Biochimica et Biophysica Acta, 408 (1975) 135-142

BBA 46998

PHOTO-INACTIVATION OF SYSTEM II CENTERS BY CARBONYL CYANIDE *m*-CHLOROPHENYLHYDRAZONE IN *CHLORELLA PYRENOIDOSA*

C. LEMASSON* and A. L. ETIENNE

Laboratoire de Photosynthèse, C.N.R.S., 91190 Gif-sur-Yvette (France)

(Received April 29th, 1975)

SUMMARY

In the presence of a high concentration of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) ($4 \cdot 10^{-6}$ M), the S_2 and S_3 dark decays are accelerated and become biphasic with a first half-time of 0.6 s. The first fast phase of the decays does not correspond to a simple reduction of S_2, S_3 back to S_0, S_1 (i.e. to an acceleration of the deactivation reaction), but to a decrease in the number of oxygen-evolving System II centers. This photo-inactivation produced by CCCP is rapidly reversible in the dark.

INTRODUCTION

According to the hypothesis of Kok et al. [1], System II requires the sequential accumulation of four positive charges before O_2 is evolved. These charges are stored on the water-splitting enzyme. According to its different oxidized forms, five states of Photosystem II can be defined: S_n ($0 \leqslant n \leqslant 4$). S_4 is rapidly reduced by H_2O back to S_0 [2]. S_2 and S_3 have a lifetime of several seconds [3, 1]. The mechanism of their disappearance in the dark is not yet elucidated. Up to now, the general consensus was to consider that when S_2 and S_3 concentrations were decreasing, there was a concomitant increase in the S_0, S_1 concentrations, the number of photoactive System II centers being constant.

Some years ago, Renger showed that several chemicals are able to accelerate the decay of the stationary yield of oxygen, under flashing light, when the flash frequency is decreased [4,5]. He attributed such an effect to an acceleration of the S_2, S_3 deactivation reactions of the water-splitting enzyme Y (the ADRY effect) i.e. their reduction either by the plastoquinone pool or by a reductant on the acceptor side of System I [6].

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCMU, 3-(3,4-dichlorophenyl)-1, 1-dimethylurea; ADRY = acceleration of the deactivation reactions of the water-splitting enzyme system Y.

* This work represents partial fulfillment of the requirements for a Thèse de Doctorat ès Sciences, Université de Paris-Sud, Centre d'Orsay (C.N.R.S. No. AO 11.430).

In the present report, we study the effect of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, one of the ADRY substances) on the oxygen evolution in whole cells of *Chlorella pyrenoidosa*. The results cannot be explained by Renger's hypothesis. They disclose a new type of chemical event which can occur in Photosystem II centers.

MATERIALS AND METHODS

The experiments are performed with *C. pyrenoidosa* grown as previously described [7]. The algae are suspended in 0.05 M phosphate buffer, pH 6.5, KCl 0.1 M. The short (5 μs) saturating flashes are produced by a Strobotac (General Radio Company). The spacing between flashes can be varied from 0.1 to 5 s. One or two preilluminating flashes can also be given before a flash sequence. The oxygen yield per flash is measured with a rate electrode identical to that described by Joliot and Joliot [8]. After differentiation and amplification the flash yields of O_2 are observed on an oscilloscope and recorded photographically. Each experiment was started with dark-adapted algae.

RESULTS

Effect of CCCP on the kinetics of S_2 and S_3 dark decays

Considering that only S_0 S_1 are stable in the dark, we used the standard procedure to study the decays [1, 3, 6]. In the case of the S_3 decay, S_3 is produced by two preilluminating flashes; then, after a variable dark time Δt, its remaining concentration is proportional to the oxygen yield Y_1 of the first flash of a following sequence. Y_2 is an approximate indicator of S_2.

In the case of the S_2 decay, S_2 is produced by one preilluminating flash and its remaining concentration after Δt is revealed by Y_2 (oxygen yield of the second flash of a following sequence).

