Plant Physiol. (1982) 69, 1418–1422
0032-0889/82/69/1418/05/$00.50/0

Photoactivation of the Water-Oxidation System in Isolated Intact Chloroplasts Prepared from Wheat Leaves Grown under Intermittent Flash Illumination[1]

Received for publication December 11, 1981 and in revised form February 16, 1982

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ABSTRACT

Photoactivation of the latent oxygen-evolving system in intact chloroplasts isolated from wheat (*Triticum aestivum* L.) leaves grown under intermittent flash illumination was investigated, and the following results were obtained: (a) The water-oxidation activity generated on illuminating the isolated intact chloroplasts was as high as that generated in intact leaves, indicating that all the machinery necessary for the activity generation is assembled within intact chloroplasts. (b) The generation of water-oxidation activity was accompanied by enhancement of the activity of diphenylcarbazide-oxidation, and both processes share the same photochemical reaction but with respective rate-limiting dark reactions of different efficiencies. (c) A23187, an ionophore for divalent cations, strongly inhibited the generation of water-oxidation activity but did not affect the activity once generated, which suggested that Mn atoms in the chloroplasts are susceptible to the ionophore before photoactivation but turn immune after photoactivation. (d) The generation of water-oxidation activity was not affected by the inhibitors of ATP formation and CO_2 fixation, but was inhibited by nitrite, methylviologen and phenylmercuric acetate which suppress or inhibit the reduction of ferredoxin in intact chloroplasts. It was inferred that some factor(s) probably present in stroma to be reduced by PSI photoreaction is involved in the process of photoactivation.

There are many reports on the reactivation of O_2 evolution in isolated chloroplasts after various inactivation treatments which preferentially block the O_2-evolving system. Yamashita and Tomita (21, 22) found that Tris-inactivated oxygen evolution is restored by simply washing the chloroplasts with reduced DCIP[2] solution, in some cases, followed by illumination in the presence of Mn^{2+}, Ca^{2+} and DTT. In these reactivation experiments with isolated chloroplasts, the coexistence of reducing agent has been shown to be the common requirement.

In reactivation experiments with whole algal cells, on the other hand, Cheniae and Martin (4) have demonstrated a good reactivation of O_2 evolution in NH_2OH-treated cells of *Anacystis nidulans* with no externally added factor. Similar photoactivation with no external factor is commonly observed for dark-grown algal cells (5), dark-grown gymnosperm leaves (11, 15) or for intermittently flashed angiosperm leaves (10, 18), when illumination is given to intact cells or leaves before isolation of chloroplasts. However, when illumination for photoactivation is applied to the chloroplasts after isolation from cells and leaves, very few cases of photoactivation of the Hill activity are reported. Oku and Tomita (16) succeeded in generating by illumination an appreciable O_2-evolving activity in isolated chloroplasts of dark-grown spruce seedlings, but the yield of the Hill activity generation was critically dependent on the presence of ascorbate at high concentrations.

A question may arise hereupon whether the process of photoactivation requires some cytoplasmic factor(s) in addition to the photochemical processes within the chloroplasts or not. In the present study, we report the characteristics of photoactivation of the latent water-oxidation system (denoted simply as photoactivation in this paper) in intact chloroplasts isolated from wheat leaves grown under flashing light. This experimental system enabled us to investigate the effect of various externally added chemicals, uncouplers, ionophores, etc, on the process of photoactivation, and the results obtained indicated clearly that all the factors necessary for the process are assembled within intact chloroplasts. It is also suggested that a factor probably present in stroma and reduced by PSI photoreaction plays an important role in the process. Based on these findings, the relationship between this factor and the reducing agent reported to be necessary for the reactivation of Tris-inactivated oxygen-evolving system is discussed.

MATERIALS AND METHODS

Plant Materials. Wheat seeds (*Triticum aestivum* L.) were sterilized with a dilute solution of Usplun (Bayer Co.) and germinated in moist vermiculite. After 6-d growth in darkness at 24 ± 1°C, the etiolated seedlings were subjected to greening under intermittent illumination with electronic white flashes. Four strobes (Kako Auto Elite II, 800 μs in flash duration) were fired simultaneously at an interval of 8 min to illuminate from above the growth bed of 30 × 40 cm^2. After 48 h of intermittent flash illumination, the leaves were harvested under dim green safe light. All the following procedures for preparation of protoplasts and intact chloroplasts were carried out under the similar green safe light.

