Effect of the CO_2-Concentration during Growth on the Oxygen Evolution Pattern under Flash Light in *Chlorella*

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Photosynthetic O_2-evolution patterns were determined in cells of *Chlorella vulgaris* 211–11 h, grown under air enriched with 2% CO_2 *(High CO_2-cells)* and under ordinary air *(Low CO_2-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 CO_2-cells* had the usual Joliot-Kok pattern of O_2 evolution whereas *High CO_2-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 CO_2-cells*. Moreover, the amplitudes of the amperometric signals in *High CO_2-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 CO_2. This was confirmed by the fact that higher concentrations of hydroxylamine had to be added to bring *Low CO_2-cells* into the maximally reduced condition namely S_{-2}, than was the case with *High CO_2-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 CO_2 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 S_{-1} 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 S_{-1} 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 CO_2 (2%) concentrations, represent a condition in which the S-state system is substantially shifted towards the S_{-1} condition. Cells grown under normal CO_2 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 CO_2/HCO_3^- effects on photosystem II, reported in the literature [6, 7].

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

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Materials and Methods

High CO_2-cells and *Low CO_2-cells* of *Chlorella vulgaris* were grown either under air containing 2% CO_2 or in normal air (330 ppm CO_2) 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.

Results

Earlier publications have shown that the content and activity of the enzyme carbonic anhydrase depends on the CO_2 partial pressure under which algae like *Chlorella* and *Chlamydomonas* are cultivated [9, 11, 12]. High CO_2 concentrations *e.g.* 2–3% suppress the synthesis of the enzyme, whereas the "normal" CO_2 partial pressure of air (330 ppm) allows high carbonic anhydrase activity [9, 11, 12]. Hence, *Chlorella vulgaris* grown under normal (330 ppm) CO_2 and algae grown under high CO_2 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 CO_2-cells*. *Low CO_2-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 CO_2-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 CO_2-cells* (Table I). The distribution corresponds in this case essentially to 70–76% S_1 and 26–30% S_0 (disregarding the obvious abnormality concerning S_3) which is in agreement with the literature. *High CO_2-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 CO_2-cells* a distribution in which 4–5% S_{-1} contribute to the initial dark population of S-states [4] whereas the *High CO_2-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 S_{-1} (Table II). In the case of Fig. 5a the contribution of S_{-1} reaches more than 40% of initial dark population. It thus looks as if the presence of high carbonic anhydrase activities prevents the S-state system from overreduction. However, ethoxyzolamide, an inhibitor of carbonic anhydrase, further

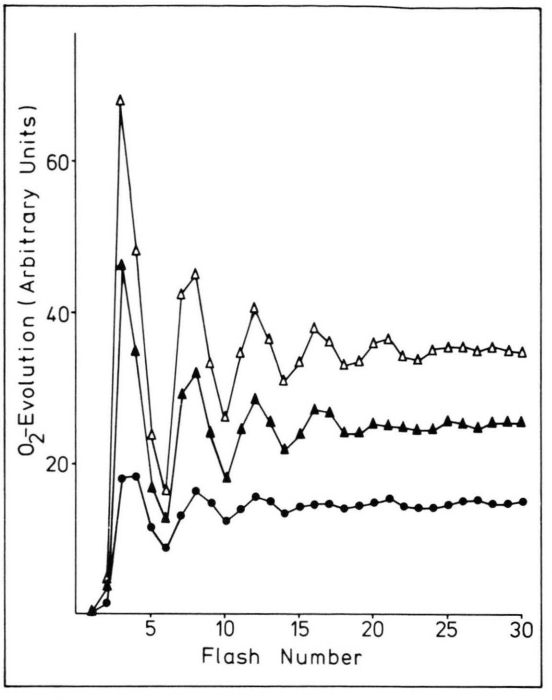

Fig. 1. Photosynthetic oxygen evolution measured as the consequence of a sequence of short (5 μsec) saturating xenon flashes in *Chlorella vulgaris*. ▲ Algae grown with 330 ppm CO_2 *(Low CO_2-cells)* which is the condition with high carbonic anhydrase activity; ● algae grown with 3% CO_2 *(High CO_2-cells)*; △ algae grown with 330 ppm but in the presence of 0.1 mM ethoxyzolamide which was added before the measurement in the electrode assay.

