Effect of the CO₂-Concentration during Growth on the Oxygen Evolution Pattern under Flash Light in *Chlorella*

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Photosynthetic O₂-evolution patterns were determined in cells of *Chlorella vulgaris* 211–11 h, grown under air enriched with 2% CO₂ (*High CO₂-cells*) and under ordinary air (*Low CO₂-cells*). Oxygen evolution in these algae was measured as consequence of short saturating light flashes with the three electrode system according to Schmid and Thibault, Z. Naturforsch. 34c, 414 (1979). It was shown that *Low CO₂-cells* had the usual Joliot-Kok pattern of O₂ evolution whereas *High CO₂-cells* exhibited a more reduced pattern characterized by the fact that maximal flash yield was observed under the 4th flash and that damping due to misses was more important than in *Low CO₂-cells*. Moreover, the amplitudes of the amperometric signals in *High CO₂-cells* were consistently lower. The observations clearly speak in favor of a more reduced condition of the S-state system, when the cells are grown under high CO₂. This was confirmed by the fact that higher concentrations of hydroxylamine had to be added to bring *Low CO₂-cells* into the maximally reduced condition namely S₂; than was the case with *High CO₂-cells*. Our observations suggest that the redox condition of the S-state system of photosystem II in *Chlorella vulgaris* is affected either by changes of CO₂ concentrations during algal growth or that the S-state system is only maintained in the 4 state Kok condition when the enzyme carbonic anhydrase is present.

**Introduction**

Thibault has repeatedly demonstrated that the oxygen evolution pattern observed, when oxygen evolution is measured as consequence of a flash series, exhibits an abnormality under the first flash [1, 2]. The abnormality in turn is the consequence of the usual interpretation mode of such flash patterns in the frame of the 4-state Kok model [3]. The abnormality disappears fully if a 5-state Kok model is assumed [4]. This observation has led Thibault to assume that in the distribution of S-states in a dark adapted sample, contribution of a more reduced dark stable state S₁ should exist, which in *Chlorella* would account for up to 17% of the S-states [4]. The state is supposed to represent a redox condition of the positive charge accumulation complex which requires the successive absorption of 5 light quanta to evolve 1 molecule oxygen. The preponderant presence of this state has been demonstrated in dark adapted *Euglena gracilis* in which up to 60% of all states were S₀ after usual dark adaptations [5]. The question remained what cellular conditions would cause such a reduction of the photosystem II complex. In the present paper we demonstrate that *Chlorella* cells, grown under high CO₂ (2%) concentrations, represent a condition in which the S-state system is substantially shifted towards the S₁ condition. Cells grown under normal CO₂ pressure (330 ppm) exhibit a normal flash pattern. It appears that the presence of the enzyme carbonic anhydrase exerts an effect on the redox condition of the S-state system. The observation is discussed in context with CO₂/HCO₃⁻ effects on photosystem II, reported in the literature [6, 7].

**Materials and Methods**

*High CO₂-cells* and *Low CO₂-cells of Chlorella vulgaris* were grown either under air containing 2% CO₂ or in normal air (330 ppm CO₂) as described earlier [8, 9]. Oxygen measurements were carried out by polarography with the three-electrode system described by Schmid and Thibault [10]. Conditions for the experiments are the same as described previously [13]. Ethoxyzolamide was the generous gift of Dr. Thilo and Co. GmbH Sauerlach, Munich, Germany.

Abbreviations: Ethoxyzolamide, 6-ethoxybenzothiazole-2-sulfonamide.

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Results

Earlier publications have shown that the content and activity of the enzyme carboxic anhydrase depends on the CO₂ partial pressure under which algae like *Chlorella* and *Chlamydomonas* are cultivated [9, 11, 12]. High CO₂ concentrations e.g. 2–3% suppress the synthesis of the enzyme, whereas the “normal” CO₂ partial pressure of air (330 ppm) allows high carboxic anhydrase activity [9, 11, 12]. Hence, *Chlorella vulgaris* grown under normal (330 ppm) CO₂ and algae grown under high CO₂ partial pressure (3%) were compared with respect to their oxygen evolution patterns. The flash sequence consisted of Xenon flashes of 5 μsec duration separated by 300 msec dark period. Fig. 1 shows that the oxygen evolution pattern is appreciably different in High and Low CO₂-cells. Low CO₂-cells give a flash sequence which corresponds to the pattern usually reported in the literature for *Chlorella*. The oscillations clearly show a periodicity of four with maximal flash yield under the 3rd flash. High CO₂-cells on the other hand show a pattern which exhibits much lower amplitudes, the sequence is strongly damped and maximal flash yield occurs under the 4th and often even under the 5th flash (see also Fig. 5). The distribution of the S-state population calculated from a fit in the 4-rank Kok model shows that a reasonable distribution can only be calculated for the Low CO₂-cells (Table I). The distribution corresponds in this case essentially to 70–76% S₀ and 26–30% S₁ disregarding the obvious abnormality concerning S₂ which is in agreement with the literature. High CO₂-cells, however, give a fit which is not acceptable. Calculation of the same data in the five-rank Kok model [2, 13] gives for both samples a perfect fit showing for the Low CO₂-cells a distribution in which 4–5% S₁ contribute to the initial dark population of S-states [4] whereas the High CO₂-cells are apparently in a condition in which more than 12 per cent of the initial dark population of S-states is in the condition S₁ (Table II). In the case of Fig. 5a the contribution of S₁ reaches more than 40% of initial dark population. It thus looks as if the presence of high carboxic anhydrase activities prevents the S-state system from overreduction. However, ethoxyzolamide, an inhibitor of carboxic anhydrase, further

