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PHOTOACTIVATION OF THE MANGANESE CATALYST OF O_2 EVOLUTION

I. BIOCHEMICAL AND KINETIC ASPECTS

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SUMMARY

Photosynthetic O_2 evolution requires a Mn complex which is activated by light. An analysis of this activation process yielded the following results:

1. In any given illumination, the time course is first order, the rate being proportional to the number of inactive O_2-evolving System II trapping centers (the quantum yield being invariant).

2. The action spectrum and the photochemical cross-section proved to be identical to that of the O_2-evolving photosystem, *i.e.* the process occurs in the reaction centers of System II.

3. In the activation process there are no interactions between neighboring reaction centers.

4. Over an intermediate intensity range the quantum efficiency is optimal and constant; at both lower and higher intensities the quantum efficiency decreases.

5. Flashing light experiments agreed with data obtained in continuous light, *i.e.* both results showed that activation is a multi (minimally two) quantum process. The product of the first photoact relaxes to a new photosensitive state of limited stability which is converted by light into a stable active O_2-evolving center.

6. Determinations were made of the limiting and decay rates of the intermediates involved.

INTRODUCTION

Removal of Mn^{2+} by growth or by mild extraction[1,2] from photosynthetic tissue leaves the primary photochemical acts (System II and System I) intact but causes the specific loss of O_2 evolution[1,2,4,6]. Activation of the capacity for O_2 production in such tissue specifically requires both Mn and light[3,7–10]. Three effects of light upon this photoactivation of the O_2-evolving reactions have been described[8]: (1) a light enhanced uptake by the cells of Mn^{2+}, which is presumably linked to photophosphorylation; (2) photoactivation *per se*, which is presumably the activation of the O_2-evolving Mn-containing catalyst; and (3) an apparent photoinhibition of photoactivation by moderate and high light intensities.

Abbreviations: Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

Previously we showed that the intracellular pool of Mn required for photoactivation could be filled in darkness without a simultaneous increase of active O_2-evolving centers[8]. An increase only occurred if light was given either during or after filling of the Mn pool. This observation permitted the separation of the effects of light upon Mn uptake from the effects upon photoactivation proper. Here we report a kinetic analysis of this process in Mn-deficient *Anacystis nidulans* cells.

METHODS

Methods for obtaining Mn-deficient Anacystis cells by growth have been described previously[7]. Cells were harvested by centrifugation at 25° after a 40–48-h growth period (20 μl packed cell volume inoculum) and resuspended (40 μg chlorophyll (Chl) per ml) in fresh Mn deficient culture medium. The Hill activity (O_2 evolution with quinone–$Fe(CN)^{3}_{6}^{-}$ as electron acceptor) was then determined. Generally the so-obtained "base rate" was 20–30 % of the rate obtained with Mn-sufficient cells or completely photoactivated Mn-deficient cells. The Mn-deficient cell suspensions were made 18 μM with respect to $MnCl_2$ and gassed with 5 % CO_2 in air for 2 h or more in total darkness. After this dark equilibration with Mn^{2+}, the cells were diluted to the desired concentration with growth medium and reassayed for O_2 evolution. In no instance did we observe an increase of O_2 evolution after equilibration (up to 8 h) in darkness of cells with Mn^{2+} (ref. 11). Following preillumination in one of several manners, O_2 evolution capacity was redetermined. The resulting increase of activity (μmoles O_2 per mg Chl per h in strong light) over the "base rate" (R_i) could be used as a measure of the extent of photoactivation, since, under the conditions of the assay procedure, no significant photoactivation occurs[8]. Preillumination and assay regimes were duplicated within each experiment when small yields of the photoactivated state were obtained. Within any given experiment, reproducibility between duplicates of $\pm$ 5 % was obtained.

Cell suspensions were stirred during preillumination in a cuvette of 0.33 cm light path. All work was done in a dark room and the cuvette was masked to eliminate any stray light.

Light measurements were made with a silicon photocell calibrated *versus* a standardized bolometer. Rates of quantum absorption were corrected for differences of geometry between the photocell and the vessel.

For preilluminations with specific wavelengths, light from a 750-Watt tungsten lamp was passed through 10 inches of water, appropriate neutral density and interference filters, and a camera shutter before being focussed onto the cuvette. For studies on the effectiveness of various wavelengths, the cell concentrations were adjusted to equivalent absorptions (20–35 % for 0.33 cm light path at all wavelengths).

Absorption measurements of cells and spinach chloroplasts were made in a split-beam integrating sphere. The same interference filters used in the absorption measurements were also employed in the preillumination of the Mn-deficient cells. These filters, 5 nm half-band width blocked to infinity on both sides of the specific wavelength, were obtained from Thin Film Products, Cambridge, Mass. Under the conditions employed the absorptions obeyed Beer's law. Typical absorption values (1 cm light path) at 620, 680 and 700 nm of 56.9, 44.7 and 6.0 %, respectively, were obtained (with correction for light scattering) for Mn^{2+}-deficient Anacystis at a con-

centration of 3.5 μg Chl per ml. In a few instances the absorption of extreme Mn-deficient cells deviated from the Beer's law relationship. This deviation was observed only at wavelengths (620 nm) exciting predominately System II and with deficient cells showing a high fluorescence yield. Such cells were not used for photoactivation studies.

