Mass Spectrometric Analysis of Oxygen Gas Exchange in High- and Low-CO\(_2\) Cells of *Chlorella vulgaris*


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Oxygen gas exchange was monitored in the unicellular green alga *Chlorella vulgaris* 211–11 h by means of a mass spectrometer equipped with a special membrane gas-inlet-system and a photosynthetic reaction vessel. CO\(_2\)-dependent \(^18\)O-uptake as well as \(^18\)O-evolution were analyzed in both High- and Low-CO\(_2\) cells. In High-CO\(_2\) cells, the \(^18\)O-uptake in the light (U\(_L\)) decreased by 65% upon addition of 3 mM NaHCO\(_3\), while \(^18\)O-evolution (E) was increased approx. 1.8 times by the same treatment. \(^18\)O-uptake in the dark (U\(_D\)) was not affected by the addition of external inorganic carbon (C\(_i\)). The addition of 3.3 mM NaHCO\(_3\) also affected U\(_L\) and E in Low CO\(_2\)-cells, however, to a minor extent. U\(_L\) under CO\(_2\)-saturating conditions was light intensity-independent up to 2 klux and 1.2 klux in High- and Low-CO\(_2\) cells, respectively.

Above these light intensities U\(_L\) increased approx. 4-fold in High- and approx. 6-fold in Low-CO\(_2\) cells. Under CO\(_2\)-limiting conditions, however, U\(_L\) increased in High-CO\(_2\)-cells even under very low light intensities, showing that photorespiratory oxygen uptake occurred even in the vicinity of the light compensation point. Under CO\(_2\)-saturating and strong light conditions U\(_L\) represented almost half of E in Low-CO\(_2\)-cells and about 30% of E in High-CO\(_2\)-cells. In Low-CO\(_2\)-cells carbonic anhydrase (CA), an inhibitor of carbonic anhydrase, enhanced U\(_L\) and suppressed E and NET under CO\(_2\)-limiting conditions, whereas the compound had only a minor effect on High-CO\(_2\)-cells.

DCMU (3 \(\mu\)M) strongly inhibited E and U\(_L\) under CO\(_2\)-saturating conditions, with the remaining U\(_L\) being smaller than U\(_L\). KCN (1 mM) and SHAM (1.5 mM) added to DCMU-treated Low-CO\(_2\) cells suppressed U\(_L\) by approx. 50%. The resulting value corresponded to half of U\(_L\). KCN also inhibited E under CO\(_2\)-saturating conditions, with U\(_L\) being strongly enhanced showing a maximal uptake at 0.4 mM KCN. Under these conditions NET was nearly zero. The effect seems to be due to an inhibition of RubisCO and an enhancement of Mehler reactions. At 0.7 mM KCN, DCMU entirely inhibited U\(_L\), but oxygen uptake appeared increased after turning the light off. This uptake corresponded to approx. 60% of U\(_L\). Whereas KCN and SHAM inhibited approx. 70% of U\(_L\), only 16% of U\(_L\) was suppressed. These results suggest that the contribution of mitochondrial respiration to U\(_L\) was negligible, since U\(_L\) seemed to be suppressed in the light under CO\(_2\)-saturated conditions. Iodoacetamide, which is an inhibitor of the Calvin cycle and thereby diverts carbon into the respiratory pathway, inhibited E and NET under CO\(_2\)-saturating conditions, but did not affect U\(_L\). This result also shows that U\(_L\) is not due to mitochondrial respiration. A hydroxylamine derivative [20, 21] which changes the ratio of the RuBP carboxylation to oxygenation activity in tobacco leaves did not affect this ratio in *Chlorella*.