In the control, the decay of S_3 (Fig. 1) is faster than that of S_2 (Fig. 2) and

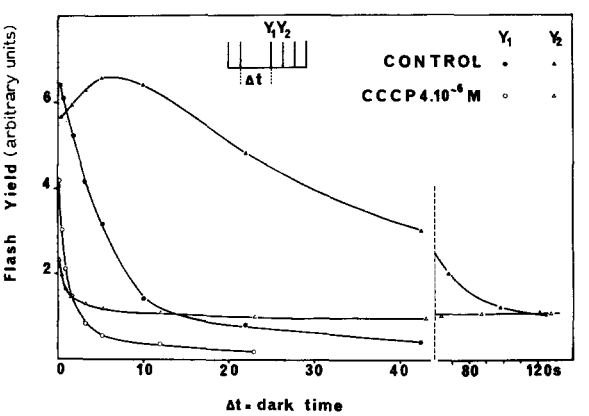

Fig. 1. Dark decay after two preillumination flashes. Oxygen yield of the first flash (Y_1) and the second flash (Y_2) in a sequence as a function of the dark time Δt. The time between flashes was 0.3 s in the control and 0.15 s in the presence of CCCP $4 \cdot 10^{-6}$ M. Control: ●, Y_1; ▲, Y_2. CCCP $4 \cdot 10^{-6}$ M: ○, Y_1; △, Y_2.

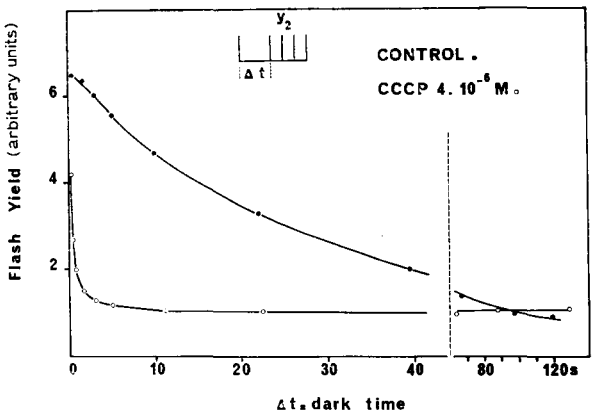

Fig. 2. Dark decay after one preillumination flash. Oxygen yield of the second flash (Y_2) in a sequence as a function of the dark time Δt. Experimental conditions are as in Fig. 1. ●, control; ○, CCCP $4 \cdot 10^{-6}$ M.

proceeds mainly through a one-step deactivation ($S_3 \rightarrow S_2$) as shown by the temporary increase in Y_2 while Y_1 decreases (Fig. 1).

The effect of CCCP on the S_3 decay depends on its concentration: in the presence of a low CCCP concentration ($\leqslant 2 \cdot 10^{-6}$ M) at which the uncoupling effect of CCCP is predominant [9], the decay is slowed down (Fig. 3). This resembles the result obtained by Renger et al. [6] on whole algae with another ADRY agent. At higher CCCP concentrations, the S_3 decay becomes highly biphasic (Fig. 1, Fig. 3). The first phase, achieved within 1 s, is much faster than the decay with no CCCP present, while the second phase remains slower than that of the control. The S_2 decay is also biphasic, the first part becoming faster and the second slower than the control (Fig. 2). In Fig. 1, one can notice that with CCCP present, there is no temporary

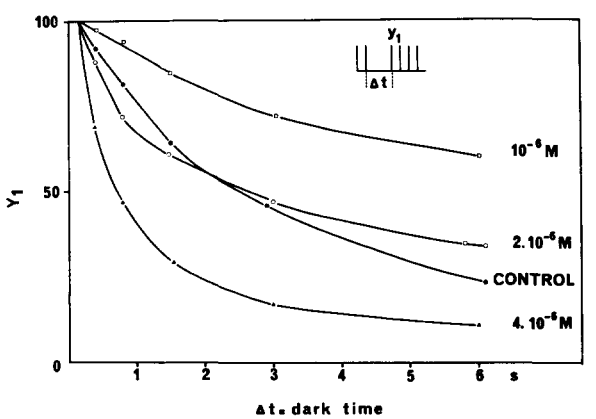

Fig. 3. Effect of various concentrations of CCCP on the S_3 decay. Oxygen yield of the first flash (Y_1) in a sequence as a function of the dark time Δt. For each decay, Y_1 maximum is normalized to 100. The spacing between flashes was 0.15 s. ●, control; □, 10^{-6} M; ○, $2 \cdot 10^{-6}$ M; ▲, $4 \cdot 10^{-6}$ M.

increase in Y_2. The slope of the fast phase (which depends in rate and amplitude on the CCCP concentration) is identical for both decays (Figs 1 and 2).

