karlsson et al 1995). No major increase in activity or amount of this CA was shown after acclimation of *C. reinhardtii* to low CO₂ conditions. So the function of this CA is obscure. In this thesis, we speculate that luminal CA is involved in controlling electron transport and ATP synthesis (Chapter 4, 5, Raven 1997; Kaplan and Reinhold 1999; Moroney and Somanchi 1999). This would imply a direct link of the CCM with the activity of the different components of the photosynthetic apparatus. In this way the need of the photosynthetic apparatus for Ci taken up by the cell might be regulated.
The Ci species which are taken up by the cell, are of course important for the build-up of the intracellular Ci concentration. When mainly CO_{2} is taken up, there is little need for a CA activity, while when HCO_{3}^{-} is also taken up in significant amounts, this has to be dehydrated to CO_{2} before it can be used by Rubisco. Of the eukaryotic algae studied so far, no species seems to be an exclusive bicarbonate or CO_{2} user, they all rather seem to use and take up CO_{2} and HCO_{3}^{-} simultaneously (Palmqvist and Badger 1994; Matsuda and Colman 1995; Chapter 2). This implies a complex functioning of the CCM to facilitate the use of these different Ci species.

*T. minimum* used in this study is of interest since its fossil remains in lacustrine sediments, as represented by its highly resistant aliphatic wall (Gorth et al. 1988), show a ratio of $^{13}$C/$^{12}$C that is significantly different from those of fossilized lipids of other algae (Freeman et al. 1990). In plant biomass the ratio between $^{13}$C and $^{12}$C is to a large extent determined by isotope discrimination which occurs during carbon fixation. In turn, this discrimination may be dependent on the supply of Ci to Rubisco, which is partially determined by the operation of a CCM. Therefore, the CCM might play a role in establishing the $^{13}$C/$^{12}$C ratio of the resulting biomass. If an organism takes up mainly HCO_{3}^{-}, a discrimination may also occur at the site of active transport. Since this transport takes place over several different membranes, a multiplication of the discrimination of the active transport mechanism may result. If the organism also creates a pool of Ci, there are several possibilities. Firstly, the pool may consist of CO_{2} in a relatively acid compartment, and the accumulated Ci can then partially diffuse out of the cell and has to be pumped back in. If this acid compartment is located within a different, alkaline, compartment, all the CO_{2}, which diffuses out, will be hydrated to HCO_{3}^{-} and then be trapped within the alkaline compartment. An active transport mechanism may then transport the HCO_{3}^{-} back into the acid compartment (Fig. 3). Both the recycling of CO_{2} and HCO_{3}^{-} results in additional isotope discrimination. Secondly, the pool may be in the form of HCO_{3}^{-} in an alkaline compartment. Then no Ci is lost, but also no Ci is available for Rubisco. A transport out of this compartment into a less alkaline compartment may then be necessary, possibly coupled to a CA again, affecting the $^{13}$C/$^{12}$C ratio (Fig. 4).

