Biochimica et Biophysica Acta, 461 (1977) 61–74

BBA 47310

IDENTIFICATION OF S_2 AS THE SENSITIVE STATE TO ALKALINE PHOTOINACTIVATION OF PHOTOSYSTEM II IN CHLOROPLASTS

J-M. BRIANTAIS[a], C. VERNOTTE[a], J. LAVERGNE[a] and C. J. ARNTZEN[b]

[a]*Laboratoire de Photosynthèse du C.N.R.S., 91190 Gif-sur-Yvette (France) and* [b]*U.S.D.A./A.R.S., Department of Botany, University of Illinois, Urbana, Ill. 61801 (U.S.A.)*

(Received November 8th, 1976)

SUMMARY

1. Chloroplasts have been preilluminated by a sequence of *n* short saturating flashes immediately before alkalinization to pH 9.3, and brought back 2 min later to pH 7.8. The assay of Photosystem II activity through dichlorophenolindophenol photoreduction, oxygen evolution, fluorescence induction, shows that part of the centers is inactivated and that this part depends on the number of preilluminating flashes (maximum inhibition after one flash) in a way which suggests identification of state S_2 as the target for alkaline inactivation.

2. As shown by Reimer and Trebst ((1975) Biochem. Physiol. Pflanz. 168, 225–232) the inactivation necessitates the presence of gramicidin, which shows that the sensitive site is on the internal side of the thylakoid membrane.

3. The electron flow through inactivated Photosystem II is restored by artificial donor addition (diphenylcarbazide or hydroxylamine); this suggests that the water-splitting enzyme itself is blocked. The inactivation is accompanied by a solubilization of bound Mn^{2+} and by the occurrence of EPR Signal II "fast".

4. Glutaraldehyde fixation before the treatment does not prevent the inactivation which thus does not seem to involve a protein structural change.

INTRODUCTION

The photochemical activity of Photosystem II in chloroplast lamellae is highly dependent upon structural organization of the photosynthetic membrane. Many chemical agents or physical treatments which disturb membrane structure (chaotropic agents [1], Tris washing [2], heat treatment [3], enzymatic iodination [4]) cause a concomitant loss of water oxidation activity. Protection against some of these inactivations is obtained by glutaraldehyde prefixation [4, 5] showing that some protein conformation change is involved.

Several studies have indicated that, on the other hand, photoinduced function-

Abbreviations: DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; DPC, diphenyl carbazide; DCPIP, dichlorophenolindophenol.

ing of the photosynthetic apparatus causes structural changes of the membrane which may involve Photosystem II components. Giaquinta and Dilley [6, 7] reported a light-induced increase in membrane reactivity to diamino benzidine sulfonate (DABS) which was dependent upon electron flux through Photosystem II. Bouges-Bocquet [8] has found evidence for conformational changes in the water-oxidizing enzymes: her studies of Kok's S states [9] turnover rates showed that one kinetic component is inhibited by prior glutaraldehyde fixation of the membrane proteins. It has also been reported that there is a light-induced inactivation of the oxidizing side of Photosystem II at alkaline pH [10]. Reimer and Trebst [11] suggested that some photoinduced conformational change in Photosystem II makes the water-splitting enzyme more labile at high pH.

In order to obtain further information about the mechanism of alkaline pH inactivation of Photosystem II, and especially to determine whether the accumulation of positive charges at the level of the water-oxidizing enzyme could be involved, we used short saturating flashes to induce the inactivation. It will be shown that the S_2 state of Photosystem II is specifically sensitive to high pH inactivation.

MATERIALS AND METHODS

Chloroplasts preparation. Broken chloroplasts were isolated from spinach, lettuce or peas, according to a procedure previously described [4], and were then resuspended in 0.4 M sorbitol, 10^{-2} M NaCl, 10^{-2} M $MgCl_2$ and 10^{-2} M Tricine, pH 7.8. They were stored in the dark at 0 °C, at a concentration corresponding to 1–3 mg chlorophyll per ml.

Photoinactivation treatment. The chloroplasts are initially in a 10 mM buffer, pH 7.8 (either Tricine or sodium phosphate) with or without 10^{-6} M gramicidin D. In order to bring the pH at about 9.25, one adds a small volume of a molar basic solution, either NaOH, glycine or carbonate buffer, pH 9.5. Then, to restore to pH 7.8, one uses again a molar buffer at pH 7.0 (Tricine or phosphate). For each experiment, a preliminary test is done to determine the quantities of buffers necessary to induce transitions to pH 9.25 and back to 7.8. The allowed range for alkaline treatment lies between 9.0 and 9.35. Below 9.0, no inactivation occurs, whereas at higher pH, a dark inactivation interferes, which becomes severely dependent on the incubation time above 9.35. This dark inactivation (pH < 9.35) varies between 10 and 50 % and has to be taken into account when photoinhibitions are calculated. The time of incubation and illumination conditions are described in the text. A General Radio Strobotac is used for flash illumination (8 μs duration). The chloroplasts at a concentration of 10 μg chlorophyll/ml are exposed to the flash light in a glass vessel with 4 mm optical path. This corresponds approximately to a monolayer of chloroplasts, and the flash saturation was checked in this geometry by oxygen yield experiments. For measurements requiring a high concentration of chloroplasts (oxygen and EPR experiments) a continuous illumination was used for photoinactivation instead of the flash which could not saturate large or concentrated samples.