The damping of the oxygen oscillations can be accounted for by a certain percentage of "misses", i.e. of transition $S_n \rightarrow S_n$ during a flash. One can imagine two ways to increase the misses: (1) By using non-saturating flashes: the number of $S_n \rightarrow S_n$ transitions will be enlarged in a random way as some traps do not receive an excitation during the flash. (2) By widening sufficiently the spacing (dt) between flashes to allow a partial achievement of the one-step deactivation reactions during the dark periods: after the $S_n \rightarrow S_{n+1}$ transition during the flash, part of the centers will undergo an $S_{n+1} \rightarrow S_n$ transition during the dark period, this succession of events resulting finally in an overall $S_n \rightarrow S_n$ transition. If the S_2 and S_3 decays, observed experimentally, are due to a one-step deactivation, the damping of the oscillations when dt is increased should occur four times faster in the presence of CCCP than in the control.

In the same way, one can predict theoretically that if a two-step deactivation ($S_3 \rightarrow S_1$, $S_2 \rightarrow S_0$) occurs for a fraction of the centers during the dark intervals of a flash sequence, an oscillation of periodicity will become apparent also (C. Lemasson and A. L. Etienne, to be published).

Therefore we varied the time between flashes to see whether the fast phase of the S_2 and S_3 decays (with CCCP present) was indeed due to an acceleration of either type of deactivation (i.e. the ADRY effect).

Oxygen sequences with various dt or with non-saturating flash intensities

For the control, when dt is increased Y_3 decreases faster than the stationary

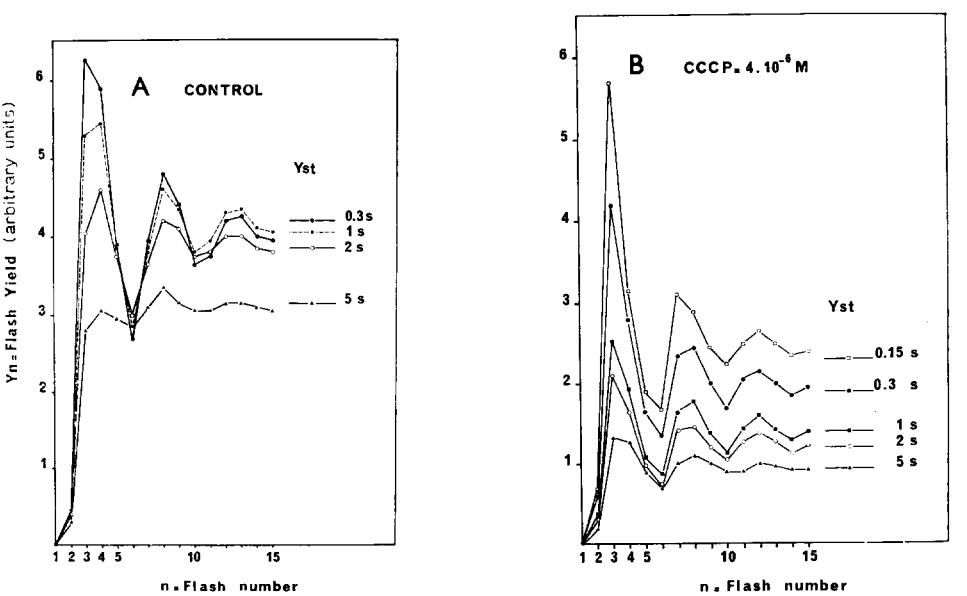

Fig. 4. Flash O_2 yield sequence after 5 min of darkness. Y_{st} represents the stationary value obtained after damping the oscillations. The spacing between flashes varies as indicated: □, 0.15 s; ●, 0.3 s; ■, 1 s; ○, 2 s; ▲, 5 s. (A) control; (B) CCCP $4 \cdot 10^{-6}$ M.