Introduction

During illumination many plants and algae exhibit the wasteful property to release part of the freshly fixed CO\(_2\), at the same time oxygen is taken up [1–3]. The phenomenon is called photorespiration and competes with the CO\(_2\)-fixation process for reducing equivalents. At the same time other CO\(_2\)-consuming reactions like Mehler type reactions [4] and mitochondrial respiration [5, 6] occur in the cells under illumination. It is obvious that photorespiratory as well as the other CO\(_2\)-consuming reactions counteract photosynthesis. Although many investigations have tried to measure Mehler reactions and mitochondrial respiration under conditions where photosynthesis and photorespiration occur i.e. in the light, the extent of their contribution is still an open question [7]. Concerning the Mehler reaction it was measured under relatively high illumination of tobacco leaves [8–11]. This approach is not suitable for algae, since the quantum yield of photosynthesis is low, and the light intensity used is too high to study the effect of CO\(_2\)-limitation [12].

Abbreviations: CA, carbonic anhydrase; C\(_i\), inorganic carbon; E, gross photosynthesis (oxygen evolution); EZA, ethoxyzymolate; High- and Low-CO\(_2\) cells, algal cells grown in air containing 3% and 0.03% CO\(_2\), respectively; NET, apparent photosynthesis; pcv, packed cell volume; U\(_L\) and U\(_D\), oxygen uptake in the dark and in the light, respectively; RubisCO, ribulose 1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose 1,5-bisphosphate; SHAM, salicylhydroxamic acid.

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shown that its importance seems to be the supply of ATP for CO₂-fixation in the chloroplasts [8—11]. On the other hand there was no Mehler reaction at all detectable in Chlamydomonas [6]. As reviewed by Graham [12], many authors believe that mitochondrial respiration is inhibited in the light in comparison to the rate in the dark, whereas others think that respiration is not affected by light. However, several recent reports using algae showed the persistence of mitochondrial respiration in the light [6, 10, 11].

Only a few methods permit the simultaneous measurement of photosynthetic O₂-evolution and photorespiratory O₂-uptake. The method used in the present study was that of mass spectrometry which permits the discrimination of O₂-uptake in the light measured as ₁⁸O₂-evolution measured as ₁⁶O₂-evolution. When algal cells like Chlorella, Chlamydomonas etc. are grown in ordinary air (Low-CO₂ cells), it is well-known that a very efficient mechanism for the utilization of Ci is induced (see reviews by [13, 14]). This mechanisms which facilitates the incorporation of Ci seems to disappear, when the algal cells are grown in air enriched with 1—5% CO₂ (High-CO₂ cells). Low-CO₂ cells exhibit a lower Kₘ (CO₂) for photosynthesis, a lower CO₂ compensation point and a weaker Warburg effect than High-CO₂ cells (see review by [14, 15]). In the case of Chlorella vulgaris, used in the present study, it was reported that the properties of Low-CO₂ cells are due to the action of the enzyme carbonic anhydrase, which is synthesized under low CO₂ tension and disappears under high CO₂ tension, facilitating CO₂ transport from the outside to the CO₂ fixation site [16]. Therefore, in the present study, we compared the activity of ₁⁸O₂-uptake in High-CO₂ cells with that in Low-CO₂ cells.

Materials and Methods

Algal cultures

Chlorella vulgaris 211—11 h was obtained from Prof. W. Kowallik (University of Bielefeld, West-Germany) and originates from the algal collection of the University of Göttingen (West-Germany). The algal cells were axenically grown at 25 °C under continuous illumination by incandescent light in an inorganic medium according to Kowallik [17]. The suspension was continuously bubbled with air enriched with 3% CO₂. After several days, the algal suspension was divided into two portions. One was kept in the same conditions (High-CO₂ cells), whereas the other portion was aerated with ordinary air that is with approx. 0.03% CO₂ (Low-CO₂ cells). These cells were harvested by centrifugation (3000 rpm for 10 min) and the pellets were resuspended in 20 mM MES-NaOH buffer (pH 6.0) to give a final algal concentration of 1 ml pcv per liter.