Glutaraldehyde fixation. Glutaraldehyde is added in the chloroplasts suspension at pH 7.8, 1 min after the addition of gramicidin; it remains in the suspension during the alkaline treatment and the activity measurements.

The fixation of chloroplasts was assayed according to Zilinskas and Govindjee

[5], by the inability to undergo any osmotic change. Complete fixation was obtained for a concentration of 0.05 % glutaraldehyde in a 10 μg chlorophyll per ml chloroplasts suspension. This glutaraldehyde to chlorophyll ratio is in agreement with that found by Zilinskas and Govindjee [5] for complete fixation.

Activities measurements. The rate of the Hill reaction is measured spectroscopically by the photoinduced changes of DCPIP absorbance at 580 nm in a Cary 14 spectrophotometer with lateral illumination, in presence, when mentioned, of $5 \cdot 10^{-4}$ M diphenyl carbazide (DPC) as an artificial donor. The actinic light is red light (Corning 2-61 filter); a blue filter (Corning 4-96) protects the photomultiplier from the actinic light.

Fluorescence induction at 685 nm is determined, at room temperature either in the absence of any addition, or in the presence of 10^{-5} M DCMU, or 10^{-2} M NH_2OH.

The yield of oxygen evolved at each flash (8 μs duration) of a series was measured with a rate electrode. Chloroplasts are used at a concentration of 500 μg chlorophyll/ml.

EPR measurements. A Bruker B-ER 420 spectrometer was used. Instrument settings : 3.2 Gauss modulation, 25 mW microwave power for Signal II spectra, 16 Gauss and 100 mW for Mn^{2+}. Chloroplasts were used at a concentration of about 4 mg chlorophyll/ml in a flat quartz cell.

RESULTS

Illumination at high pH

The experiment is basically the same as Reimer and Trebst's [11] except for flash instead of continuous illumination. Chloroplasts (10 μg chlorophyll/ml) with gramicidin D 10^{-6} M are brought from pH 7.8 to 9.25 (see Materials and Methods).

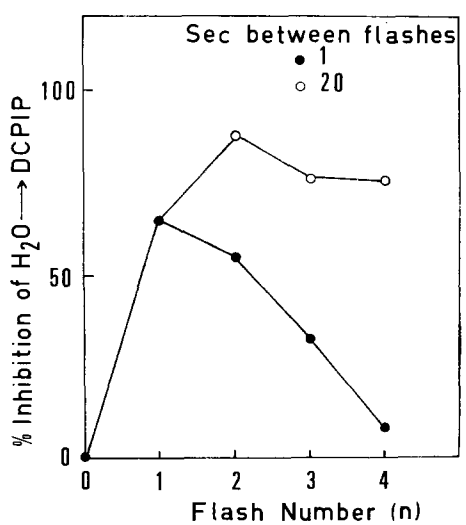

Fig. 1. Inhibition of the H_2O-DCPIP reaction in limiting light, as a function of the number of flashes during the pretreatment (see text).

1 min later they are exposed to n short saturating flashes, then, after two more minutes, brought back to pH 7.8. Fig. 1 shows the inhibition of the Hill reaction as a function of n, for two time intervals between flashes (Δt, 1 and 20 s). For $\Delta t = 1$ s the maximum inhibition ($\approx 65\,\%$) occurs after the first flash. The inactivation then decreases with the subsequent flashes.

A similar situation where one or two flashes induce a larger effect than 3 or 4 has already been described by Babcock and Sauer [12] showing that EPR signal II is formed from states S_2 and S_3 of Photosystem II charge accumulating device and not from states S_0 and S_1. Taking Kok et al. [9] scheme, neglecting double hits and misses, and assuming that 25 % of the dark-adapted centers are in the S_0 state, 75 % in the S_1 state, one gets after one flash: 25 % S_1, 75 % S_2; after two flashes, S_2 = 25 %, S_3 = 75 %; after three flashes S_3 = 25 % S_0 = 75 %, and so on. Therefore, the result of Fig. 1 for $\Delta t = 1$ s strongly suggests that state S_2 is the target of high pH inactivation.