Flash illumination was provided by 2 Amglo U-35-o lamps at a distance of about 0.5 cm on opposite sides of the cuvette. A Shott OG-3 cut-off filter was interposed between the lamps and the cuvette. The flashes were obtained with 0.5 μF capacitors charged to 2000 V yielding 1 J per flash of 2 μsec half duration. Neutral density filters were used to vary the flash intensity.

Preliminary experiments indicated that the increased O_2 evolution capacity resulting from preillumination remained constant in darkness over a period of at least 6 h. In general, assay of the photoactivated state was made within 30 sec to 15 min following preillumination. The "base rate" O_2 evolution capacity of cells maintained in darkness sometimes declined as much as 20–30 % after 5–6 h equilibration with Mn^{2+}. In most instances the described experiments were completed before the onset of this decline of "base rate".

RESULTS

Requirement for preequilibration of cells with Mn^{2+}

A previous report[7] described the autocatalytic behavior of the photoactivation process. These experiments encountered two secondary complications: (1) we were not certain that the intracellular Mn^{2+} pool had been filled; and (2) photoactivation was observed as the increasing rate of photosynthesis with time of illumination. However, a marked induction phase of photosynthesis complicated interpretation. This latter complication was eliminated by assaying O_2 evolution capacity with quinone–$Fe(CN)^{3}_{6}{}^{-}$ as electron acceptor. In this system the rate of O_2 evolution shows no lag and remains constant for at least several minutes.

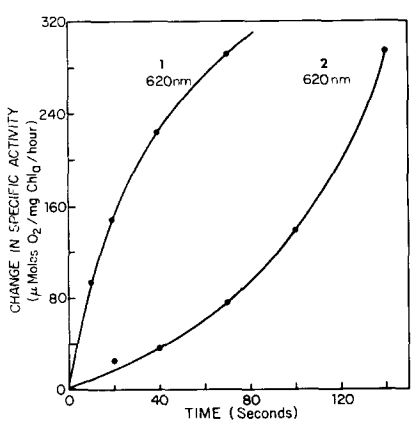

Fig. 1. Time course of photoactivation as affected by preequilibration of cells in darkness with Mn^{2+}. Cells were resuspended (40 μg Chl per ml) in growth medium containing no added Mn^{2+}. Cells of Curves 1 and 2 were equilibrated with 18 μM $MnCl_2$ in darkness for 120 and 5 min, respectively, before illumination with 620 nm light. Rate of quantum absorption into 7 μg Chl was 15 neinsteins/min. Original "base rate" in strong light was 124 μmoles O_2 per mg Chl per h.

Curves 1 and 2 in Fig. 1 show the time course of photoactivation of Mn-deficient cells in weak 620 nm light. For Curve 1 the cells were preequilibrated (2 h) in darkness with Mn^{2+}. No time lag was observed and the process follows a first order time course. Curve 2 was observed when Mn was added at the start of the illumination, *i.e.* without prior equilibration of the Mn pools. This time course shows a distinct lag and we ascribe its "autocatalytic" kinetics to a rate limiting step of Mn^{2+} influx. These data show that simple kinetics and maximum quantum efficiency of photoactivation are obtained only after the intracellular pool of Mn required for photoactivation has been filled through dark processes.

Assay of photoactivation

(*a*) *Relation between* v_{max} *and* v. To assay the percent of the System II traps which are in the active state, one could measure the quantum yield of O_2 evolution, the maximum rate in strong light (v_{max}), or the maximum flash yield of O_2. The last measurement is most direct but the determination of v_{max} is technically the simplest and most sensitive. Moreover, in the presence of quinone–$Fe(CN)_6^{3-}$ photoactivation in weak light is inhibited only about 50 % but is completely inhibited in strong continuous light. Finally, the quantum yield, a more difficult assay, does not unambiguously reflect the number of active O_2 evolving System II traps because of possible energy transfer between units. In Chlorella[12] and chloroplasts[13] the relation between the quantum yield and the flash yield of O_2 is non-linear such that, in general, a 2-fold effect on quantum yield is reflected by a 4-fold effect on flash yields. Consequently in this work we used the assay of v_{max}.

In principle, v_{max} is codetermined by the concentration of active O_2 evolving

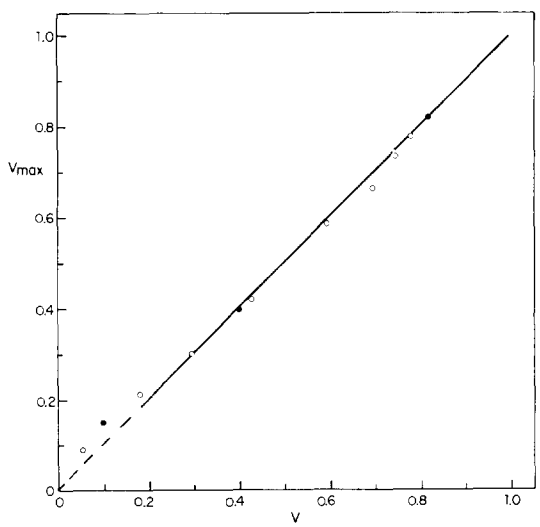

Fig. 2. Relationship between rate of O_2 evolution in strong light (v_{max}) *vs.* rate in weak light (v) Data of open circles were obtained by photoactivating NH_2OH extracted cells (2 mM NH_2OH containing 200 μg Chl per ml for 15 min at 20°) for various times. After each period of preilluminations, rates of O_2 evolution in strong and weak light (rate linear with intensity) were measured. The v_{max} "base rate" of non-preilluminated extracted cells was 35 μmoles O_2 per mg Chl per h. Data of closed circles were obtained similarly except Mn^{2+}-deficient cells obtained by growth were used. Original v_{max} "base rate" was 110 μmoles O_2 per mg Chl per h.