![Fig. 1. Photosynthetic oxygen evolution measured as the consequence of a sequence of short (5 μsec) saturating xenon flashes in *Chlorella vulgaris*. ▲ Algae grown with 330 ppm CO₂ (Low CO₂-cells) which is the condition with high carboxic anhydrase activity; • algae grown with 3% CO₂ (High CO₂-cells); △ algae grown with 330 ppm but in the presence of 0.1 mM ethoxyzolamide which was added before the measurement in the electrode assay.](image-url)

<table>
<thead>
<tr>
<th>Sequence</th>
<th>S₀</th>
<th>S₁</th>
<th>S₂</th>
<th>S₃</th>
<th>Misses</th>
<th>Double hits</th>
<th>Δ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>High CO₂-cells</td>
<td>46.0</td>
<td>61.3</td>
<td>-9.3</td>
<td>2</td>
<td>20</td>
<td>8.2</td>
<td>3.2</td>
</tr>
<tr>
<td>Low CO₂-cells</td>
<td>30.1</td>
<td>71.6</td>
<td>-2.5</td>
<td>0.8</td>
<td>15</td>
<td>4</td>
<td>1.0</td>
</tr>
<tr>
<td>Low CO₂-cells + Ethoxyzolamide</td>
<td>26.8</td>
<td>76</td>
<td>-3.5</td>
<td>0.7</td>
<td>15</td>
<td>4</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Table I. S-State population in *Chlorella vulgaris* calculated from a fit in the 4-state Kok Model.

% Δ, relative quadratic deviation.
Table II. S-State population in *Chlorella vulgaris* calculated from a fit in the 5-state Kok Model.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>$S_{-1}$</th>
<th>$S_1$</th>
<th>$S_2$</th>
<th>$S_3$</th>
<th>Misses $\alpha$ (in per cent)</th>
<th>Double hits $\gamma$</th>
<th>$\Delta%$</th>
</tr>
</thead>
<tbody>
<tr>
<td>High CO$_2$-cells</td>
<td>12.5</td>
<td>38.4</td>
<td>51.5</td>
<td>-3.3</td>
<td>1.1</td>
<td>15.6</td>
<td>5.8</td>
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<tr>
<td>Low CO$_2$-cells</td>
<td>4</td>
<td>29.1</td>
<td>67.5</td>
<td>-1.3</td>
<td>0.7</td>
<td>14.1</td>
<td>3.8</td>
</tr>
<tr>
<td>Low CO$_2$-cells + Ethoxyzolamide</td>
<td>5.5</td>
<td>25.4</td>
<td>70.3</td>
<td>-1.8</td>
<td>0.5</td>
<td>14</td>
<td>3.5</td>
</tr>
</tbody>
</table>

$\Delta\%$, relative quadratic deviation. Note that the fit as verified with $\Delta\%$ is much better in the five rank model than in the four rank model of Table I.

increases this property by increasing not only oxygen amplitudes (Fig. 1) but lessening the damping, which means a reduction of misses. This might mean that the conformation of the enzyme protein exerts some influence on the structure of photosystem II. The redox state of the oxygen evolving apparatus in *Low* and *High* CO$_2$-cells can already be deduced from the O$_2$-evolving transient in a 30 sec light pulse (Fig. 2). *High* CO$_2$-cells show a clearly reduced fast oxygen gush (Fig. 2B) in comparison to *Low* CO$_2$-cells (Fig. 2A). Since $S_{-1}$ is supposed to be a dark stable state which accumulates in dark and hence in the course of long dark adaptations, a suitable shorter dark adaptation of *High* CO$_2$-cells should yield a normal flash pattern. Fig. 3 clearly demonstrates that a flash sequence, fired 1 min after the previous one, yields a normal flash pattern. Dark relaxation of 5 minutes leads to the reduced initial condition. Since $S_{-1}$ is the redox condition of the positive charge accumulation complex in which 5 light quanta have to be absorbed for the evolution of 1 molecule of oxygen, a successful transition from $S_{-1} \rightarrow S_0$ would require 1 flash. Fig. 4 shows that if a flash sequence with *High* CO$_2$-cells is
Fig. 4. Photosynthetic oxygen evolution measured as consequence of a flash sequence in High CO₂-cells. a. Dark relaxed sequence (20 min); b. the algal cells received one single flash after 5 min dark relaxation. Thereafter the complete flash sequence was fired after 1 min of dark relaxation.