When the time between flashes is 20 s, a large inactivation has had time to develop on the S_2 centers between the first and second flash. To these inactivated centers, the second flash adds its contribution (centers initially in S_0, plus these S_2 centers which escaped inactivation and had time to deactivate to S_1 before the second flash). Thus when the Δt is long enough, the inactivation builds up during the sequence contrary to the pattern observed at short Δt values.

Actually, at pH 9.3 the S states distribution during a flashes sequence certainly differs from the one we assumed above. At alkaline pH, according to Wraight et al. [13], no oxygen is evolved, thus showing that some reaction between S_3 and oxygen evolution is inhibited.

In order to avoid this difficulty and to handle the S states in better known conditions we changed the experimental procedure to that described in Fig. 2, where

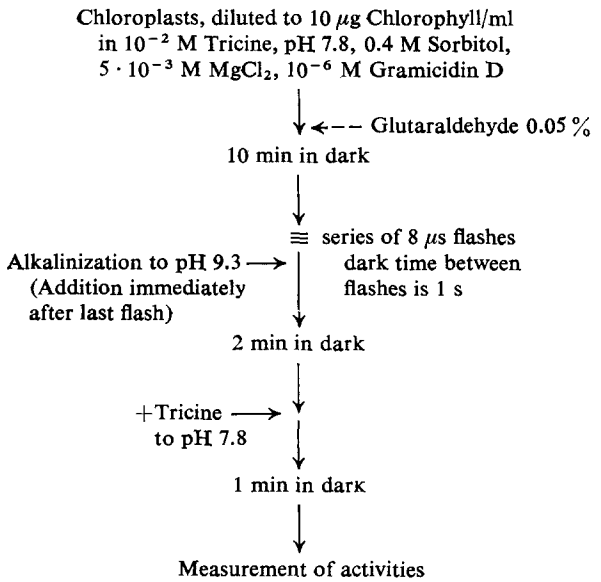

Fig. 2. Experimental procedure for the alkaline treatment.

the chloroplasts are given suitable preillumination at pH 7.8 just before injecting the alkaline buffer. Thus, in most of the following experiments the procedures involving light occur at pH 7.8, while the alkaline excursion takes place in complete darkness.

Flashes preillumination at pH 7.8

Following the experimental procedure described in Fig. 2, Fig. 3a shows the inhibition of the $H_2O \rightarrow$ DCPIP reaction in saturating light vs. the number of preillumination flashes. Fig. 3b gives the oxygen evolved at flash $n+2$ in an experiment with an untreated sample. This oxygen yield Y_{n+2} is an approximate measurement of the amount of state S_2 present after the n^{th} flash, i.e. just before injecting the alkaline buffer in the experiment of Fig. 3a. The similarity of the sequences of Figs. 3a and 3b confirms that the high pH inactivation involves state S_2. If state S_3 was equally sensitive, the maximum inhibition would occur after two preilluminating flashes. Fig. 3a shows, however, a greater inhibition after two flashes than would be expected from the Y_{n+2} sequence. A likely explanation is that S_3 centers formed after two flashes deactivate through S_2 during high pH incubation thus yielding some additional inactivation.

Reimer and Trebst [11] have shown that high pH inactivation does not affect

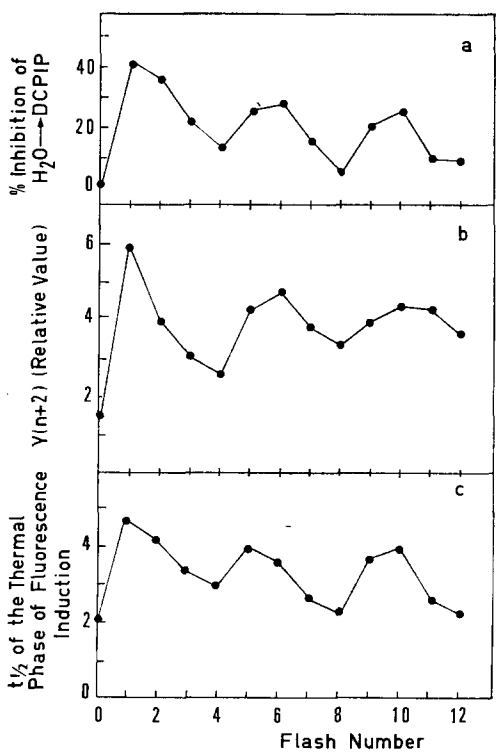

Fig. 3. (a) Inhibition of the H_2O-DCPIP reaction in saturating light as a function of the number of preillumination flashes, according to procedure of Fig. 2. (b) Oxygen evolved at flash $n+2$ in an untreated sample. (c) $t_{\frac{1}{2}}$ of the F_1-F_m phase of fluorescence induction as a function of the number of preillumination flashes, according to the procedure shown in Fig. 2.