Measurement of oxygen gas exchange

₁⁸O₂-uptake and ₁⁶O₂-evolution was monitored using a mass spectrometer (Finnigan MAT, Bremen, West-Germany) equipped with a special membrane-gas inlet system and a photosynthetic reaction vessel made of stainless steel, fitted with a plexiglass lid. The reaction vessel with a volume of 7.5 ml was used without gas phase. A gas-permeable but water-impermeable polyvinylchloride membrane (size 7 cm²) was placed on the plastic screen at the bottom of the vessel. During measurements the reaction medium was stirred magnetically. For illumination a projector lamp providing an intensity of 50 klux (162 W × m⁻²) was used. The temperature was kept at 30 °C. NaHCO₃ solutions, as in inorganic carbon source, and inhibitors were injected with a microsyringe through a capillary of the lid. In the system, the sensitivity and the response time are considerably increased by connection of the reaction vessel directly to the ion source. The gas dissolved in the reaction medium is separated by the membrane from the space of the ion source thus by-passing the normal inlet system which permits an only slow and insensitive response due to the large internal volume [18]. Using the mass spectrometer, changes in ₁⁸O₂ (mass 36) and ₁⁶O₂ (mass 32) were simultaneously measured. Experiments were initiated by injection of algae or ₁⁸O₂ gas. After injection of ₁⁸O₂ the algal suspension was kept in the dark until the ₁⁸O₂ and ₁⁶O₂ concentration in the suspension had reached the steady level.

O₂-Uptake rate (U) and gross O₂-evolution (E) were calculated from the following equations as reported by [6].

\[ U(\text{mV} \times \text{min}^{-1}) = (\Delta[M36], - k[M36],)(([M32], + [M36],))/([M36],) \]

\[ E(\text{mV} \times \text{min}^{-1}) = (\Delta[M32], - k[M32],) + U([M32],/[M32], + [M36],)^{-1} \]

where \( k \) is the rate constant of the O₂-decrease due to oxygen consumption by the mass spectrometer. The rate constant measured in the absence of algae was
0.020 min\(^{-1}\). \([M_{36}]_t\) and \([M_{32}]_t\) represent the signal (mV) of mass 36 and mass 32 at time \(t\), respectively. \(\Delta\) means the difference of signals between two successive values. The values of signals (mV) were calibrated to the amount of \(^{18}\)O\(_2\) and \(^{16}\)O\(_2\) in \(\mu\text{mol}\) on the basis of the equilibrium with ordinary air (230 \(\mu\text{M}\) at 30 \(^\circ\)C).

**Results**

Fig. 1 shows the effect of the external CO\(_2\) concentration (Ci) on \(E\), \(U\) and NET in High-CO\(_2\) cells of *Chlorella vulgaris* 211—11 h. \(E\) and NET increased with the CO\(_2\) concentration up to 0.6 mM and saturated above this concentration. In contrast to this, the uptake in the light (\(U_L\)) was nearly constant up to 0.1 mM and decreased beyond this concentration by 65% reaching a steady state level at 0.6 mM. In Low-CO\(_2\) cells \(E\), \(U\) and NET did not show a clear steady dependence on the concentration of external CO\(_2\) (Fig. 2). To the contrary increasing the external CO\(_2\) concentration rather seems to inhibit \(E\), NET and \(U_L\). This seems to be due to an internal CO\(_2\) accumulation in the cells and to a facilitated CO\(_2\) supply caused by the action of carbonic anhydrase (CA) in Low-CO\(_2\) cells. In both High- and Low-CO\(_2\) cells, \(U_D\) was not affected by external CO\(_2\) concentration (Fig. 1 and 2). When light intensity increased in the presence of 3 mM external CO\(_2\) in High-CO\(_2\) cells, \(U_L\) was constant up to 2 klux and increased after this lag with light intensity, whereas \(E\) and NET had a linear dependency on light intensity up to approx. 3 klux (Fig. 3A) and approached light saturation, that is a steady level above this intensity. Under CO\(_2\)-limiting (no addition of external Ci) conditions, a remarkable increase of \(U_L\) was observed (Fig. 3B) even under low light intensity. In the light intensity curve \(E\) outran \(U_L\) at about 1 klux under both CO\(_2\)-saturating and -limiting conditions. \(E\) reached at 40 klux an almost three times higher value than \(U_L\) under CO\(_2\)-saturating conditions but was only 10% higher than that of \(U_L\) under CO\(_2\)-limiting conditions in High-CO\(_2\) cells (Fig. 3). The light intensity curve of Low-CO\(_2\) cells is shown in Fig. 4. Here \(U_L\) was nearly constant up to 1.5 klux and increased above this light intensity much steeper in Low-CO\(_2\) cells (Fig. 4) than in High-CO\(_2\) cells.
Fig. 3. Effect on light intensity on $E$, $U_L$ and NET measured under $CO_2$-saturating (A) and $CO_2$-limiting (B) conditions in High-$CO_2$ cells of *Chlorella vulgaris* 211–11 h. The concentration of external $CO_2$ during photosynthesis was 3.3 mM. The concentration of dissolved $O_2$ changed from 314 to 332 pM during the experiment.