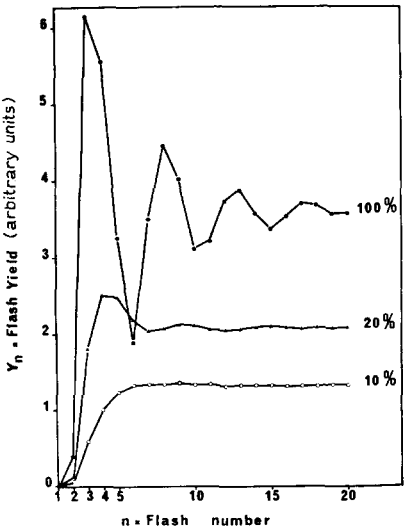

Fig. 5. Effect of flash intensity on O_2 yield sequence after 200 s of darkness. ●, 100 %; ▲, 20 %; ○, 10 %. The spacing between flashes was 0.3 s.

yield Y_{st} (Fig. 4). This means that the S_3 decay kinetics are not the same at the onset of illumination and in stationary conditions. We checked that it is also true with chloroplasts (unpublished results).

This observation can reconcile the fast deactivation curves published by various authors [1, 3, 6] and the constant value of Y_{st} observed by Renger for large dt between flashes [4, 5].

For small dt values in the presence of CCCP, the first striking point is that Y_3 is equivalent to that of the control, whereas Y_{st} is much smaller than that of the control: $Y_3/Y_{st} = 1.6$ for the control and 2.5 in the presence of CCCP (Fig. 4). Y_2 is larger than in the control and so is the ratio Y_3/Y_4. It is also obvious that the damping of the oscillations does not occur more rapidly with CCCP present than in the control (compare Fig. 4A and 4B). Moreover, the oscillations are still conspicuous for dt as large as 5 s and the periodicity remains the same.

When the $S_n \rightarrow S_n$ transitions in the control are increased by lowering the flash intensity with a constant dt value of 0.3 s, a complete damping is attained for a Y_{st} value around 0.4 Y_{st} under saturating flashes (Fig. 5). An equivalent Y_{st} value with CCCP present is observed for dt = 1 s between flashes and in this case, the damping of the oscillations seems to be equivalent to that of the control with dt = 0.3 s (compare Figs 4B and 5).

Therefore we have the feeling that the fast phases of the S_n decays with CCCP present can be due neither to a one-step deactivation nor to a two-step deactivation. It needs another explanation which can be deduced from a mathematical analysis of the oxygen sequences.

Mathematical computations on oxygen sequences

In the frame of the linear model of Kok et al. [1], there are different ways to

compute the percentage of misses α and double hits β (transitions $S_n \rightarrow S_{n+2}$ during the flash).

One is a computer-assisted least square-fitting procedure between a theoretical sequence and the experimental one [10]. The number of parameters are reduced by an arbitrary choice based on other experiments [11, 12]. The initial values of S_2 and S_3 are set equal to zero [1]. No misses are considered on the $S_0 \rightarrow S_1$ and $S_1 \rightarrow S_2$ transitions [10, 12, 13] and no double hits on S_2 and S_3 [14]. The double hits are considered equal on S_0, S_1. With our curve-fitting procedure [10], equal misses on S_2, S_3, or only one miss on either S_2 or S_3, give equivalent curve fittings; therefore the misses are arbitrarily distributed on one or two states before each computation. The curve-fitting procedure allows then a determination of α, β, the initial S_1/S_0 ratio and the relative concentration of Photosystem II active centers: $\Sigma_{n=0}^{3} S_n$.

When dt is varied, α for the control is always higher than with CCCP present (Fig. 6) (as already noted by one of us in ref. 11). The increase of α with increasing dt values is larger in the control than in the presence of CCCP, where it remains fairly constant. Table I shows an increase in β and S_1/S_0 when CCCP is present.