TABLE I

RATE OF DCPIP PHOTOREDUCTION

Flash number	Rate of DCPIP photoreduction	
	H_2O	+DPC
0	100*	100
1	35	75

* 100 = 600 μmol DCPIP reduced per h per 1 mg chlorophyll.

the Hill reaction when the artificial System II donor diphenylcarbazide (DPC) is used. This is confirmed by the data of Table I which shows that DPC addition increases the rate of DCPIP photoreduction in the inactivated samples, although not restoring 100 % of the activity. This is consistent with the assumptions that DPC is a less efficient donor than water and that it does not replace the natural donor unless the water-oxidizing system has been damaged, e.g. by alkaline inactivation.

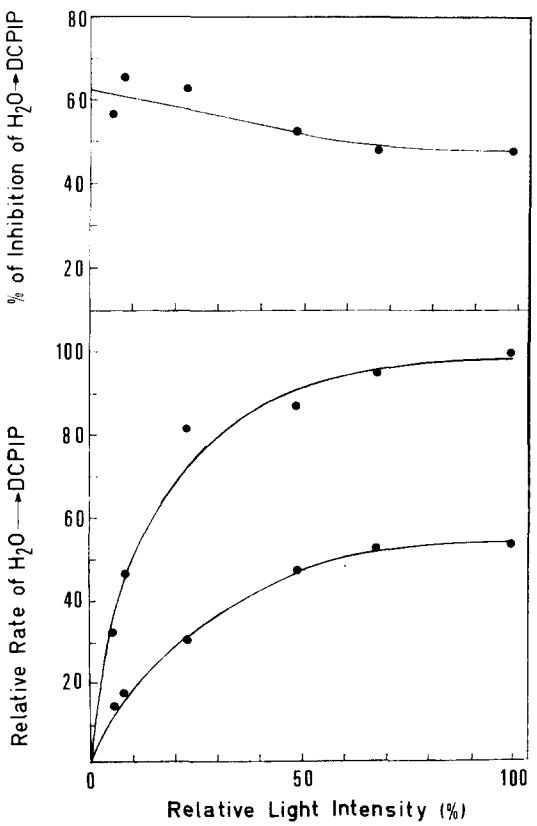

Fig. 4. Rate of DCPIP photoreduction for 0 and 1 preillumination flash samples as a function of the light intensity during the assay.

When no gramicidin D is added, preillumination by one flash does not cause any inactivation, which shows that only the internal pH of the thylakoid is involved.

In Fig. 4 is shown the rate of DCPIP photoreduction for 0 and 1 preillumination flash samples as a function of the light intensity during the assay. It can be seen that the inhibition is roughly constant in limiting or saturating light intensity conditions.

Oxygen yield sequence of a partially inactivated sample

For this experiment, instead of using preilluminating flashes (which might be non-saturating for a concentrated chloroplasts suspension), a 1 min continuous illumination with strong white light was used before the pH rise. One expects then to reach the steady state 25 % concentration for every S state and thus a percent inactivation between 25 and 50 if S_3 states deactivate to S_2 during the alkaline incubation. After 1 min at high pH the sample was brought back at pH 7.8, and then assayed for oxygen evolution during a sequence of flashes. Compared to an untreated control, the sequence displayed a 50 % inactivation but otherwise the oscillating pattern was essentially the same, showing no change in periodicity or damping characteristics.

Deactivation of the pH-sensitive state

When the time between one flash illumination and the alkalinization is varied one obtains the deactivation curve (in darkness, at pH 7.8) of the high pH inactivation sensitive state (Fig. 5). The kinetics of this deactivation has a $t_{\frac{1}{2}}$ of 45 s which fits nicely S_2 deactivation measured on these chloroplasts (as the yield of O_2 evolved at a second flash of a series of 2 vs. the dark time interval after one flash).

Kinetics of the alkaline inhibition

We have so far implicitly assumed that the inactivation kinetics of state S_2 at pH 9.25 is fast compared to S_2 natural deactivation. That this is so is shown in

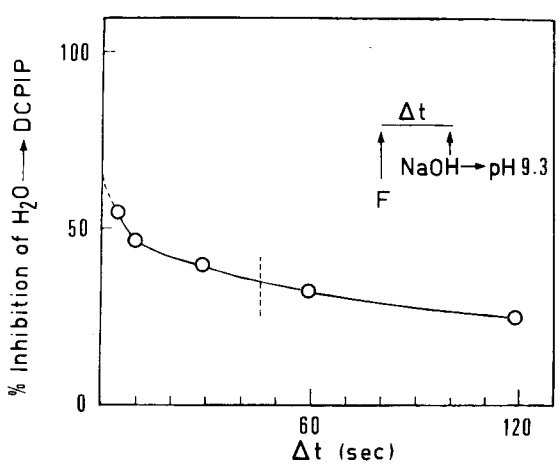

Fig. 5. Dark relaxation of the alkaline pH-sensitive species.