Preparation of Protoplasts. Protoplasts were prepared from the intermittently illuminated leaves according to the method of Edwards *et al.* (6). The leaves were cut into segments (0.5 to 1.0 mm in width) and incubated in an enzyme medium containing 0.5 M sorbitol, 1 mM $CaCl_2$, 2% (w/v) Cellulase (Onozuka R-10) and 0.3% (w/v) pectinase (Macerozyme R-10) and 0.05% (w/v) BSA

[1] Supported by the research grant on 'Solar Energy Conversion by Means of Photosynthesis' given by the Science and Technology Agency of Japan (STA) to The Institute of Physical and Chemical Research (RIKEN).

[2] Abbreviations: DCIP, 2,6-dichlorophenolindophenol; DPC, diphenylcarbazide; DCCD, *N,N'*-dicyclohexylcarbodiimide; CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; SH, sulfhydryl; MV, methylviologen; PMA, phenylmercuric acetate.

(pH 5.5). The incubation was continued for 4 h at 26°C in darkness and the crude protoplasts were collected by centrifugation at 100*g* for 5 min. The protoplasts were then purified by sucrose/sorbitol discontinuous density gradient centrifugation at 100*g* for 5 min, collected by centrifugation at 400*g* for 5 min, and then resuspended in a small volume of a medium containing 0.33 M sorbitol, 5 mM NaCl, 0.2 mM K_2HPO_4, and 50 mM Hepes-NaOH (pH 7.5).

Preparation of Intact and Broken Chloroplasts from Protoplasts. The protoplasts were disrupted by passing twice through a 20 μm nylon mesh attached to a plastic syringe. The crude chloroplasts were precipitated by centrifugation at 4,300*g* for 40 s and resuspended in a small volume of the same medium. Intact and broken chloroplasts were separated by discontinous Percoll density gradient centrifugation. The crude chloroplast supension was layered on top of a gradient consisting of 10, 40, 60, and 75% (v/v) Percoll (Pharmacia Fine Chemicals, Upsala) dissolved in a medium containing 0.33 M sorbitol, 5 mM NaCl, 0.2 mM K_2HPO_4, and 50 mM Hepes-NaOH (pH 7.5), and centrifuged in a fixed angle rotor (Hitachi, RP-18-3) at 4,000*g* for 15 min. Two green bands appeared between 10 and 40% phase and between 40 and 60% phase. The bands were collected separately with a pipette and stored at 0°C. The upper band contained broken chloroplasts with damaged envelope and the lower band contained intact chloroplasts as judged under a phase contrast microscope. The separated intact chloroplasts were used for experiments within 1 h after preparation.

Preparation of Broken Chloroplasts from Leaves. Wheat leaves were ground in a mortar with an ice-cold medium containing 0.33 M sorbitol, 5 mM NaCl, 0.2 mM K_2HPO_4, 1 mM $MgCl_2$, and 50 mM Hepes-NaOH (pH 7.5). The homogenate was filtered through a 77 μm nylon mesh and centrifuged at 4,000*g* for 10 min. The precipitate was suspended in the same medium.

Photoactivation. For the photoactivation experiment with isolated protoplasts or chloroplasts, the samples suspended in a medium containing 0.33 M sorbitol, 5 mM NaCl, 0.2 mM K_2HPO_4, and 50 mM Hepes-NaOH (pH 7.5) at a Chl concentration of 10 μg/ml were illuminated at 25°C with continuous light from an incandescent slide projector (150 w) passing through an orange filter (Toshiba, VO-56) and a 4-cm layer of 4% $CuSO_4$ solution at an intensity of 250 $\mu w/cm^2$. For the experiment with intact leaves, the flashed leaves were spread on a filter paper moistened with distilled H_2O and illuminated from above with orange light as described above. In the light-intensity dependence experiments, the intensity was reduced by neutral density filters (Nihon Shinku, ND filters).