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

Sequence	S_0	S_1	S_2	S_3	Misses α (in per cent)	Double hits γ	Δ %
High CO_2-cells	46.0	61.3	−9.3	2	20	8.2	3.2
Low CO_2-cells	30.1	71.6	−2.5	0.8	15	4	1.0
Low CO_2-cells + Ethoxyzolamide	26.8	76	−3.5	0.7	15	4	1.1

% Δ, relative quadratic deviation.

Table II. S-State population in *Chlorella vulgaris* calculated from a fit in the 5-state Kok Model.

Sequence	S_{-1}	S_0	S_1	S_2	S_3	Misses α (in per cent)	Double hits γ	Δ %
High CO_2-cells	12.5	38.4	51.5	−3.3	1.1	15.6	5.8	1.9
Low CO_2-cells	4	29.1	67.5	−1.3	0.7	14.1	3.8	0.6
Low CO_2-cells + Ethoxyzolamide	5.5	25.4	70.3	−1.8	0.5	14	3.5	0.6

% Δ, relative quadratic deviation. Note that the fit as verified with Δ% 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

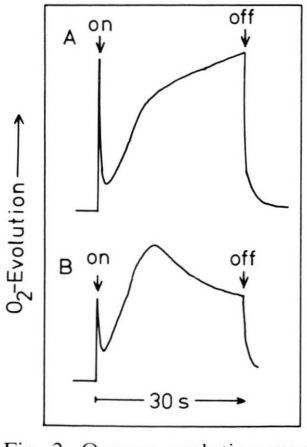

Fig. 2. Oxygen evolution measured during a 30 sec light pulse. A) *Low CO_2-cells* which have high carbonic anhydrase activity; B) *High CO_2-cells*.

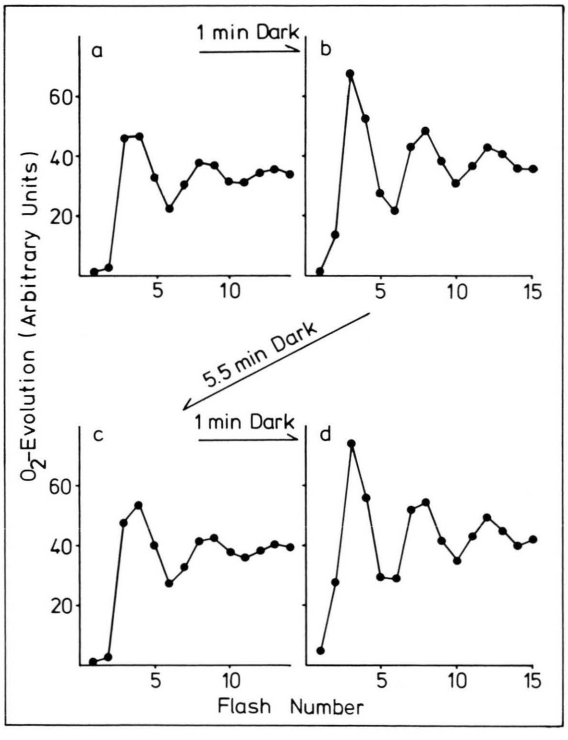

Fig. 3. Photosynthetic oxygen evolution measured as consequence of a flash sequence in *Chlorella* cells grown with 3% CO_2 *(High CO_2-cells)*. a. Dark relaxed sequence (5 min or more); b. sequence fired after 1 min dark relaxation; c. sequence obtained after 5.5 min of dark relaxation of sequence b.; d. sequence obtained after 1 min dark relaxation of c. The figure shows that *High CO_2-cells* are in a more reduced condition than *Low CO_2-cells*.