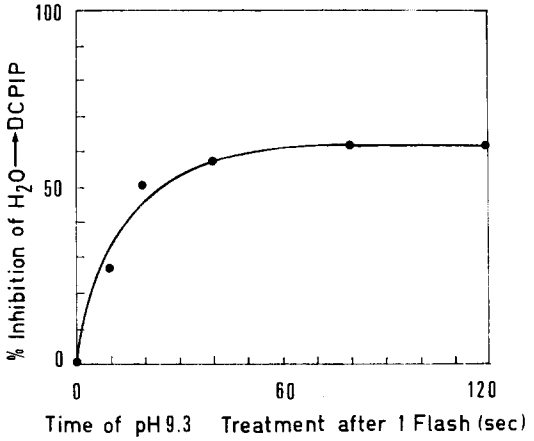

Fig. 6. Kinetics of the alkaline inactivation.

Fig. 6 in which the incubation time at high pH is varied (for convenience, the one flash illumination is given here at pH 9.3). Every point is calculated by comparing the activity of the illuminated sample to that of a dark sample which has remained for the same time at pH 9.3. The maximum inactivation is reached in about 40 s, the half-time is about 7 s.

Effect of o-phenanthroline

Reimer and Trebst [11] reported that *o*-phenanthroline caused some protection against the alkaline inactivation. This would be surprising if S_2 is the pH-sensitive state for it is well known that with this inhibitor as with DCMU, Photosystem II cycles around a unique photochemical step:

$$S_iQ \underset{\text{dark}}{\overset{\text{light}}{\rightleftharpoons}} S_{i+1}\, Q^-$$

where Q stands for the primary acceptor (DCMU-type inhibitors block its normal reoxidation by plastoquinones), and S_i is the S state of the center in the dark when DCMU is added. As the reverse dark reaction is slow, a moderate light intensity is sufficient to maintain a high steady-state concentration of centers in the $S_{i+1}Q^-$ (high fluorescent) state. Thus if in the presence of DCMU one illuminates continuously a sample at high pH, one expects an inactivation of those centers which were in state S_1 in the dark, i.e. about 75 %.

We did such an experiment illuminating for 1 min at pH 9.3 samples with or without *o*-phenanthroline. After pH neutralization, all the samples are centrifuged, resuspended in a medium with 0.2 M zinc acetate (in order to complex any remaining *o*-phenanthroline [14] and the DCPIP photoreduction is then assayed. A 92 % inhibition was obtained for samples without *o*-phenanthroline, and 60 % for these with *o*-phenanthroline, which is thus pretty close to the expected dark concentration of state S_1.

Fluorescence induction

Fig. 7 shows the fluorescence kinetics for 0 and 1 flash-pretreated samples

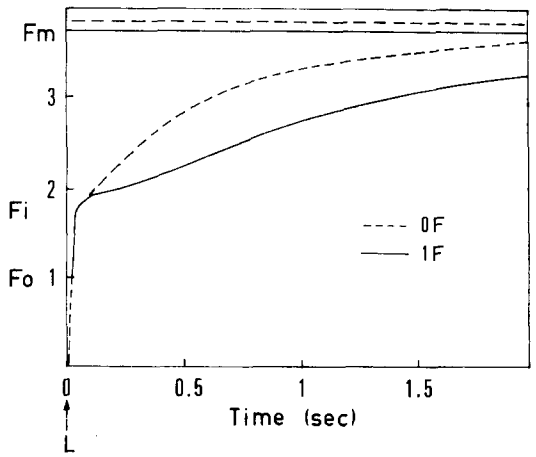

Fig. 7. Fluorescence induction for 0 and 1 flash-pretreated samples (according to Fig. 2). Chlorophyll concentration, 10 μg chlorophyll/ml.

(according to Fig. 2 procedure again). One has the usual fast phase from the F_0 dark adapted level to F_i, followed by the slower $F_i - F_p$ (here called "F_m") rise which accompanies the plastoquinone pool reduction. It can be seen that high pH inactivation affects strongly the rate of this phase, leaving otherwise the fast phase and the levels F_0, F_i and F_m mainly unchanged. This holds too with a greater number of preilluminating flashes, and Fig. 3c shows a plot of the $t_{\frac{1}{2}}$ of the slow fluorescence rise vs. the number of flashes. The rate of this fluorescence phase behaves quite similarly to that of the DCPIP photoreduction (Fig. 3a).