Activity Measurement. Chloroplasts were osmotically disrupted by adding 20 volumes of distilled H_2O just before activity measurements, and the activity of DCIP photoreduction with water or DPC as electron donor was assayed at 25°C with a recording spectrophotometer (Shimadzu, UV-300). Actinic light was the red light from an incandescent slide projector (500 w) passing through a red filter (Toshiba, VR-65), a heat absorbing filter (Nihon Shinku, Cold filter-B) and a 10-cm layer of water and focused onto the optical cell at a saturating intensity of 130 mw/cm^2. The standard medium for the assay contained 0.33 M sorbitol, 5 mM NaCl, 0.2 mM K_2HPO_4, 1 mM $MgCl_2$, 20 mM methylamine, 20 μM DCIP, and 50 mM Hepes (pH 7.5), and 1 mM DPC was added when used. Chl concentration was determined by the method of Arnon (1).

RESULTS

Table I summarizes the generation of the Hill activity by the photoactivation effected in various chloroplast preparations. In the experiment, continuous light for photoactivation was given to chloroplasts at different stages of the procedure for preparing intact chloroplasts from wheat leaves grown under flashing light.

Table I. *Hill Activity Generation Effected by Photoactivation in Various Chloroplast Preparations*

Continuous light (20 min) for photoactivation was given to chloroplasts at different stages of the procedure for preparing intact chloroplasts from wheat leaves grown under intermittent flashes.

Sample for Illumination	Activity			
	Before illumination		After illumination	
	Water-oxidation	DPC oxidation	Water-oxidation	DPC oxidation
	μmol DCIP/mg Chl·h			
Intact leaves	2	75	205	205
Protoplasts	0	70	180	150
Intact chloroplasts	5	100	170	175
Broken chloroplasts	0	75	0	40

Irrespective of the sample source, all thylakoid preparations obtained from nonilluminated leaves, protoplasts, intact or broken chloroplasts showed no activity of DCIP photoreduction with water as electron donor (denoted in this paper as water-oxidation activity), while showing appreciable activities with DPC as electron donor (denoted as DPC-oxidation activity). When, however, the leaves, protoplasts, or intact chloroplasts were illuminated with continuous light before chloroplast preparation, the thylakoids prepared from these samples showed remarkable water-oxidation activities, and the DPC-oxidation activity was also enhanced by a factor of about 2. The water-oxidation activities thus generated by illumination in protoplasts and intact chloroplasts were as high as the activity generated by illumination of intact leaves. These results clearly indicate that photoactivation proceeds normally in intact chloroplasts as well as in protoplasts and intact leaves, and that all the factors necessary for the process are assembled within intact chloroplasts. By contrast, the illumination of broken chloroplasts did not induce water-oxidation activity at all but rather lowered the activity of DPC-oxidation. This indicates that the intactness of chloroplasts is essential for the photoactivation *in vitro*, and may also imply that some factor(s) in stroma is involved in the process.

Figure 1 shows the course of generation of water-oxidation activity and enhancement of DPC-oxidation activity during illumination of intact chloroplasts. Before illumination, both activities of water- and DPC-oxidation were 4 and 54 μmol DCIP/mg Chl·h, respectively. On illumination, the water-oxidation activity was rapidly induced and reached the saturation level of about 100 to 200 μmol DCIP/mg Chl·h after 20 min (curve A). The DPC-oxidation activity was also enhanced by illumination and reached the same saturation level as that of water-oxidation activity (curve B). The saturation level fluctuated between 100 and 200 μmol DCIP/mg Chl·h depending on the preparation of intact chloroplasts, but the two activities repeatedly showed the same saturation level independent of the preparation. The actvities thus generated or enhanced by illumination were stable during the subsequent dark incubation at 0°C at least for 4 h.

Figure 2 shows the light-intensity dependence of the rate of photoactivation, in which the increment of the water- and DPC-oxidation activities during the first 10 min of illumination was plotted against the intensity of illumination. Both rates were steeply increased with increasing the light intensity and were saturated at the same intensity of about 70 $\mu w/cm^2$, but the saturation level of the increment of water-oxidation activity was reproducibly higher than that of DPC-oxidation activity (curve A). This implies that the overall quantum yield for the enhancement of DPC-oxidation activity is limited by some reason at a