centers and a rate limiting dark reaction, $k_d : v_{max}$ = [active centers]k_d. To ascertain the relationship between relative quantum yield (v) and v_{max}, we measured initial rates of both parameters during the photoactivation of Mn-deficient cells obtained either by growth or by NH_2OH extraction[1,4]. This relationship is shown in Fig. 2 (Mn deficiency by growth and NH_2OH extraction, closed and open circles, respectively). Except for some possible deviation below an 80 % decrease of quantum yield, any increase of v_{max} was reflected by a proportionate increase of v. Such results therefore demonstrate parallelism between v_{max} and v during photoactivation. We conclude that v_{max} is a valid measurement for assessment of the increase of active O_2 evolving centers resulting from photoactivation.

(*b*) *Effect of increasing Mn deficiency upon kinetics of photoactivation; time course of photoactivation is first order.* For technical reasons, it is virtually impossible to routinely obtain Mn deficient cells of equivalent "base rate". Moreover, with increasing Mn deficiency pigment changes often occur in Anacystis[2,14] which complicate evaluation of v_{max} based only upon Chlorophyll. In our analyses of photoactivation we used illumination with widely different wavelengths, intensities and time distributions and algae with variable degrees of deficiencies.

In Fig. 3 we assessed the effect of variable degrees of deficiencies induced by growth at different Mn levels on the flash induced time-course of photoactivation. In other experiments (not shown) variable degrees of deficiencies were obtained by partial photoactivation with weak 620 nm light of cells cultured at one Mn level. The results obtained in either type experiment proved consistent with each other.

In Fig. 3 the effect of extreme (Curve 1), moderate (Curve 2) and low Mn deficiency (Curve 3) induced by growth has been examined on the time-course of photoactivation induced by flashes. The flashing regime had no effect on the O_2 evolution capacity of Mn-sufficient cells.

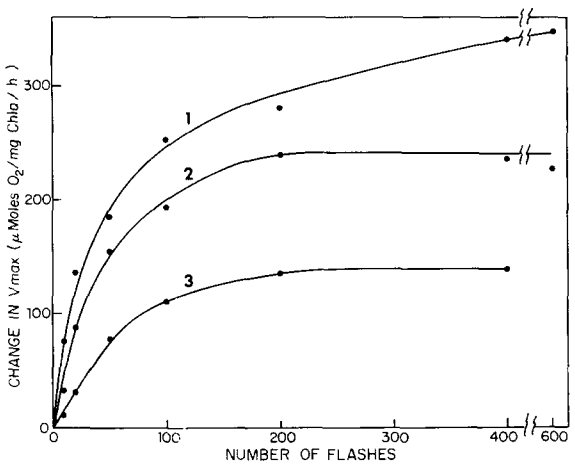

Fig. 3. Effect of increasing Mn depletion on the flash induced photoactivation of Mn^{2+}-deficient Anacystis. Cells of Curves 1, 2 and 3 were cultured in Mn-depleted growth medium supplemented with zero, $1 \cdot 10^{-8}$ M and $4 \cdot 10^{-8}$ M $MnSO_4$, respectively. Flash periodicity was 0.6 sec followed (25 msec) by a delay flash. Values on the abscissa are the total number of saturating flashes. Relative absorption values (in 30% bovine serum[21]), at 620 nm for equivalent amounts of Chl for Mn-sufficient and cells of Curves 1, 2 and 3 were 1.0, 4.3, 3.2 and 1.8, respectively. "Base rate" in strong light of unphotoactivated cells of Curves 1, 2 and 3 were 365, 400 and 215 μmoles O_2 per mg Chl per h.

With increasing number of flashes the yield of the photoactivated states for Curves 1, 2 and 3 of Fig. 3 increased and eventually attained changes of v_{max} of 345, 240 and 135 μmoles O_2 per mg Chl per h, respectively.

As expected, the magnitude of the maximum obtainable amount of the photoactivated state was a function of the initial extent of the deficiency. With correction for differences in absorption (620 nm) between the different cells, the final specific activity of O_2 evolution for the three different Mn-deficient cells became approximately equivalent (within 10 %). Ignoring the last 10 % of Curve 1, essentially the same number of flashes were required to produce half conversion to the active state for all three cultures regardless of the differences in extents of deficiencies. Moreover, as indicated in Fig. 4 homogeneous apparent first-order kinetics were obtained for all three curves.