When the artificial donor NH_2OH is added (10^{-2} M), no difference is observed between the 0 and 1 flash samples (Table II). Hydroxylamine is (like DPC)

TABLE II

EFFECT OF 10^{-2} M NH_2OH ADDITION ON THE RATE OF THE $F_i - F_m$ PHASE

Flash number	$t_{\frac{1}{2}}$ $F_i - F_m$ phase (s)	
	No addition	$+NH_2OH$ (10^{-2} M)
0	0.65	0.42
1	0.92	0.42

TABLE III

FLUORESCENCE CHARACTERISTICS IN 10^{-5} M DCMU-POISONED CHLOROPLASTS

The F values are in arbitrary units.

Flash number	F_0	F_m	$t_{\frac{1}{2}}$ (ms)
0	100	297	20
1	100	300	23.5

a less good donor than the endogenous one, but (unlike DPC), it replaces this natural donor. This explains why NH_2OH cancels the oscillating inactivation pattern although DPC could only damp it.

In the presence of DCMU (10^{-5} M) the kinetics of the 0 and 1 flash samples are essentially the same (see Table III). The well known effect of DCMU on fluorescence kinetics is to suppress the slow phase by extending the fast one up to the maximum level: this fluorescence rise displays essentially one photochemical act (reduction of the primary acceptor) whereas the slow phase involves about 10 times more photochemical turnover (reduction of the secondary acceptor pool when the DCMU block is absent).

Effect of glutaraldehyde fixation

In Table IV are reported the activities and fluorescence characteristics (without any addition) in unfixed and fixed chloroplasts. The fixation is done before the inactivating treatment (Fig. 2). An inhibitory effect of flash is still observed, but fluorescence inductions show that in glutaraldehyde-fixed chloroplasts maximum fluorescence F_m is greatly decreased. In glutaraldehyde-fixed chloroplasts as in unfixed chloroplasts, the addition of 10^{-2} M NH_2OH suppresses the inactivation, and so does 10^{-5} M DCMU.

TABLE IV

EFFECT OF GLUTARALDEHYDE PREFIXATION

Glutaraldehyde (%)	0		0.05	
	0 flash	1 flash	0 flash	1 flash
$H_2O \rightarrow$ DCPIP rates	100 %	27 %	41 %	12 %
Inhibition (%)	73		71	
F_0	100	100	100	100
F_m	280	275	280	175

EPR measurements

Most treatments which affect the Photosystem II donor side between water and the site(s) for artificial donation by DPC, hydroxylamine, etc., are known to cause solubilization of Mn^{2+} which seems to play a key role in the water-splitting mechanism. Tris washing [2], heat [3], NH_2OH washing [15], low pH treatment [16] inhibit water oxidation (but not Photosystem II activity with an artificial donor), collapse the EPR Signal II u (according to Babcock and Sauer [17] nomenclature), cause a new light induced EPR Signal II ("fast") and solubilize some Mn^{2+} which becomes EPR detectable. The loss of Mn^{2+} is thought to explain the water-splitting enzyme inactivation which in turn accounts for the occurrence of Signal II f that Babcock and Sauer [17] attribute to an oxidized intermediate on the donor side of Photosystem II.

In order to check whether alkaline photoinactivation behaves in a similar way, we ran EPR spectra of inactived and control samples. However, the buffers used for the pH transitions proved to have a quenching effect on the Mn^{2+} EPR

signal and we therefore used the following procedure: a fresh chloroplasts batch with 10^{-6} M gramicidin D is parted into three samples that we shall call C, D and L. D and L are alkalinized for 5 min, D in darkness, L with continuous light on. D and L are brought back to pH 7.8 and the three samples are spun down. They are washed once in the usual suspension medium except for low (1 mM) Tricine concentration, in which they are resuspended. The concentrations and DCPIP photoreduction activity of the three samples are then measured and they are assayed in EPR for Signal II (in darkness, with continuous light on, and in darkness again), and for the Mn^{2+} signal. The samples are then heated at 58 °C for 30 min and Mn^{2+} EPR spectra run again. The difference ΔMn between the EPR detected Mn^{2+} after and before heating is taken as the amount of bound Mn^{2+} present in each sample before heating.

The results are shown in Fig. 8 where ΔMn, and the amplitude of Signal II f are plotted versus the activity. The inactivation is indeed accompanied by a loss of bound Mn^{2+} and by the appearance of Signal II f, but unexpectedly, whereas ΔMn and Signal II f are linearly correlated to each other (not shown), they are not linearly dependent on the activity.