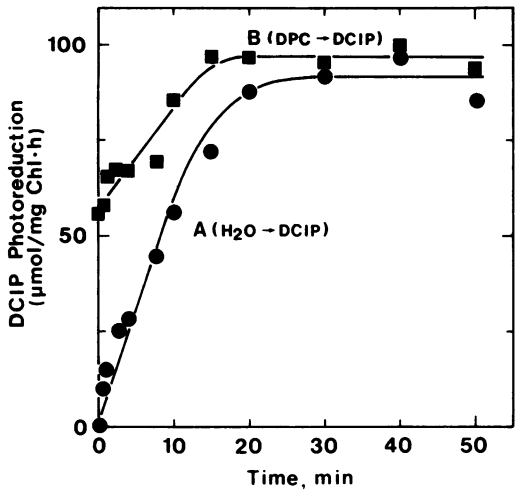


FIG. 1. Time course of photoactivation induced by continuous light illumination of the intact chloroplasts isolated from wheat leaves grown under intermittent flash illumination, shown in terms of generation of water-oxidation activity (curve A, ●) and enhancement of DPC-oxidation activity, (curve B, ■).

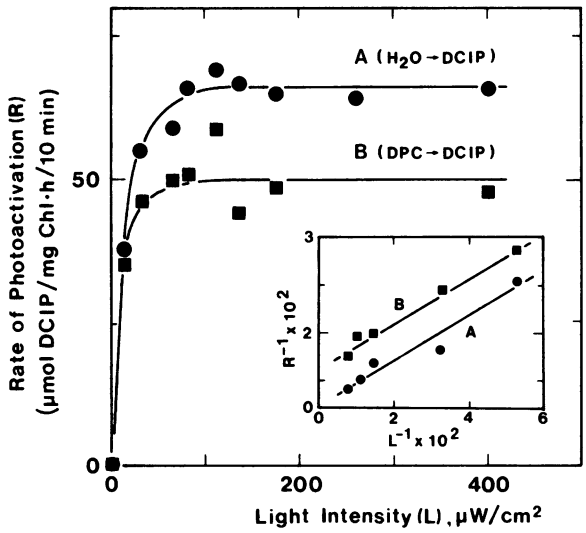


FIG. 2. Light-intensity dependence of the rate of water-oxidation activity generation (curve A, ●) and of DPC-oxidation activity enhancement (curve B, ■) during photoactivation in isolated intact chloroplasts. The rates were estimated from the activity increments in the first 10 min of continuous illumination. Small figure in the margin shows the double reciprocal plot of curves A and B.

lower level than that for the induction of water-oxidation activity (curve B).

The difference in the overall quantum yield between the two activity increments was analyzed based on the method by Lumry and Spikes (13). The small figure in the margin of Figure 2 shows the reciprocal plot of the rate of photoactivation *versus* the reciprocal of light intensity. Both plots for water- and DPC-oxidation activities resulted in straight lines with the same inclination but with different intersecting points on the ordinate. According to the theory by Lumry and Spikes, the intersecting point on the ordinate stands for the reciprocal of the maximum value of the rate-limiting dark reaction, and the inclination of the line stands for the reciprocal of the quantum yield of the photochemical process. Accordingly, it is deduced from these two straight lines that both activations of water- and DPC-oxidation activities share the same photochemical process(es) with the same quantum yield, but

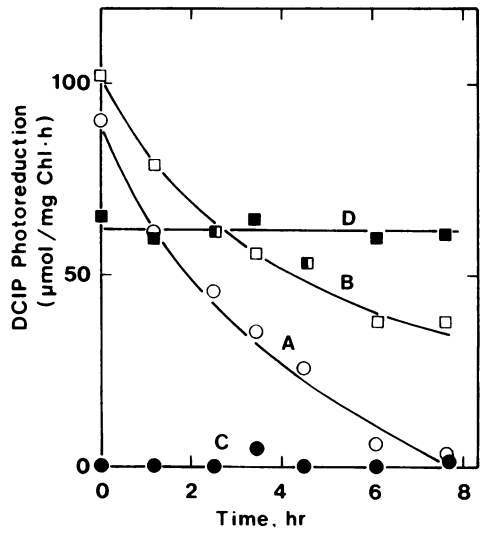


FIG. 3. Effect of aging on the competence of photoactivation in intact chloroplasts. The intact chloroplasts were incubated at 0°C for the indicated periods before subjected to photoactivation by 20-min continuous illumination. Curve A (○), water-oxidation activity after illumination; curve B (□), DPC-oxidation activity after illumination; straight line C (●), water-oxidation activity before illumination; straight line D (■), DPC-oxidation activity before illumination.