Fig. 4. Effect of light intensity on $E$, $U_L$ and NET measured under $CO_2$-saturating conditions in Low-$CO_2$ cells of *Chlorella vulgaris* 211–11 h. The concentration of external $CO_2$ during photosynthesis was 3.3 mM. The concentration of dissolved $O_2$ changed from 314 to 332 pM during the experiment.

(Fig. 3 A). A remarkable difference between High- and Low-$CO_2$ cells was that $U_L$ under both $CO_2$ and light-saturating conditions reached almost half the value of $E$ in Low-$CO_2$ cells, while $U_L$ in High-$CO_2$ cells was always several times smaller than $E$ under the same conditions (Fig. 1–4). Ethoxyzolamide (EZA, 50 μM), an inhibitor of carbonic anhydrase, suppressed NET, $E$ and $U_L$ by 76–78% under $CO_2$-limiting conditions but the subsequent addition of 10 mM NaHCO$_3$ further decreased $U_L$ and strongly enhanced NET in High-$CO_2$ cells. In Low-$CO_2$ cells, EZA (50 μM) suppressed $E$ by 48%, $U_L$ by 74% and NET by 20%. This effect was changed by the subsequent addition of 10 mM NaHCO$_3$ to 68%, 58% and 79% of the control in $E$, $U$ and NET, respectively (Table I). It should be borne in mind that in these experiments 30% of the inhibition was due to the inhibition of photochemical reactions [19]. The inhibition of $E$ by EZA appeared to be due to the inhibition of the $CO_2$-supply from the outside to the $CO_2$-fixation site by inhibition of the enzyme car-
bonic anhydrase (CA). E seemed to be completely inhibited by DCMU whereas U_D was not inhibited by DCMU (Fig. 5, Table II). The addition of 1 mM KCN and 1.5 mM SHAM upon this assay suppressed U_D by approx. 70% (Table II). U_L was enhanced by the addition of KCN showing a peak at 0.4 mM (Fig. 6), while E appeared to be inhibited with increasing KCN concentrations. At 0.7 mM KCN, E and U_L showed almost the same rate. The addition of DCMU at this stage lowered U_L to nearly zero. In the presence of 1 mM KCN, U_D still maintained approx. 50% of its original activity (Table II). This data might suggest that mitochondrial respiration is rather suppressed in the light (Table II). Fig. 7 shows the effect of iodoacetamide, which is an inhibitor of triose phosphate dehydrogenase, on E, U and NET under CO_2-saturating conditions in High-CO_2 cells. Approx. 60% of E was inhibited but U_L as well as U_D were not affected by this inhibitor. A hydroxylamine derivative (HA 368) which is an inhibitor of the Warburg effect, observed in tobacco leaves, causes conformational changes of the enzyme RubisCO [20, 21], suppressed both E and U_L, but NET was kept almost constant (Fig. 8).