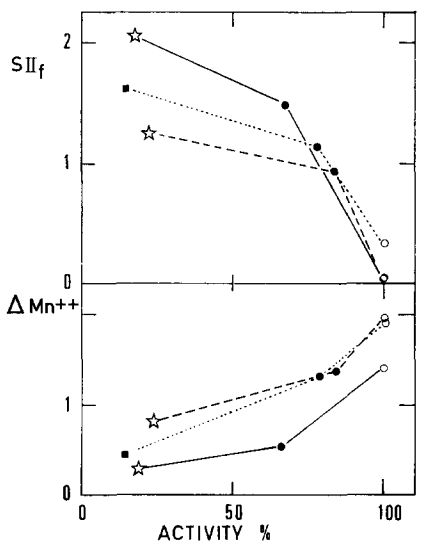

Fig. 8. ΔMn^{2+} and Signal II f versus activity ($H_2O \rightarrow$ DCPIP). ○, control; ●, 5 min at pH 9.3 in the dark; ■, 20 min at pH 9.3 in the dark, ★, 5 min at pH 9.3 in the light. The three sets of curves represent three different experiments.

In the same figure the result of a similar experiment is shown except that no sample has been photoinactivated, only the alkalinization duration varied (5 and 20 min). A very similar ΔMn vs. activity dependence is found, suggesting that the role of light is to merely accelerate the alkaline inactivation.

DISCUSSION

The evidence presented in this paper shows that light causes a dramatic increase in the rate of inactivation of Photosystem II donor side at pH 9.3 and that this

increase can be explained by the specific sensitivity of state S_2 to alkalinization. We cannot rule out that state S_3 may be alkaline sensitive to some extent too, but an analysis of data such as those of Fig. 3 shows that S_3 should be about three times less sensitive than S_2. This is by far an upper limit because the S_3 centers may deactivate to S_2 and be inactivated in this state.

It is not clear whether the S_2 sensitivity could account even for the (slower) inactivation in darkness at pH 9.3. The occurrence of oxygen on the second flash of a sequence may not be entirely due to double hitting and may imply that centers have a non-zero probability to be found in state S_2 in the dark adapted state.

The degree of inhibition seems to depend upon the number of inactivated centers rather than on a degree of inhibition on all centers. This is suggested by the fact that the O_2 flash sequence is only modified by a factor of scale, and by the insensitivity of the inhibition to the light intensity during the assay (Fig. 4). In first approximation, the percent inactivation can be interpreted like a percent inactivated centers' the other centers being unaffected.

The fact that inactivation necessitates the presence of gramicidin (if the alkaline incubation time is to be short) locates this process on the internal side of the thylakoid membrane, which is to be expected for a phenomenon involving Photosystem II donor side. The inactivation does not appear in the DPC – DCPIP reaction, nor in the NH_2OH – plastoquinones reaction studied through the fluorescence slow rise. The inhibition thus strikes between the charge accumulating device and Photosystem II centers. The following considerations allow a slightly more precise functional localization: inactivation does not cause a significant change of the fluorescence rise in the presence of DCMU. Thus a one step photochemical reaction is unaffected, whereas the inactivation appears in reactions which involve several oxidation steps on the donor side: oxygen evolution, steady-state Hill reaction, or reduction of the plastoquinone pool. The inactivated centers therefore still possess at least a one equivalent secondary donor (generally denoted Z) and most likely a second one (Y), for centers with only Z would probably undergo a fast charges recombination ($Z^+Q^- \rightarrow ZQ$) which does not occur in our DCMU experiment, whereas Y would allow a better stabilization of the oxidizing equivalent.

An interesting point appears in the fluorescence curves such as in Fig. 7, that is, the constancy of the maximum level even though the half-time to reach this level is controlled by the alkaline inactivation. This means that even the Q belonging to inactivated centers become eventually reduced. Three possible explanations can be envisaged:

(i) The acceptor Q of inactivated centers is reduced by the active centers' Q^- through interaction with the plastoquinone pool, which is a thermodynamically likely situation. Probably because of the secondary acceptor B [18] role in the redox exchanges with plastoquinones, the reduction of Q through plastoquinones is known to necessitate light (which is of course present in our fluorescence experiment). It is well known that although no direct interaction takes place between the Q's of different centers, a communication between the electron transfer chains occurs through the plastoquinone pool [19–21].

(ii) One may on the contrary involve the donor side, if the active/inactive donor systems are not rigidly bound to each center's chlorophyll-acceptor complex but can visit several centers, contrary to Kok's model. Such an hypothesis is not

untenable (Lavergne, J., unpublished, and ref. 22). A consequence of the inactivation would be to remove some "donor particles" from the pool, which in such models should induce a modification of the damping characteristics of the oxygen sequence. As mentioned, no modification of this kind was observed.

(iii) The assumption of an all or none inactivation can be questioned. Inactivated centers may have a secondary electron donation slowed down which favours the competition by the back reaction ($Y^+Q^- \rightarrow YQ$). These centers would be functional but with a lower probability than normal centers (a quite low probability indeed to fit our data). They would thus be able to reduce their Q provided a sufficient time of illumination is given.

Although hypothesis i seems the more likely, a participation of mechanism iii cannot be ruled out.