Based on the considerations elaborated above, we embarked on a study of the CCM in *T. minimum*, and were fortunate to be able to apply mass spectrometry (MS) for most of the assays. With this apparatus it is possible to measure simultaneously the different isotope masses in an algal suspension during various treatments. The reaction cuvette is connected to the MS via a semi-
permeable membrane inlet system which allows gases to diffuse from the suspension into the analyser of the MS (Fig. 5). After calibration, changes in concentration of either oxygen or carbon dioxide can be detected. Since bicarbonate cannot be measured directly by a mass spectrometer in the set-up we used (Fig. 5) we had to use a disequilibrium model developed by Badger et al (1994). This model uses the equilibrium constants for CO$_2$ and HCO$_3^-$ at a certain pH to determine the amount of HCO$_3^-$ which has been taken up by the cells. With this constant it is possible to calculate from the measured [CO$_2$], the percentage of total Ci which is either CO$_2$ or HCO$_3^-$ at a certain pH and ionic strength. For this we measured the changes in [O$_2$] and [CO$_2$] in the dark and during illumination and the rapid efflux of CO$_2$ at the beginning of the dark period (Fig. 6). This efflux is due to a certain amount of HCO$_3^-$ which is inside the cells, and is not yet fixed when the light is turned off. From the oxygen uptake in the dark we can determine the amount of CO$_2$ which the cells produce both in the dark and in the light. When we take this together with the concentration of CO$_2$ at the beginning and at the end of the light period, we are able to calculate the amount of HCO$_3^-$ used by the cells. By this method we were able to estimate changes in HCO$_3^-$ concentrations and rates of HCO$_3^-$ uptake. We kept the algal suspension at the relative high pH = 8 to be sure that the major part of Ci available was HCO$_3^-$ and to minimize passive uptake of CO$_2$ through a pH gradient.
CA activity was measured mass spectrometrically according to Silverman (1982) by measuring $^{18}$O exchange from doubly labelled CO$_2$ ($^{13}$C $^{18}$O $^{18}$O). Doubly labelled HCO$_3^-$ was added to the buffer to a final concentration of 1 mM. When chemical equilibrium was reached aliquots of cells or thylakoids were added and changes in the signals of $^{13}$C $^{18}$O$_2$ (molecular mass = 49), $^{13}$C $^{18}$O $^{16}$O (molecular mass = 47) and $^{13}$C $^{16}$O$_2$ (molecular mass = 45) were recorded and subsequently calculated as log enrichment (log $(49/(45+47+49)) \times 100$). To calculate CA activity the rate of decrease of $^{13}$C $^{18}$O$_2$ was compared with the non-catalysed rate of depletion of $^{18}$O from labelled CO$_2$, and one unit of enzyme activity was defined as a 100% stimulation of the non-catalysed depletion rate (Badger and Price 1989).

Finally, we could measure the electron flow in the thylakoid membranes by first removing all the oxygen from the algal suspension and then adding $^{18}$O$_2$ (molecular mass of 36). When an active electron transport was present, the evolution of normal $^{16}$O$_2$ (molecular mass of 32), occurred due to water splitting. As the water oxidase is the only mechanism known to evolve O$_2$, measurements of the O$_2$ evolution is a direct measure of light-generated electrons. Because of the total lack of $^{16}$O$_2$ at the beginning of the experiments, this is a very sensitive way to detect the activity of the photosystems.
Fig. 5: Schematic depiction of a mass spectrometer and its function as was used during the work for this thesis.

Fig. 6: Typical time course of changes in the CO₂ and O₂ concentrations in the dark and upon illumination measured by mass spectrometer.
The scope of this thesis is to determine the different components of the CCM in both Tetraedron minimum and Chlamydomonas noctigama, alias C. monoica. T. minimum typically occurs in alkaline lacustrine habitats (Starr 1954; Kováčik 1975). This already suggests that this alga can take up C\textsubscript{i} in the form of HCO\textsubscript{3} efficiently. We will speculate about the role of the identified components of the CCM in stable carbon isotope discrimination. Chlamydomonas was chosen for comparison because it has an inducible CCM, which has been described extensively (eg. Amoroso et al. 1998).

In Chapter 2 we show that both algae have an inducible CCM. We also demonstrate that T. minimum mainly takes up HCO\textsubscript{3} and makes a large internal pool of C\textsubscript{i}, which is in contrast with C. noctigama, which mainly absorbs CO\textsubscript{2}.

In Chapter 3 we show that the active transport of C\textsubscript{i} in these algae is not only limited to the plasma membrane, but that also the chloroplast plays an important role in the regulation of the uptake of C\textsubscript{i}.

In Chapter 4 we describe the identification and localization of a CA in the thylakoid lumen of the chloroplast which is associated with photosystem II.

In Chapter five we describe the possible function of this CA.

Finally we provide a summary about what is now known about the CCM in both T. minimum and C. noctigama and will discuss the mode of action of the CCM and the possible regulatory effects.
References


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Introduction


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