Table II. *Effect of A23187, an Ionophore for Divalent Cations, on Photoactivation in Isolated Intact Chlorplasts*

Water-oxidation activity generated in the first 10 min of illumination was listed. Postaddition means that the chemicals were added after illumination for photoactivation.

Condition	Water-Oxidation Activity
	μmol DCIP/mg Chl·h
Nonilluminated	2
Illuminated (10 min)	
Control	104
+ EDTA (0.1 mM)	110
+ EDTA and A23187 (10 μM)	0
+ EDTA and A23187 and $MnCl_2$ (5 mM)	20
+ EDTA and A23187 (10 μM, postaddition)	105

involve respective rate-limiting dark reactions with different efficiencies.

Figure 3 shows the effect of aging of the intact chloroplasts on photoactivation. The sample intact chloroplasts were stored after preparation for various durations at 0°C in darkness before being subjected to photoactivation by 20-min illumination. The competence of photoactivation declined with aging in terms of both water- and DPC-oxidation activities and, after 8-h aging, both activities could no more be activated by illumination (curves A and B). The DPC-oxidation activity observed for nonilluminated samples did not change during the aging of 8 h but was rather decreased by illumination below the initial level before illumination when the aging period is prolonged. Probably, the photochemical reaction in the absence of electron donor in the latent water-oxidation system no more capable of being activated because of aging resulted in some destruction of the system.

Table II summarizes the effect of A23187 (17), an ionophore for divalent cations having high specificity for Mn^{2+} ions, on the photoactivation in intact chloroplasts. In the experiment, EDTA (0.1 mM) was added to the reaction mixture in order to trap the divalent cations released out of the intact chloroplasts by the action of the ionophore. The presence of A23187 at a concentra-

Table III. *Effect of Inhibitors of ATP Formation on Photoactivation in Isolated Intact Chloroplasts*

Water-oxidation activity generated in the first 8 min of illumination was listed.

Condition	Water-Oxidation Activity
	μmol DCIP/mg Chl·h
Nonilluminated	2
Illuminated (8 min)	
Control	76
+ DCCD (50 μM)	70
+ Oligomycin (50 μM)	70
+ Gramicidin D (30 μM)	74
+ CCCP (2 μM)	46
+ Methylamine (30 mM)	91
+ NH_4Cl (30 mM)	103
+ CH_3COONa (30 mM)	74

Table IV. *Effect of Various Types of CO_2-Fixation Inhibitors on Photoactivation in Isolated Intact Chloroplasts*

Water-oxidation activity generated in the first 10 min of illumination was listed.

Condition	Water-Oxidation Activity
	μmol DCIP/mg Chl·h
Nonilluminated	2
Illuminated (10 min)	
Control	104
+ KCN (0.5 mM)	105
+ $NaNO_2$ (5 mM)	36
+ MV (0.2 mM)	33
+ PMA (50 μM)	0

tion of 10 μM combined with EDTA during illumination completely inhibited the generation of water-oxidation activity. Inasmuch as A23187 added after illumination did not affect the water-oxidation activity generated beforehand, the elimination of Mn^{2+} from the intact chloroplasts must have caused the inhibition. When an excess amount of Mn^{2+} (5 mM) was present in the A23187/EDTA system during illumination, the water-oxidation activity was slightly but significantly induced. This result is consistent with the above interpretation for the action of A23187 on photoactivation. The low restoration of about 20% of the control may imply that the photoactivation requires one or more divalent cations other than Mn^{2+}. The requirement of Ca^{2+} in addition to Mn^{2+} has been reported by Yamashita and Tomita (21) for the photoactivation of water-oxidation activity in Tris-inactivated chloroplasts.

Table III shows the effect of various chemicals affecting ATP formation on the photoactivation in intact chloroplasts. The generation of water-oxidation activity was not affected by DCCD (50 μM) or oligomycin (50 μM), which are known to inhibit ATP formation by affecting ATPase. Among the uncouplers tested, Gramicidin D (30 μM) did not affect the rate either, while methylamine (30 mM) and NH_4Cl (30 mM) were stimulatory. This stimulation seems to result from the pH rise in intrathylakoid space or in stroma, a shift toward the optimum pH of 7.8 for photoactivation as reported by Oku and Tomita (16). CH_3COONa (30 mM), which is known to lower the pH in intact chloroplasts, on the other hand, did not affect the process. CCCP (2 μM) was inhibitory for the photoactivation. Judging from the effects of the other uncouplers tested, this inhibition does not seem to result from the uncoupling function of this chemical. Possibly, the interference of this chemical with SH groups (12) might be responsible for this inhibition.