With increasing values of dt, the ΣS seems to increase slightly in the control, whereas it drops drastically in the presence of CCCP when dt varies from 0.1 to 1 s.

Another way to estimate the numerous parameters of the recurrence law defined by Kok et al. is a matrix analysis developed by Lavorel [15]. This analysis discloses interesting properties which are averaged out in the curve-fitting procedure described above.

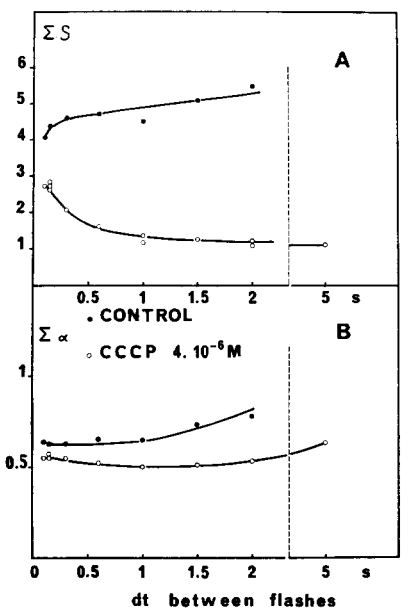

Fig. 6. Curve-fitting results obtained with the experimental data of Fig. 4A and B. (The computation was done with the misses on the $S_3 \rightarrow S_0$ transition.) (A) ΣS represents the total concentration of photosystem II centers calculated for each sequence with different dt between flashes. ●, control; ○, CCCP $4 \cdot 10^{-6}$ M. (B) $\Sigma\alpha$ represents the misses calculated for each sequence with different dt between flashes. ●, control; ○, CCCP $4 \cdot 10^{-6}$ M.

TABLE I

SEQUENCE PARAMETERS CALCULATED BY THE CURVE-FITTING PROCEDURE

Experimental data of Fig. 4.

CCCP concentration	0	$4 \cdot 10^{-6}$ M
dt between flashes	0.3 s	0.15 s
Double hits β	0.03	0.11
Initial ratio S_1/S_0	75/25	90/10

With CCCP present, (the details of the analysis will soon be published) the matrix analysis shows clearly that during a flash sequence the number of photoactive System II centers decreases. Its decay is very fast for small dt values; it is slower when dt is increased. For dt values exceeding 1 s, the number of photoactive centers remains very constant during the whole flash sequence. These variations in the number of active centers during a flash sequence are the cause of the variations of the ΣS values (Fig. 6). (The ΣS values are averaged on a whole sequence and give therefore only a mean value which depends on the number of flashes taken into account for the curve-fitting procedure).

The other findings of the first computation are confirmed by this analysis.

DISCUSSION AND CONCLUSION

Depending on its concentration, CCCP is known to have various effects on the photosynthetic electron transport [5, 9, 16]. We will focus our attention on its effects on the oxygen evolution in algae. Considering Fig. 3, 2 types of CCCP effects are distinguishable: a stabilizing effect on S_2 and S_3 and an accelerating effect on S_2 and S_3 which exist only for a high concentration of CCCP ($\geqslant 2 \cdot 10^{-6}$ M). It is known that CCCP inhibits the back reaction in algae and chloroplasts: it inhibits the luminescence emission (ref. 11, and Haveman, J. and Lavorel, J., unpublished results) and suppresses the fluorescence decay remaining in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) [16, 17]. This can explain why, in algae, the S_2 and S_3 deactivations are slowed down since the back reaction is usually considered as one of the normal deactivation pathways [7], the cycle induced by the ADRY substances in chloroplasts [6] does not seem to exist in whole algae.

The specific effect of a high concentration of CCCP is to superimpose a very fast phase on the slow S_2, S_3 decays observed at lower concentrations.