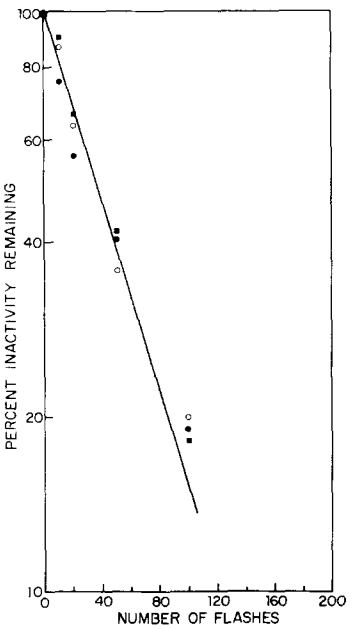

Fig. 4. First-order plot of conversion of inactive to active O_2 evolving centers *versus* number of flashes. Data were obtained from Fig. 3. Symbols for cells cultured at $1 \cdot 10^{-8}$ M, $4 \cdot 10^{-8}$ M and without addition of $MnCl_2$ to the growth medium are open circles, closed squares, and closed circles, respectively, For other details, see text.

Fig. 5 shows the yield of O_2 centers resulting from photoactivation in four different rates of continuous 620 nm light absorption. The time course proved essentially first order over the 50-fold intensity range.

The results of Figs. 3–5 thus indicate that, although the rate constant or quantum yield may vary widely depending upon rate of quantum absorption and/or number of inactive O_2 yielding centers, photoactivation is a first order process. Accordingly, the general equation for conversion of the inactive to active O_2 centers can be described by: $R_t = R_i e^{-\Phi \alpha I t}$ where R_t is the v_{max} rate after photoactivation during time t, R_i the initial v_{max} rate, α the fractional absorption of the intensity (I) and Φ the quantum yield.

The data of Figs. 3–5 suggest: (1) the rate of photoactivation is proportional to the number of inactive O_2 producing centers; (2) for any set of conditions the quantum yield is constant; and (3) activation of an O_2 center is unaffected by the state of neighboring centers.

These results imply that it is possible to obtain a meaningful estimate of the effect of an illumination pattern by measuring two values of v_{max}: one before (R_i) and one after (R_t) an exposure to this illumination. By normalizing R_i and R_t in terms of R_{max} one can compute the specific rate constant (k). The condition, $R_i = 0$ (no active O_2-evolving centers), cannot be attained in practice through growth deficiency without

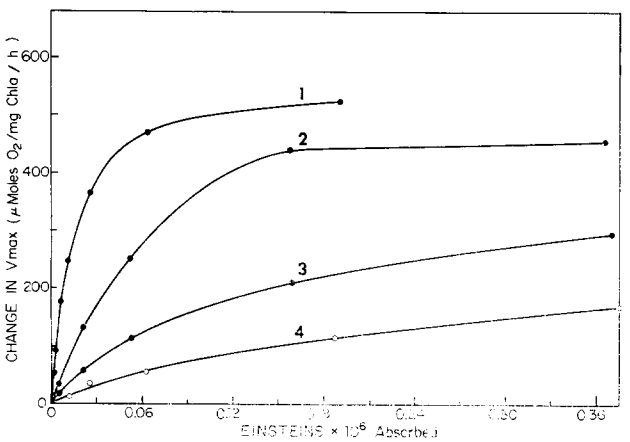

Fig. 5. The effect of increasing rates of quantum absorption (620 nm) on the yield of photoactivation. Rates of quantum absorption by 6.3 μg Chl (total) for Curves 1, 2, 3 and 4 were 13.2, 61.9, 312 and 738 neinsteins absorbed per min, respectively. "Base rate" (v_{max}) of original cells was 250 μmoles O_2 per mg Chl per h. Relative quantum efficiency for each intensity is given by the initial slope of the corresponding curves.

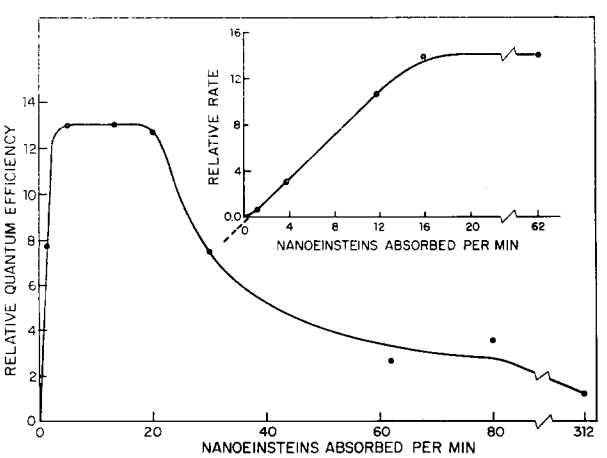

Fig. 6. Effect of increasing light intensity on relative quantum efficiency and rate of photoactivation. Relative quantum efficiency as a function of rate of 620 nm quantum absorption was determined as outlined in legend of Fig. 5. Each point represents the average of 4 experiments with cells of essentially similar "base rate" (v_{max} = 240–257 μmoles O_2 per mg Chl per h). In the insert of Fig. 6 the relative rate of photoactivation of data of Fig. 6 is plotted *versus* rate of 620 nm quantum absorption.

deleterious secondary effects. However, knowing the value of R_i and R_{max}, the reaction velocity of the photoactivation process for $R_i = 0$ is readily obtained.

Effect of intensity on quantum yield and rate of photoactivation; a multi-quantum process

In Fig. 6 the initial slopes of curves such as those shown in Fig. 5 are plotted versus the rate of 620 nm absorption. Each point on the curve represents the average of 3–4 determinations with cells of essentially the same degree of Mn^{2+} deficiency and equivalent "base rates". The results reveal that the quantum efficiency of photoactivation is constant and optimal only over a narrow range of intensities. At both lower and higher intensities the yield declines.