The apparently similar location of the alkaline inactivation with that of other treatments led us to check whether any Mn^{2+} released and Signal IIf occurrence accompanied the inactivation. We found that such was the case although the amount of Mn^{2+} released and number of active centers are not linearly related, whereas Signal IIf and Mn^{2+} are (to each other). The Mn^{2+} result may be interpreted on the grounds that there are several Mn^{2+} per Photosystem II centers, some of which not strictly required for Phostosytem II activity. However, the lack of a linear relationship between inactivation and Signal IIf is more difficult to understand if the Babcock and Sauer [17] interpretation of Signal IIf is correct. Further experiments are obviously needed to clarify this point.

Alkaline (photo) inactivation thus appears essentially similar to other treatments, in that it seems to be caused by a solubilization of bound Mn^{2+} required for the water-splitting enzyme activity. It has been reported [23] that illumination accelerates the inactivation by Tris and it would be interesting to check whether a sensitivity of S_2 is implied there too.

The cause of the specific sensitivity of the S_2 state to alkaline inactivation remains to be discussed. It does not seem to involve a protein structural change, as glutaraldehyde prefixation does not prevent inhibition. However, a structural change of lipids, caused by a certain distribution of +charges and entailing exposure of the water-splitting enzyme to alkaline attack, may be involved. A similar action on lipid rather than on protein structure may explain the comparatively low temperature (40 °C) sufficient to cause thermal inactivation of the water-oxidizing system.

Eventually another hypothesis is that of a direct sensitivity of the twice oxidized form to high pH, without involving a structural change: $S^{2+} + OH^-$ yielding an inactive form.

ACKNOWLEDGEMENTS

The skillful technical assistance of Mrs M. Picaud is gratefully acknowledged. We wish to thank Dr. A. L. Etienne for helpful discussions. This research was supported in part by a NATO grant (SA. 5.2.05/2005 (76) MDL) to J.M.B., C.V. and C.J.A.

REFERENCES

1 Lozier, R., Baginsky, M. and Butler, W. L. (1971) Photochem. Photobiol. 14, 323–328
2 Yamashita, T. and Butler, W. L. (1968) Plant Physiol. 43, 1978–1986
3 Yamashita, T. and Butler, W. L. (1968) Plant Physiol. 43, 2037–2040
4 Arntzen, C. J., Vernotte, C., Briantais, J-M. and Armond, P. (1974) Biochim. Biophys. Acta 368, 39–53
5 Zilinskas, B. A. and Govindjee (1976) Z. Pflanzenphysiol. Bd. 77, 302–314
6 Giaquinta, R. T. and Dilley, R. A. (1974) Proceedings of the 3rd International Congress of Photosynthesis Research (Avron, M., ed.), Vol. II, pp. 883–895, Elsevier, Amsterdam
7 Giaquinta, R. T. and Dilley, R. A. (1975) Biochemistry 14, 4392–4396
8 Bouges-Bocquet, B. (1973) Biochim. Biophys. Acta 292, 772–785
9 Kok, B., Forbush, B. and McGloin, M. (1970) Photochem. Photobiol. 11, 457–475
10 Harth, E., Reimer, S. and Trebst, A. (1974) FEBS Lett. 42, 165–168
11 Reimer, S. and Trebst, A. (1975) Biochem. Physiol. Pflanz. 168, 225–232
12 Babcock, G. T. and Sauer, K. (1973) Biochim. Biophys. Acta 325, 483–503
13 Wraight, C. A., Kraan, G. P. B. and Gerrits, N. M. (1972) Biochim. Biophys. Acta 283, 259–267
14 Kautsky, H., Appel, W. and Amann, H. (1960) Biochem. Z. 332, 277–292
15 Cheniae, G. M. and Martin, I. F. (1972) Plant Physiol. 50, 87–94
16 Pulles, M. P. J., Van Gorkom, H. J. and Verschoor, G. A. M. (1976) Biochim. Biophys. Acta 440, 98–106
17 Babcock, G. T. and Sauer, K. (1975) Biochim. Biophys. Acta 376, 329–344
18 Bouges-Bocquet, B. (1974) Biochim. Biophys. Acta 314, 250–256
19 Duysens, L. N. M. (1972) Proceedings of the 2nd International Congress of Photosynthesis Research (Forti, ed.), Vol. I, pp. 19–25, Junk, The Hague
20 Siggel, V., Renger, G., Stiehl, H. H. and Rumberg, B. (1972) Biochim. Biophys. Acta 256, 328–335
21 Williams, W. P. (1972) Proceedings of the 2nd International Congress of Photosynthesis Research (Forti, ed.), Vol. I, pp. 745–752, Junk, The Hague
22 Lavorel, J. (1976) FEBS Lett. 66, 164–167
23 Cheniae, G. and Martin, I. F. (1976) Plant Physiol. Meeting, Abstract 132