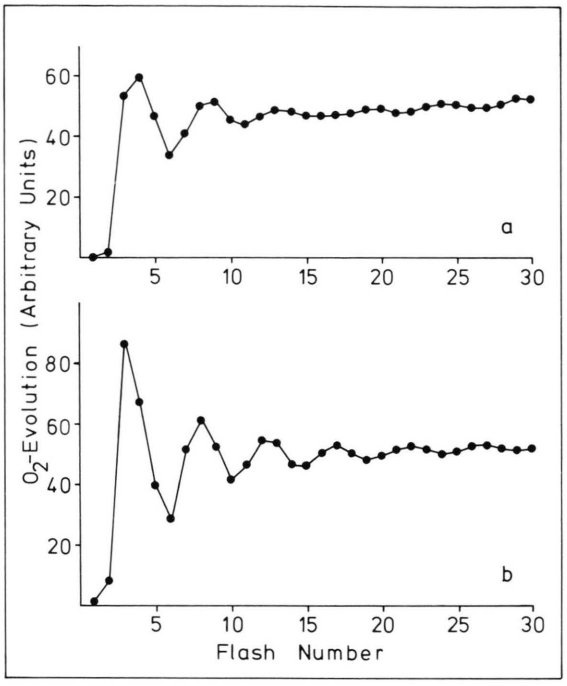

Fig. 4. Photosynthetic oxygen evolution measured as consequence of a flash sequence in *High CO_2-cells*. a. Dark relaxed sequence (20 min); b. the algal cells received one single flash after 5 min dark relaxation. Thereafter the complete flash sequence was fired after 1 min of dark relaxation.

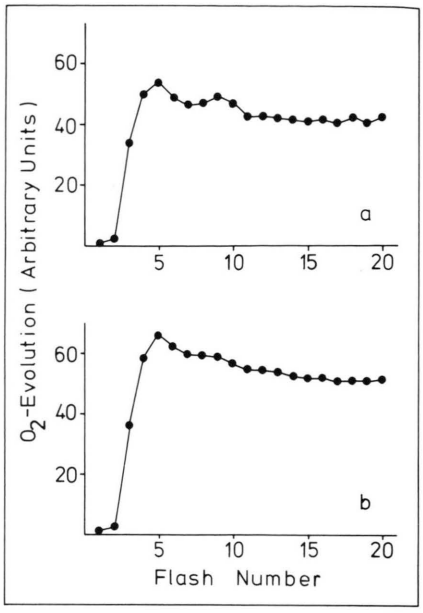

Fig. 5. Effect of hydroxylamine on the oxygen evolution pattern of *High CO_2-cells*. a. Control without additions; b. sequence in the presence of 40 μM hydroxylamine.

preceded by 1 flash 1 minute prior to the sequence a normal flash pattern is obtained. In *High CO_2-cells* the state S_{-1} seems to be a condition which by strong reducing agents such as hydroxylamine cannot be pushed to S_{-2}. For unknown reasons the pattern in Fig. 5a is practically final. This observation was also true for *Euglena* [5]. However, *Low CO_2-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 S_{-2} and hydroxylamine is assumed which requires one additional light quantum for its separation. The resulting state S_{-2} thereafter requires the successive absorption of 6 light quanta for the evolution of one molecule of oxygen [4, 14]. When *High CO_2-cells* are transferred to the normal CO_2-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% CO_2 to 330 ppm