A plot of rate *vs.* intensity (Fig. 6, inset) is S shaped, showing a lag in very weak light, a range of linearity (constant quantum yield), and saturation in moderate light (about one-tenth the intensity needed for photosynthetic saturation). These data reveal that photoactivation is complex: (1) the initial lag in very weak light indicates that photoactivation is a multi-quantum process involving unstable intermediates; (2) the early light saturation implies the involvement of one or more slow rate limiting dark steps.

Subsequent sections will show that we are dealing with events in the trapping centers of System II. Therefore, assuming an abundance of one System II trap per 300 Chl and equal distribution of the absorbed 620 quanta between Systems II and I, we calculate from data of Fig. 6 that the quantum efficiency is half maximal at high intensities when each trapping center receives a quantum about every 80 msec. This 80 msec presumably reflects the reaction time of a rate limiting dark step. On the other hand, in weak intensities the quantum efficiency is decreased to one-half at a flux of 1 *hν*/min per 6.3 Chl or 1 excitation per System II trap every 2.3 sec. This presumably reflects the decay time of an unstable intermediate which is made by one light step and must be further processed in another light reaction.

Optimal quantum yield

The data of Fig. 6 showed that the rate constant of photoactivation is proportional to intensity only over a relatively narrow range of rates of quantum absorption. In this range of intensity quanta arrive in rapid enough succession so that the decay of unstable intermediate(s) is of little consequence and slow enough so that the rate limiting step(s) does not interfere. Consequently, over a $\leqslant 5$-fold intensity range the rate of the process is exclusively determined by intensity and the $I \times t$ relation is valid, *i.e.* the amount of conversion is proportional to the number of quanta absorbed regardless of the rate of absorption and the quantum yield is optimal. Accordingly, with minimum assumptions, the minimum number of quanta for conversion of an inactive to an active O_2-yielding center can be ascertained.

Data of Figs. 7A and B show a half-time of photoactivation of about 23 sec for a rate of quantum absorption of 14 neinsteins/min by 7 nmoles Chl. This corresponds to a total absorption of 0.77 quantum per Chl *a* to convert one-half of the original inactive O_2 centers to active O_2 evolving centers. Assuming a concentration of System II traps of 1/300 Chl and that one-half of the quanta (620 nm) are directed to System II, we calculate, without correction of R_i to R_0, that about 200 quanta per System II are required for converting an inactive O_2 center to an active one.

Determination of the amount of functional Mn per System II has yielded values of 6–8 Mn for Scenedesmus[2,3] and 5–6 Mn for spinach chloroplasts[1]. Similar values (6–12 Mn per System II) were obtained for Anacystis from attempts to titrate the minimal amount of Mn required for photoactivation[8]. However, previous data[1,4]

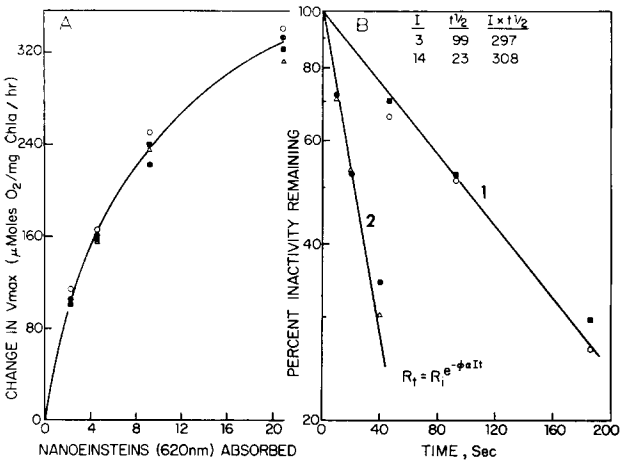

Fig. 7. Optimal quantum yield of photoactivation. Mn-deficient cells (3.9 μg Chl per ml) were suspended in growth medium containing $1.8 \cdot 10^{-5}$ M $MnCl_2$ and gassed in darkness with water saturated 5% CO_2 in air for 2 h. Aliquots (1.8 ml) then were preilluminated with 620-nm light for times indicated on the ordinate of Fig. 7B. For the two separate experiments shown, the rates of quantum absorption corresponded to 3 (open circles, closed squares) and 14 (open triangles, closed circles) neinsteins/min. The "base rates" of the cells used in the two experiments shown were essentially equivalent (150 μmoles O_2 per mg Chl per h).

indicate that only 4 Mn per System II of the heterogeneous Mn pool of System II is intimately associated with the O_2 yielding center. With the assumptions that only the Mn associated with the O_2 center is involved in photoactivation and that each of these Mn undergoes a 2-equivalent photochemical oxidation, we calculate from the data of Figs. 7A, 7B, that about 25 quanta need be absorbed into each System II unit for the incorporation of each Mn associated with the O_2 center.

The experiments reported here, were spread-out over a course of several years involving many different cultures. Though many of these cultures had equivalent R_i values and all yielded evidence for a multi-quantum process for photoactivation, we occasionally observed yields from a given illumination regime which were 2–3 times the yields routinely observed. This variable has not been resolved. In any event, photoactivation is a low quantum yield process.