Table IV summarizes the effect of various types of CO_2 fixation inhibitors on the activity generation in intact chloroplasts. Inhibition of Calvin-Benson cycle enzymes by KCN (5 mM) did not cause any inhibition of the photoactivation. By contrast, NO_2^- (5 mM) or MV (0.2 mM) considerably inhibited the activity generation. Since these chemicals are known to inhibit CO_2 fixation by preferentially accepting electrons from PSI, the results suggest that the electron transport on the reducing side of PSI plays an essential role in the process of photoactivation. PMA (50 μM), a typical inhibitor for ferredoxin and ferredoxin-NADP oxidoreductase, also inhibited the activity generation. Possibly, some factor reduced with ferredoxin as catalyzer is required for the process of photoactivation.

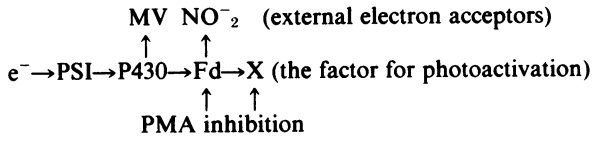
DISCUSSION

In the present study, it was shown that the latent water-oxidation system in isolated intact chloroplasts prepared from wheat leaves grown under intermittent flashes can be activated by simply illuminating the isolated chloroplasts with continuous light with no externally added factor. The activity generated by illuminating the isolated intact chloroplasts fluctuated around 200 μmol DCIP/mg Chl·h, but these values were almost comparable to the activity generated by illuminating the intact leaves. This fact indicates that all the machinery necessary for normal photoactivation is assembled within intact chloroplasts and no cytoplasmic factor is required (Table I).

As opposed to intact chloroplasts, no activity generation could be observed for broken chloroplasts. The broken chloroplasts used in the present study were obtained by the same procedure as for the intact chloroplasts and separated by Percoll density gradient centrifugation. Judging from the mild preparation procedure used and also from observation under microscope, the reason for the loss of photoactivation competence of the broken chloroplasts cannot be due to the damage in thylakoids but seems likely to be attributed to the leaking of stroma. We may, hence, assume that some factor(s) contained in stroma is required for photoactivation to proceed normally. This factor may not be the cations such as Mn^{2+} and Ca^{2+}, since the factor is a redox substance which may be reduced in competition with NO_2^- or MV as discussed in the paragraph below.

As indicated in Table IV, NO_2^- and MV inhibited the photoactivation in intact chloroplasts. As to the role of electron transport in photoactivation, it has been inferred that photoactivation requires some oxidized equivalents on the oxidizing side of PSII, based on the observation that the photoactivation in algal cells and intact leaves was sensitive to DCMU (3, 10). From this point of view, the inhibitory effect of NO_2^- and MV shown in the present study seems remarkable in that the generation of water-oxidation activity was affected under the condition which allowed electron transport through both photosystems to accumulate oxidizing equivalents on the oxidizing side of PSII. Considering that both of those two chemicals are efficient electron acceptors of PSI, the former via ferredoxin assisted by nitrite reductase (14) and the latter via P430, one of the early electron acceptors of PSI (7), we may assume that a redox factor is involved in the process of photoactivation; possibly the reduced form of the factor is required for the photoactivation process in addition to the photooxidation of Mn^{2+} on the oxidizing side of PSII (5), and the redox state of the factor is competitively regulated in the presence of NO_2^- or MV, as shown by the scheme below. This view is consistent with the above assumption that some soluble factor in stroma is involved in the process, and does not conflict with the previous observation that DCMU is inhibitory for photoactivation. The necessity of such reduced factor may also be related with the observation by Oku and Tomita (16) that the photoactivation in broken chloroplasts of dark-grown spruce leaves requires high concentrations of ascorbate. Possibly, the ascorbate can partially substitute the function of the postulated factor, or acts as antioxi-

dant to keep the factor reduced.