![Fig. 5. Effect of DCMU on E, U_L and NET measured under CO_2 saturating conditions in High-CO_2 cells of Chlorella vulgaris 211–11 h. The concentration of external CO_2 during the measurement was 6.4 mM. The concentration of dissolved O_2 changed from 211 to 153 μM during the experiment.](image1)

![Fig. 6. Effect of KCN on E, U_L and NET measured under CO_2 saturating conditions in High-CO_2 cells of Chlorella vulgaris 211–11 h. The concentration of CO_2 during photosynthesis was 3 mM. The concentration of dissolved O_2 changed from 295 to 336 μM.](image2)
Table II. Effect of KCN, SHAM and DCMU on $U_L$ and $U_D$ in High-CO$_2$ cells of Chlorella vulgaris 211–11 h.

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Condition</th>
<th>Rate of $^{18}$O$_2$-uptake [$\mu$mol x ml pcv$^{-1}$ x h$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control</td>
<td>$778$ (100%)</td>
</tr>
<tr>
<td></td>
<td>+ 1 mM KCN</td>
<td>$66$ (56%)</td>
</tr>
<tr>
<td></td>
<td>+ 1 mM KCN + 1.5 mM SHAM</td>
<td>$650$ (84%)</td>
</tr>
<tr>
<td>II</td>
<td>Control</td>
<td>$456$ (100%)</td>
</tr>
<tr>
<td></td>
<td>+ 2.6 $\mu$m DCMU</td>
<td>$145$ (32%)</td>
</tr>
<tr>
<td></td>
<td>+ 2.6 $\mu$m DCMU + 1 mM KCN + 1.5 mM SHAM</td>
<td>$70$ (15%)</td>
</tr>
<tr>
<td></td>
<td>+ 1 mM KCN + 1.5 mM SHAM</td>
<td>$229$ (105%)</td>
</tr>
</tbody>
</table>

No external CO$_2$ was added in Exp. I, whereas 6.4 mM NaHCO$_3$ were added in Exp. II. Light intensity was 40 klux. $O_2$-concentration during the experiment was near the ordinary air-level.

Fig. 7. Effect of iodoacetamide on E, $U_L$ and NET measured under CO$_2$ saturating conditions in High-CO$_2$ cells of Chlorella vulgaris 211–11 h. The concentration of external CO$_2$ during the measurement was 10 mM. The concentration of dissolved $O_2$ changed from 202 to 180 $\mu$m during the experiment.

Fig. 8. Effect of a hydroxylamine derivative (HA 368) on E, $U_L$ and NET measured without addition of external CO$_2$ in High-CO$_2$ cells of Chlorella vulgaris 211–11 h. The concentration of dissolved $O_2$ changed from 309 to 248 $\mu$m during the experiment. Light intensity was 40 klux (162 W x m$^{-2}$).
Discussion

In the present paper we show that, when oxygen gas exchange is measured in the light, the ratio of E/U₁ is 1.3 under the condition without any addition of external CO₂, but 3.5 with 3 mM external CO₂ in High-CO₂ cells. In Low-CO₂ cells, the same ratio is 2.6 without external CO₂ and 2.4 with 3.2 mM external CO₂ (Fig. 1 and 2). If the O₂-uptake, which is dependent on the external CO₂ concentration, is defined as photorespiratory O₂-uptake, it appears that 35% of U₁ under CO₂-limiting conditions is due to photorespiratory O₂-uptake in High-CO₂ cells (Fig. 1). In the case of Low-CO₂ cells, it was not possible to exactly calculate the photorespiratory O₂-uptake from Fig. 2, but the extent seems to be smaller than that of High-CO₂ cells. At any rate it appeared that U₁ was higher than in High-CO₂ cells, independent on the external CO₂-concentration given during the experiment (Fig. 2), which might be due to the activity of Mehler type reactions. From the literature it is well-known that Low-CO₂ cells exhibit in photosynthesis a high affinity for CO₂ due to the action of the enzyme carbonic anhydrase whereas High-CO₂ cells have only a very low carbonic anhydrase activity [16]. From this it can be expected that E will be suppressed whereas U₁ will be increased upon addition of the carbonic anhydrase inhibitor ethoxyzolamide, since the inhibitor is supposed to block the CO₂ supply and as a consequence increases the O₂-supply to RubisCO. Table I generally supports this notion, but it appears that the inhibitory effect of ethoxyzolamide on E was stronger than its stimulatory effect on U₁.