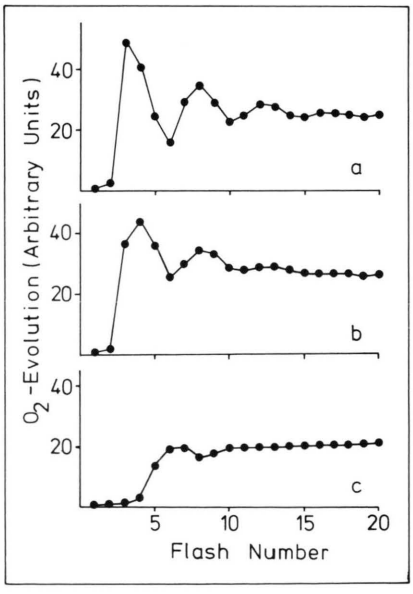

Fig. 6. Effect of hydroxylamine on the oxygen evolution pattern of *Low CO_2-cells*. a. Control; b. in the presence of 40 μM hydroxylamine and c. in the presence of 240 μM hydroxylamine which shows full reduction of the S-state system with maximum flash yield under the 7th flash *i.e.* reduction to the S_{-2}-condition.

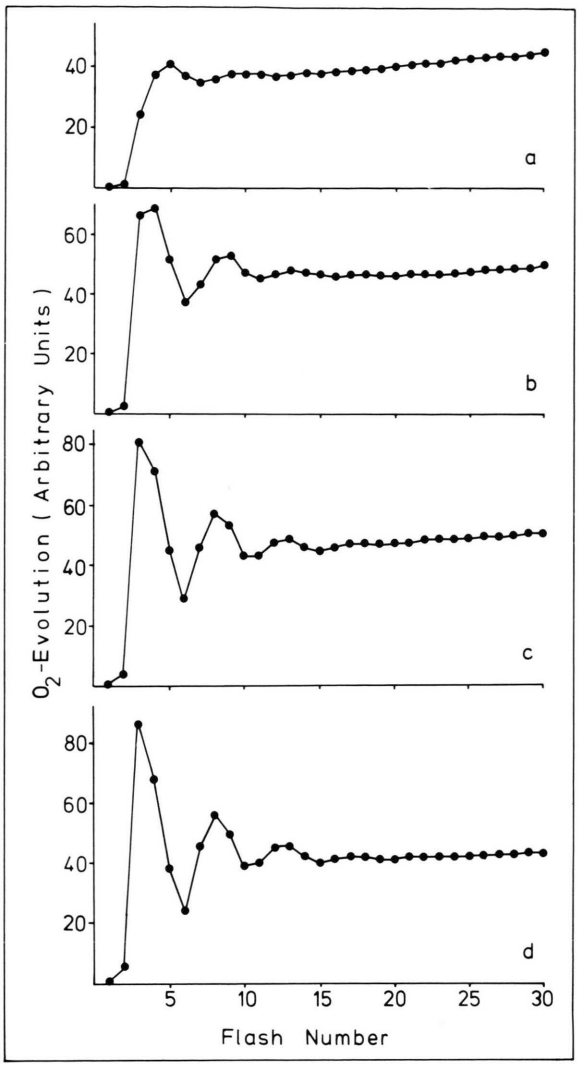

Fig. 7. Modification of the photosynthetic O_2-evolution pattern by the transfer of *High CO_2-cells* to low CO_2 (0.03%) concentration. a. *High CO_2-cells;* b., c. and d. 1 h, 3.3 h and 5 h after transfer, respectively.

CO_2 the oxygen evolution pattern starts to became 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 CO_2. In the latter case it should be possible to bring our observations in line with reports of the literature on CO_2 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 CO_2-cells* in which no carbonic anhydrase is present normal diffusion of CO_2 into the chloroplast might indeed lead to high HCO_3^- 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% CO_2 (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 CO_2-cells* (n.b. the condition with presence of high carbonic anhydrase levels!) the effect of ethoxyzolamide, 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 CO_2 via a bicarbonate shuttle to the site of CO_2 fixation, in the sample with ethoxyzolamide the CO_2 concentration in the immediate interface between the thylakoid membrane and the stroma should be considerably decreased. This could mean that the high local CO_2 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 CO_2 in the ratio of 1 CO_2 per reaction center is necessary for photosystem II activity [7]. Also, a competition be-

tween CO_2 and O_2 for reducing equivalents in the fashion described by Radmer and Kok [17] is not contained in our measurements. In this case the O_2-evolution pattern should contain a rapid O_2-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.

Acknowledgement

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