Another observation in this study that PMA completely inhibited the photoactivation seems also to be related to the above view. Since PMA is a typical inhibitor for SH-groups, the chemical will block the function of ferredoxin in intact chloroplasts (8), which may result in complete inhibition of photoactivation according to the above scheme. PMA is also known to affect the coupling factor of chloroplast phosphorylation, CF_1. However, CF_1 does not seem to be involved in the process, since DCCD, Oligomycin, Gramicidin and methylamine did not inhibit the photoactivation in intact chloroplasts (Table III). As opposed to Gramicidin and methylamine, CCCP inhibited the photoactivation in intact chloroplasts. Judging from the fact that Gramicidin and methylamine, typical uncouplers, were not inhibitory, the inhibition by CCCP is not due to the uncoupling function but may be causing from some other function of this chemical. It is reported that CCCP reacts with DTT to lose its uncoupling effect in bacterial membranes (12). It seems possible to assume in this context that the postulated factor, X, is an SH-bearing substance being reactive with CCCP to lose its function in the photoactivation. It seems also worth while to note that some enzymes for Calvin-Benson cycle, such as NADP-linked glyceraldehyde-3-P dehydrogenase and fructose-1,6-diphosphatase are inactive in darkness but are activated on illumination by a soluble factor in stroma having SH-groups, thioredoxin, reduced via ferredoxin by PSI photoreaction (19, 20). It may be reasonable to assume a similar mechanism for the photoactivation of the water-oxidation system, correlating the factor postulated with thioredoxin.

As shown in Table II, A23187, an ionophore for divalent cations, completely inhibited the photoactivation. This result is consistent with the established view that Mn atoms are incorporated during photoactivation into the proper sites on the water-oxidation enzymes (2, 4, 21). The clear difference in the effect of A23187 shown in this study, inhibitory when present during photoactivation, but not inhibitory at all when added after photoactivation, suggests that Mn atoms are free or loosely bound to the enzyme before photoactivation to be susceptible to the ionophore, but become tightly bound to the enzyme to turn resistant to the ionophore after photoactivation. The inhibition by A23187 was restored by the presence of excess Mn^{2+}, but the restoration was partial, about 20% of the control. This low restoration may have resulted from the shortage of some divalent cations other than Mn^{2+}, which are extracted together with Mn^{2+} out of chloroplasts or thylakoids by the action of the ionophore, as reported by Yamashita and Tomita (21, 22) that Ca^{2+} is needed for the reactivation in Tris-inactivated chloroplasts. We observed that high concentration of Mn^{2+} is rather inhibitory for photoactivation and the coexistence of Ca^{2+} restores this inhibition caused by Mn^{2+}. Such examination is under way to be published elsewhere.

As shown in Table II, uncouplers except CCCP did not affect the photoactivation. This result is not always consistent with the previous observations by Cheniae and Martin (3) or Yamashita and Tomita (22) that the photoactivation in Mn-deficient algal cells or in Tris-inactivated chloroplasts (at the high pH of 8.8), respectively, was blocked by uncouplers. They inferred that uncouplers suppress the transport of Mn^{2+} through the cell membrane or thylakoid membrane to reach the binding site to form active water-oxidation enzymes. When we consider the data in the present study referring to their interpretation, the insensitivity to uncouplers shown in this study seems to suggest that Mn atoms necessary for the photoactivation in intact chloroplasts are located in the vicinity of the water-oxidation enzyme to be activated, and the transport of Mn through thylakoid membranes is not involved in the process.

It was also shown in the present study that DPC-oxidation activity is enhanced during photoactivation in parallel with the generation of water-oxidation activity. This is consistent with the previous observation (9), and may imply that the photoactivation is not a phenomenon limited within the functional development of the latent water-oxidation enzyme but simultaneously involves some changes in the electron transport activities of the secondary electron donors located between PSII and the water-oxidation system. The function and role of the secondary electron donors of PSII during illumination for photoactivation will be the problems to be pursued, since these donors appear to supply a certain amount of electrons to reduce the factor postulated and discussed in the present study.