Effectiveness spectrum of photoactivation

Knowledge of the kinetic order and the rate-intensity relation (Fig. 6, inset) of photoactivation allow the unambiguous determination of the effectiveness spectrum. In the experiments of Fig. 8 cell concentration was adjusted to yield equivalent absorption at each wavelength. The intensity of 620 nm light was adjusted to yield a maximal rate of photoactivation but still within the intensity range where the rate was proportional to intensity. The intensities of other monochromatic luminating beams were adjusted to yield rates of quantum absorption equivalent

to that obtained with the 620-nm beam. Similar results were obtained in experiments where the intensities of monochromatic illuminating beams (620–700 nm) were adjusted to yield equivalent rate constants of photoactivation in the linear region of the intensity curve. A plot of the observed relative quantum yield values *vs.* wavelength is given in Fig. 8, Curve 3.

Fig. 8 also illustrates the quantum yield profiles for O_2 evolution by Mn-sufficient, and Mn-deficient Anacystis (Curves 1 and 2, respectively). The low efficiency of the deficient cells precluded measurements at wavelengths greater than 650 nm.

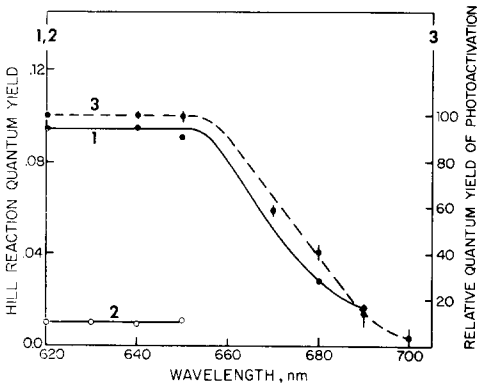

Fig. 8. Wavelength dependency of O_2 evolution and photoactivation of Anacystis. Curves 1 and 2 describe the effect of wavelength on the quantum yield of O_2 evolution of Mn-sufficient and deficient Anacystis cells, respectively. Values on the ordinate for Curves 1 and 2 are expressed in terms of moles O_2 per mole absorbed quanta. Curve 3 shows the effect of wavelength on the relative quantum yield of photoactivation of Mn-deficient cells. "Base rates" (v_{max}) for the Mn-sufficient and -deficient cells were 610 and 100 μmoles O_2 per mg Chl per h. For other details see text and METHODS.

With either Mn-sufficient Anacystis cells, the efficiency of the O_2 evolution was relatively constant between 620 and 650 nm (predominately System II) despite a 9.5-fold difference in the absolute yield values.

Comparison of Curves 3 (photoactivation) and 1 (O_2 evolution) reveals that the quantum yield profile of photoactivation closely resembles that of O_2 evolution. Both show a flat region between 620 and 650 nm where quanta are directed primarily to System II and both show a "red-drop" at wavelengths $>$ 650 nm. At 680 and 700 nm the yields are respectively 40 and 4 % of the yield at 620 nm. Previously GERHARDT AND WIESSNER[19] reported that 700 nm light was totally ineffective for photoactivation of Anacystis. We suspect that the rate–intensity relation of Fig. 6 complicated their experiments.

We made an additional measurement in blue light (447 nm) and found the yield to be 42.5 % of that at 620 nm. This value is in agreement with earlier reports of the efficiency spectrum of photosynthesis[15]. It allows us to exclude any special "blue-light" effect which might be sensitized by pigments other than photosynthetic ones. We conclude that the quantum yield profiles of photosynthetic O_2 evolution and photoactivation are nearly identical and that the two processes are sensitized by the pigment system of System II.

Effect of flash intensity; photoactivation occurs via the reaction center of System II

In the previous section, we showed that photoactivation was sensitized by the pigments of System II. The question then arises whether these pigments cooperate in "units" for photoactivation; *i.e.* whether the primary conversion is carried out by the quantum trapping centers of System II. This appears to be a reasonable hypothesis since earlier work[1,6] indicated that Mn deficiency does not greatly impair the primary photochemical charge separation by System II.