The responses of E, U and NET on the light intensity in High- and Low-CO₂ cells are shown in Fig. 3 and 4 and fit into observations reported in the literature [22]. E measured under CO₂-saturating conditions was very sensitive to light intensity, especially in the weak light region up to 3 klux. In contrast to this, oxygen uptake in the light (U₁) was insensitive to light up to 1.5–2 klux under the same conditions (Fig. 3A and 4), whereas U₁ depended on the light intensity under CO₂-limiting conditions (Fig. 3B). This data might mean that CO₂-dependent O₂-uptake in the light, i.e. photorespiratory O₂-uptake, also occurs under very weak light conditions even in the vicinity of the light compensation point. The rate of the U₁ portion, insensitive to light, was the same as that of U_D. Under CO₂- and light-saturated conditions U₁ was 3.3 times higher in High-CO₂ cells and 6 times higher in Low-CO₂ cells than U_D (Fig. 3A and 4). Addition of DCMU, an inhibitor of photo-system II plus KCN plus SHAM, inhibitors of RubisCO and mitochondrial respiration, strongly suppressed U₁ whereas the addition of KCN plus SHAM alone did not substantially affect U₁ (Table II). The rate of the remaining portion of U₁ was enhanced by switching the light off (Table II, Fig. 5 and 6). This data shows that mitochondrial O₂-uptake was inhibited in the presence of these inhibitors in the light, showing that mitochondrial respiration is probably suppressed in the light. From this it is concluded that most of U₁ under CO₂- and light-saturating conditions is due to Mehler type reactions, whereas under CO₂-limiting and light-saturating conditions U₁ was a mixed composition of Mehler type reactions and photorespiratory O₂-uptake. The stimulatory effect of KCN on U₁ seems to be an inhibitory effect on RubisCO, as reported by Siegel et al. [23], which means that the stimulation of U₁ was mainly due to a Mehler reaction and/or to the reaction which supplies ¹⁸O₂ to glycolate even in the presence of KCN, as reviewed by Beck [24].

The effect of iodoacetamide, an inhibitor of triose phosphate dehydrogenase in the Calvin Benson Cycle, on U₁ under CO₂-saturating conditions, as shown in Fig. 7, also suggests that mitochondrial respiration was at least not enhanced in strong light and that most of U₁ was due to Mehler type reactions.

As already mentioned above, the occurrence of mitochondrial respiration in the light has been a matter of controversy. Earlier reports have suggested that mitochondrial respiration was inhibited by ATP production in illuminated chloroplasts [12, 25]. In contrast to this, recent reports suggested that mitochondrial respiration is maintained during illumination [6, 11]. The existence of Mehler type reactions, i.e. the direct photoreduction of O₂ using energy derived from the photosystems in intact cells also remains a matter of discussion [7], although reports of the literature have shown that Mehler reactions might play an important role for the ATP supply in CO₂-fixation in intact chloroplasts [7, 8]. Recently, for example, Brechignac and André [10] have been able to demonstrate a significant involvement of Mehler type reactions in the red macroalga Chondrus crispus whereas Peltier and Thibault [6] reported that no Mehler reactions occurred in
Chlamydomonas. In entire tobacco plants Ishii and Schmid [26] have shown that the $^{18}\text{O}_2$-uptake in the light is due to photorespiration and Mehler type reactions in the wild type $N. \text{tabacum}$ var. John William's Broadleaf, whereas $^{18}\text{O}_2$-uptake in the light was exclusively due to photorespiration and contained no Mehler type reactions in the chlorophyll-deficient mutant $Su/su$. As a result, the present study with Chlorella subscribes to the opinion that mitochondrial respiration is suppressed in the light, at least under CO$_2$-saturating conditions, and that Mehler reactions, whose activity take place in intact cells are enhanced under strong light.

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