The CCCP-induced fast decays of S_2 S_3 have been shown not to correspond to a simple acceleration of the deactivations of S_2 and S_3: they do not induce an increased damping (as should a one-step deactivation) nor a periodicity of two (two-step deactivation) in the oxygen sequence when the flash period allows a partial achievement of the decays in the dark between flashes. The mathematical analysis of the oxygen sequences show that instead of an accelerated deactivation of S_2 and S_3 back to S_0, S_1, the fast part of the decays corresponds to a decrease in the number of oxygen-evolving System II centers: CCCP seems to produce a temporary inhibition of the centers in their S_2 and S_3 states. The half time of the interaction of CCCP with S_2 and S_3 (leading to an inactive state: S_i) is given by the half time of the fast decay

$\approx$ 0.6 s for $4 \cdot 10^{-6}$ M CCCP. After two preilluminating flashes, the study of the dark time Δt needed for the recovery of maximal values of Y_3 and Y_4 during a following flash sequence gives an idea of the time needed for the inhibition to be released in the dark. The recovery time is equivalent to that needed for a complete deactivation in a control experiment. It shows that the inactive state S_i is reduced back to active S_0, S_1 states in the dark. We do not know the nature of the inactive state S_i. In spite of the fact that the fast disappearance of S_2 and S_3 induced by a high concentration of CCCP does not correspond to a concommitant increase of the S_0, S_1 concentration, the positive charges are rapidly unable to react back with Q^- (reduced primary acceptor) as already said above.

The inhibition caused by CCCP on the oxygen-evolving system in algae can be called a photo-inactivation for it needs light to occur and is reversible in the dark. In chloroplasts, in addition to the true ADRY effect well described by Renger [4, 5, 6], preliminary experiments show that in some cases the same mechanism can occur.

The photo-inactivation described in the report might be correlated in some way to the CCCP effect on the electrical field decay, to its effect on the cytochrome b_{559} potential and oxido-reduction state [16] or to its effect on the carotenoids [19]. Speculations concerning these eventual correlations have already been published [18] and we have not yet investigated these points.

REFERENCES

1 Kok, B., Forbush, B. and McGloin, M. (1970) Photochem. Photobiol. 11, 457–475
2 Joliot, P., Hoffnung, M. and Chabaud, R. (1966) J. Chim. Phys. 10, 1423–1441
3 Joliot, P., Joliot, A., Bouges, B. and Barbieri, G. (1971) Photochem. Photobiol. 14, 287–305
4 Renger, G. (1971) Z. Naturforsch. 26b, 149–153
5 Renger, G. (1972) Biochim. Biophys. Acta 292, 786–795
6 Renger, G., Bouges, B. and Delosme, R. (1973) Biochim. Biophys. Acta 292, 796–807
7 Lemasson, C. and Barbieri, G. (1971) Biochim. Biophys. Acta 245, 386–397
8 Joliot, P. and Joliot, A. (1968) Biochim. Biophys. Acta 153, 625–634
9 Heytler, P. C. and Pricard, W. W. (1962) Biochem. Biophys. Res. Commun. 7, 272–275
10 Etienne, A. L. (1974) Thèse de Doctorat d'Etat, Paris
11 Etienne, A. L. (1975) Proc. 3rd Int. Congr. Photosynth. Res. (1974), (Avron, M. ed.), Vol. 1, pp. 335–343, Elsevier, Amsterdam
12 Duysens, L. N. M., Den Haan, G. A. and Van Best, J. A. (1975) Proc. 3rd Int. Congr. Photosynth. Res., 1974 (Avron, M. ed.), Vol. 1, pp. 1–12, Elsevier, Amsterdam
13 Delrieu, M. J. (1974) Photochem. Photobiol. 20, 441–454
14 Bouges-Bocquet, B. (1973) Biochim. Biophys. Acta 292, 772–785
15 Lavorel, J. (1975) J. Theor. Biol. in the press
16 Cramer, W. A. and Böhme, H. (1972) Biochim. Biophys. Acta 256, 358–369
17 Etienne, A. L. (1974) Biochim. Biophys. Acta 333, 320–330
18 Renger, G. (1975) Proc. 3rd Int. Congr. Photosynth. Res., 1974 (Avron, M. ed.), Vol. 1, pp. 127–144, Elsevier, Amsterdam
19 Yamashita, K., Konishi, K., Itoh, M. and Shibata, K. (1969) Biochim. Biophys. Acta 172, 511–524