LITERATURE CITED

1. ARNON DI 1949 Copper enzymes in isolated chloroplasts: Polyphenol oxidase in *Beta vulgaris*. Plant Physiol 24: 1-5
2. BLANKENSHIP RE, GT BABCOCK, K SAUER 1975 Kinetic study of oxygen evolution parameters in Tris-washed, reactivated chloroplasts. Biochim Biophys Acta 387: 165-175
3. CHENIAE GM, IF MARTIN 1969 Photoactivation of manganese catalyst in photosynthetic oxygen evolution. Plant Physiol 50: 87-94
4. CHENIAE GM, IF MARTIN 1972 Effects of hydroxylamine on photosystem II. II. Photoactivation of the NH_2OH destruction of O_2 evolution. Plant Physiol 50: 87-94
5. CHENIAE GM, IF MARTIN 1973 Absence of oxygen-evolving capacity in dark-grown *Chlorella*: the photoactivation of oxygen-evolving centers. Photochem Photobiol 17: 441-459
6. EDWARDS GE, SP ROBINSON, NJC TYLER, DA WALKER 1978 Photosynthesis by isolated protoplasts, protoplast extracts, and chloroplasts of wheat: Influence of orthophosphate, pyrophosphate and adenylates. Plant Physiol 62: 313-319
7. HIYAMA T, B KE 1971 A new photosynthetic pigment, "P430", its possible role as a primary electron acceptor of photosystem I. Proc Natl Acad Sci USA 68: 1010-1013
8. HONEYCUT PC, DW KROGMANN 1972 Inhibition of chloroplast reactions with phenylmercuric acetate. Plant Physiol 49: 376-380
9. INOUE Y, Y KOBAYASHI, E SAKAMOTO, K SHIBATA 1974 Action spectrum for photoactivation of the water-splitting system in plastids of intermittently illuminated wheat leaves. Physiol Plant 32: 228-232
10. INOUE Y, Y KOBAYASHI, E SAKAMOTO, K SHIBATA 1975 Multiple flash activation of the water-splitting system in intermittently illuminated wheat leaves. Plant Cell Physiol 16: 327-336
11. INOUE Y, S FURUTA, T OKU, K SHIBATA 1976 Light-dependent development of thermoluminescence, delayed emission and fluorescence variation in dark-grown spruce leaves. Biochim Biophys Acta 449: 357-367
12. KABACK HR, JP REEVES, SA SHORT, FJ LOMBARDI 1974 Mechanism of active transport in isolated bacterial membrane vesicles. XVIII. The mechanism of action of carbonylcyanide-*m*-chlorophenylhydrazone. Arch Biochem Biophys 160: 215-222
13. LUMRY R, JD SPIKES 1959 The mechanism of the photochemical activity of isolated chloroplasts. V. Interpretation of the rate parameters. Plant Physiol 34: 293-300
14. NEYRA CA, RH HAGEMAN 1974 Dependence of nitrite reduction on electron transport in chloroplasts. Plant Physiol 54: 480-483
15. OKU T, G TOMITA 1976 Photoactivation of oxygen-evolving system in dark-grown spruce seedlings. Physiol Plant 38: 181-185
16. OKU T, G TOMITA 1980 Photoactivation of the latent O_2-evolving center in chloroplasts isolated from dark-grown spruce seedlings. Physiol Plant 48: 99-103
17. REED PW, HA LARDY 1972 A23187, a divalent cation ionophore. J Biol Chem 347: 6970-6977
18. REMY R 1973 Appearance and development of photosynthetic activities in wheat etioplasts greened under continuous or intermittent light—evidence for water-side photosystem II deficiency after greening under intermittent light. Photochem Photobiol 18: 409-416
19. WOLOSIUK RA, BB BUCHANAN 1977 Thioredoxin and glutathione regulated photosynthesis in chloroplasts. Nature (Lond) 226: 565-567
20. WOLOSIUK RA, BB BUCHANAN 1978 Activation of chloroplast NADP linked glyceraldehyde-3-phosphate dehydrogenase by the ferredoxin/thioredoxin system. Plant Physiol 61: 669-671
21. YAMASHITA T, G TOMITA 1974 Effects of manganese, calcium, dithiotheitol and bovine serum albumin on the light-reactivation of Tris-washed chloroplasts. Plant Cell Physiol 15: 59-82
22. YAMASHITA T, G TOMITA 1976 Light-reactivation of (Tris-washed)-DPIP-treated chloroplasts: manganese incorporation, chlorophyll fluorescence, action spectrum and oxygen evolution. Plant Cell Physiol 17: 571-582