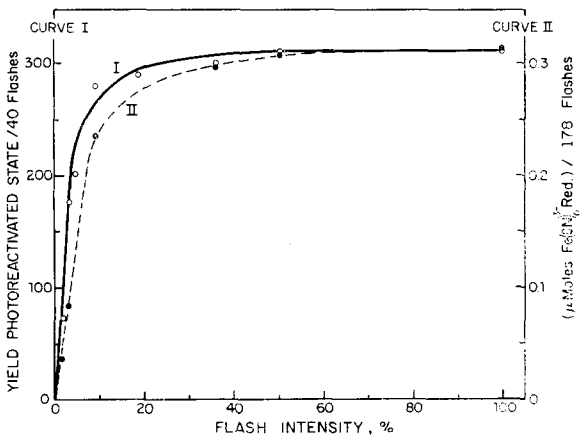

Fig. 9. Comparison of the effect of flash intensity on photoactivation of Anacystis cells *versus* O_2 evolution by spinach chloroplasts. In these experiments cell and chloroplast concentrations were adjusted so that the respective absorptions at 620 and 650 nm were identical. The chloroplasts used in these experiments showed a quantum requirement (650 nm) of 16 $h\nu/O_2$ with $Fe(CN)_6^{3-}$ as electron acceptor and a yield of 1 O_2/2800 Chl_{total} with saturating flashes of 50 msec periodicity. This periodicity yielded 80% of optimal flash yield of O_2. $Fe(CN)_6^{4-}$ was determined with the bathochrome reagent[16]. The reaction mixture (1.5 ml) contained in μmoles: Tricine–NaOH, pH 7.5, 75; $Fe(CN)_6^{3-}$, 2.4; methylamine, 45; and chloroplasts equivalent to 37 μg Chl. A total of 1200 flashes was used. For photoactivation the flash periodicity was 0.6 sec followed (25 msec) by a delay flash (see Fig. 10). The units on the abscissa for Curve I are expressed in μmoles O_2 per mg Chl per h per 40 flashes total. "Base rate" of original cells was 180 μmoles O_2 per mg Chl per h.

In the experiments of Fig. 9 the effect of flash intensity upon yield of photoactivation of Anacyctis cells and yields of O_2 ($Fe(CN)_6^{3-}$ reduction) of spinach chloroplasts is compared. Anacystis cells could not be used in both experiments because the endogenous reductants of the cells yielded high levels of reduced $Fe(CN)_6^{3-}$ in dark controls. Cell and chloroplast concentrations were adjusted so that the respective absorptions at 620 and 650 nm were identical.

The flash regime used with spinach chloroplasts (Curve II, Fig. 9) was close to optimal for O_2 evolution (50 msec spacing between flashes yielded 80 % of the maximum flash yield). These chloroplasts showed a quantum requirement at 650 nm of 16 $h\nu/O_2$ with $Fe(CN)_6^{3-}$ as the electron acceptor and a yield of 1 O_2/2800 Chl with saturating flashes. As shown by Curve II, Fig. 9, half the maximal yield of O_2 was obtained with chloroplasts with 5 % of maximal flash intensity.

In the experiment of Curve I, Fig. 9, photoactivation of Mn-deficient Anacystis cells was measured using a flash regime which was optimal for this process (see later section). Photoactivation was half-maximal at 3 % of the maximal flash intensity.

Although the comparison made here is only approximate, the agreement (within a factor of 2) between the two curves with respect to flash intensity shows that the two different processes have similar photochemical cross-sections. Photoactivation, like O_2 evolution, is therefore sensitized by "units" of a few hundred collaborating pigments.

Analysis of dark steps using flashing light

In previous sections, it was shown that photoactivation occurs with flashing light. This observation permitted an analysis of the dark steps implicated by the results in Fig. 6. Curve 1 (Fig. 10) shows the yields induced by sequences of brief saturating flashes separated by different dark times. The number of flashes presented in such experiments was kept minimal such that a maximum of 20–30 % conversion of inactive centers was obtained. Accordingly, we viewed as much as possible the initial, relatively linear part of the restoration curve (see Figs. 3 and 5).

The data show that with increasing dark time the yield of O_2 evolving capacity increased from a very low value to a maximum at a dark interval of 0.6–0.8 sec. With further increase of the spacing, the yield declined so that with dark time of 8 sec only a small, though finite, amount (4 units, corresponding to 0.74 % of the maximum possible number of units of the experiment of Fig. 10) of activation is obtained.

Curve 1 (Fig. 10) reflects the light curve of Fig. 6 and confirms the conclusions made from that experiment. When the flashes are spaced too far apart (which is essentially equivalent to the continuous light intensity being too weak), no activation occurs. This result implies that more than one photoevent is needed and that the effect of the first quantum event disappears. From the descending portion of Curve 1 (Fig. 10) a decay half-time of 1.6 sec was calculated while from Fig. 6 we estimated about 2 sec. The ascending portion of Curve 1 (Fig. 10) shows a half-rise time of 0.2 sec, a value similar to the estimated rate limitation of about 0.1 sec in the experiment of Fig. 6.

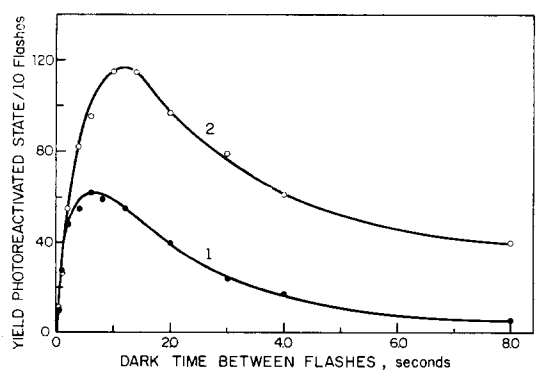

Fig. 10. Effect of dark time between flashes on the yield of flash induced photoactivation. The units of yield of the photoactivated state on the ordinate are expressed in μmoles O_2 per mg Chl per h. Curve 1 shows the yields induced by 10 saturating flashes separated by the dark times given on the abscissa. Curve 2 was obtained similarly except paired flashes (Δt invariant of 15 msec for the delay flash) was used. For comparative purposes the yields for Curve 2 are expressed in units per 10 flashes of variant dark time spacing. Original "base rate" and fully activated rate (R_{max}) were 282 and 820 μmoles O_2 per mg Chl per h, respectively.