preceded by 1 flash 1 minute prior to the sequence a normal flash pattern is obtained. In High CO₂-cells the state S₁ seems to be a condition which by strong reducing agents such as hydroxylamine cannot be pushed to S₂. For unknown reasons the pattern in Fig. 5a is practically final. This observation was also true for Euglena [5]. However, Low CO₂-cells are gradually reduced to a final condition described already earlier by Bouges-Bocquet [14] in which maximal flash yield is observed under the 7th flash (Fig. 6c). In the presence of excess hydroxylamine complex formation between S₂ and hydroxylamine is assumed which requires one additional light quantum for its separation. The resulting state S₂ thereafter requires the successive absorption of 6 light quanta for the evolution of one molecule of oxygen [4, 14]. When High CO₂-cells are transferred to the normal CO₂-conditions (330 ppm), the synthesis of the enzyme carbonic anhydrase is induced, as reported in the literature [9, 11, 12]. Fig. 7 shows that one hour after the transfer from 2% CO₂ to 330 ppm

Fig. 5. Effect of hydroxylamine on the oxygen evolution pattern of High CO₂-cells. a. Control without additions; b. sequence in the presence of 40 µM hydroxylamine.

Fig. 6. Effect of hydroxylamine on the oxygen evolution pattern of Low CO₂-cells. a. Control; b. in the presence of 40 µM hydroxylamine and c. in the presence of 240 µM hydroxylamine which shows full reduction of the S-state system with maximum flash yield under the 7th flash i.e. reduction to the S₂-condition.
CO₂ the oxygen evolution pattern starts to become normal and reaches the preponderant 4 state Kok conditions after approx. 3 hours.

Discussion
The described observations refer to two growth conditions which result in two types of Chlorella cultures which differ in their content of carbonic anhydrase [9, 11]. The observed effect on the S-state system might theoretically be due to two things: The presence or the absence of the enzyme carbonic anhydrase as such might influence the redox environment of the positive charge accumulation complex, or the phenomenon is caused by the substrates of the enzyme i.e. by either bicarbonate or CO₂. In the latter case it should be possible to bring our observations in line with reports of the literature on CO₂ or bicarbonate effects on the donor side of photosystem II [15, 16]. According to Stemler [7] high concentrations of bicarbonate are supposed to inhibit photosynthesis especially when the pH is around 8. In High CO₂-cells in which no carbonic anhydrase is present normal diffusion of CO₂ into the chloroplast might indeed lead to high HCO₃⁻ levels on the inside face of the thylakoid membrane. We feel, however, that a Stemler-type of inhibition can be excluded since growth conditions under 2–3% CO₂ (which is the condition with no or low carbonic anhydrase activity) do not really lead to an inhibition but rather to a more reduced condition of the “positive charge accumulation complex” which can be overcome by one single light flash (Fig. 4). In the presence of high amounts of the enzyme protein the photosystem II reaction center is apparently in the condition of conformation which requires the successive absorption of only 4 light quanta to evolve one molecule of oxygen (Fig. 1). This “structural influence” is clearly visible when in Low CO₂-cells (n.b. the condition with presence of high carbonic anhydrase levels!) the effect of ethoxzolamide, a carbonic anhydrase inhibitor, is compared to the condition in which the enzyme is not present (Fig. 1). In contrast to Stemler and Jursinic [15] this carbonic anhydrase inhibitor does not inhibit photosystem II or at least not the S-state system. To the contrary, presence of the inhibitor stimulates or increases the amperometric signal amplitudes in the flash sequence considerably. If the function of carbonic anhydrase is to funnel CO₂ via a bicarbonate shuttle to the site of CO₂ fixation, in the sample with ethoxzolamide the CO₂ concentration in the immediate interface between the thylakoid membrane and the stroma should be considerably decreased. This could mean that the high local CO₂ concentrations provided by carbonic anhydrase activity inhibit photosystem II. We do not favor this possibility since it would really be the opposite of Stemler’s observation who described that CO₂ in the ratio of 1 CO₂ per reaction center is necessary for photosystem II activity [7]. Also, a competition be-
between CO₂ and O₂ for reducing equivalents in the fashion described by Radmer and Kok [17] is not contained in our measurements. In this case the O₂-evolution pattern should contain a rapid O₂-uptake [10] which we did not observe. In conclusion an influence of the enzyme protein on the conformation of the reaction center of photosystem II seems more acceptable.

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