Each point in Fig. 10 is the cumulative effect of many flashes. We felt that easier interpretable results might be obtained by separating the fast and the slow steps, *i.e.* by using flash regimes in which a long spacing was alternated with a short one. Fig. 11 shows an experiment in which sequences of flash pairs were used. The invariant dark time between pairs was 2 sec.

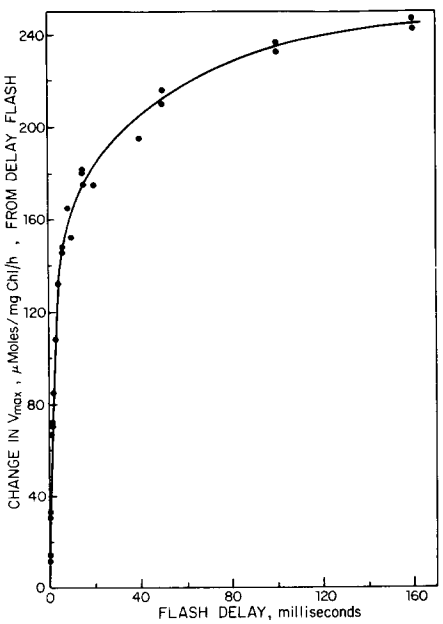

Fig. 11. Effect of delay flash on yield of flash induced photoactivation as affected by the dark interval between the single and paired flash. The difference in yield between single and paired flashes is plotted on the ordinate *versus* the dark time between paired flashes. For each sample, 20 single flashes of 2 sec periodicity were used. A second flash, delayed in the time given on the abscissa, was superimposed on the first flash periodicity to yield paired flashes. Each point was obtained with a new sample. With 150 msec spacing of the delay flash, the yields obtained with the delay flashes were about 3-fold greater than yields obtained with the single flashes of 2 sec periodicity. The "base' and R_{max} rates of the cells were 361 and 1020 μmoles O_2 per mg Chl per h. After 20 flashes ($\Delta t = 0$) the rate was 503 μmoles O_2 per mg Chl per h.

Separation of the two flashes of the pair (Δt) was varied form 0 (single flash) to 150 msec. Compared to that of the single flash ($\Delta t = 0$) the yield of the pairs ($\Delta t = 150$ msec) was 3.1-fold higher at a 2-sec invariant dark time between flash pairs. These data reveal another reaction, much shorter than the two others already described.

This same relatively rapid relaxation is seen in Curve 2 of Fig. 10. These data were obtained in the same way as those of Curve 1, the difference being that instead of single flashes, flash pairs were given (spacing of the pair invariant, 15 msec). While compared to the single flashes, the pairs had little effect in the region of short dark times (< 0.2 sec), the yield was about doubled at t_d values of 0.5–1 sec and increased up to 10-fold with the longest (8 sec) dark time. At this longest dark time the yield from the paired flashes is approximately equal to the maximal yield obtained with optimum spacing of single flashes.

DISCUSSION

We define Mn photoactivation as that process in which quantum events lead to the incorporation of Mn into the O_2 evolving center of Photosystem II and to its capacity to evolve O_2. This definition is based upon the following observations: (1) The primary photochemical charge separation by the System II trap is essentially independent of the reactions associated with O_2 evolution[1,5,6,18]. (2) O_2 evolution requires besides a functional System II trap, an active Mn catalyst. (3) During photoactivation light is required for the incorporation of Mn^{2+} into the O_2-evolving enzyme which results in its capacity to evolve O_2 (to be published).

In the studies on the kinetics of Mn photoactivation reported here, we have used the blue-green alga *Anacystis nidulans*. However, this photoactivation of the O_2-evolving enzyme is not unique to this organism and has been observed in several green algae[3,10] as well as spinach plants[17]. We therefore believe this process occurs generally in all O_2-evolving tissue. We might extrapolate one step further and advance the hypothesis that this photoactivation occurs also in *de novo* synthesis of photosynthetic tissue. By varying the concentration of inactive Mn catalyst either directly by growth deficiency or by NH_2OH extraction[1,4], the rate of photoactivation proved to be proportional to the number of inactive O_2-evolving centers. Thus, under any given illumination regime, the rate of conversion of inactive to active O_2-evolving centers is $-dc/dt = kc$, where c is the concentration of inactive O_2-evolving centers. This implies that each inactive O_2 center is restored by its own quantum trapping center, *i.e.* interactions between neighboring active and inactive centers are small or absent.

The evidence, presented here or previously[3,8,9,19], indicates that Mn photoactivation is sensitized by Photosystem II exclusively: (1) the quantum yield profiles for Mn photoactivation and O_2 evolution are equivalent; (2) the photochemical cross-section (the size of the photosynthetic unit) of both processes is essentially identical; (3) Mn photoactivation and O_2 evolution are equally sensitive to 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and (4) Mn activation is observed in mutant No. 8 of Scenedesmus[20], an organism devoid of Photosystem I (unpublished data).

Since the process is not inhibited by uncouplers of photophosphorylation[8,9], it appears that the minimal requirements for Mn photoactivation are: (1) a functional System II trapping center; (2) Mn^{2+}; and (3) an electron transport chain, independent of Photosystem I and energy coupling via phosphorylation.

To account for the observed kinetics we need to assume minimally the involvement of two dark reaction steps, two sequential photo steps, and that one photochemically formed intermediate has limited stability.

A model which satisfies the presented data will be described in detail in a forthcoming papaer.

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