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Studies on the Photoactivation of the Water-Oxidizing Enzyme[1]

I. PROCESSES LIMITING PHOTOACTIVATION IN HYDROXYLAMINE-EXTRACTED LEAF SEGMENTS

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ABSTRACT

In weak yet optimal light intensity, complete photoactivation of the water-oxidizing enzyme in NH_2OH-extracted wheat (*Triticum aestivum*, var Oasis) leaf segments could be obtained only after long dark preincubation. Photoactivation was not affected by ethylenediaminetetraacetate or inhibitors of photophosphorylation and protein synthesis, but was partially inhibited by a divalent cation ionophore. Complete photoactivation required ligation of ~4 Mn by the water oxidizing enzyme.

Without dark preincubation, photosystem II (PSII) was susceptible to weak light photoinhibition resulting in: (a) 50% maximum decrease in photooxidation of artificial electron donors by PSII: (b) increased times for the variable fluorescence rise (with 3-(3,4-dichlorophenyl)-1,1-dimethyl urea): (c) abolishment of photoactivation: and (d) the imposition of sensitivity to inhibitors of photophosphorylation and 70S but not 80S protein synthesis on subsequent light-dependent recovery from photoinhibition and recovery of O_2 evolution. Decrease in susceptibility to photoinhibition and increase in rates of photoactivation resulting from dark preincubations proved closely correlated. Neither protein synthesis nor increases in abundances of thylakoid Mn^{2+} and Ca^{2+} were required for escape from photoinhibition. However, photoactivation of the water-oxidizing enzyme in NH_2OH-extracted *Chlamydomonas* occurred in absence of dark preincubation and protein synthesis. Results are discussed in the context of disassembly/reassembly/resynthesis of specific PSII polypeptides.

The active form of the water-oxidizing S-State complex of PSII is a Mn-containing enzyme (reviewed in 2, 7, 42, 46). The early data (42) indicated that such Mn occurred as a tetra-polynuclear complex, a result subsequently confirmed from studies either with chloroplasts (3, 18, 59) or more recently with Triton-prepared O_2 evolving PSII membranes (6). Some results (13, 25, 55), however, argue for a binuclear Mn complex within the water-oxidizing S-State enzyme.

Analysis of the interrelationship between O_2 evolving capacity, PSII polypeptides, and abundances and/or stability of the Mn complex have been made using specific algal mutants (6, 33, 47) and Triton derived O_2 evolving membranes (6, 26, 37). These various approaches have yielded the suggestive general conclusion that the active O_2 evolving Mn complex is somehow ligated and/or shielded by the 17-, 23-, 33-, and 34-kD polypeptides (1, 6, 33, 37). Though the actual topography of the water oxidizing Mn-S-State enzyme remains unknown, studies with NH_2OH, derivatives of NH_2OH, and chemical analogs of NH_2OH tend to suggest that the Mn-S-State enzyme is cryptic (6, 42, 43), possibly buried within the thylakoid membrane. This idea is reinforced from the studies showing the existence of latent, metastable proton pools in PSII (23, 28, 52).

Irrespective of the controversy over the number of Mn atoms in the active S-State enzyme complex, the functions of the 17-, 23-, 33-, and 34-kD PSII polypeptides and the topography of the enzyme complex, it seems clear that the active Mn-S-State water oxidizing complex is formed or assembled *in vivo* only via the photoactivation process (9–11, 22, 39, 40). This process has been defined (42) as a multiquantum process driven by PSII quantum events leading to Mn ligation into the inactive apo-S-State complex with the conversion of the inactive complex into an active water oxidizing S-State complex.

This multiquantum process has been observed with Mn-deficient algae made Mn-sufficient (9), NH_2OH extracted algae (10), dark grown *Chlorella* capable of synthesis in darkness of the pigments of photosynthesis, PSI and PSII trapping centers and electron transport components (11), wheat leaves greened under widely spaced flash regimes leading to the synthesis of the pigments of photosynthesis, PSI and PSII trapping centers and electron transport components but not the active water oxidizing S-State complex (22), and Tris-extracted spinach chloroplasts reactivated by $DCIPH_2$[3] washings (56).

Additionally, light requirements for the appearance of V_{O_2} have been noted for Mn-deficient higher plants made Mn-sufficient (20), spruce needles greened in darkness (38), some cold sensitive higher plants following cold stress regimes (31, 34), Tris-acetone extracted spinach chloroplasts (57), and intact chloroplasts from wheat leaves greened under widely spaced flashes (39, 40). It is not at all clear that the same biochemical process(es) is involved in all these instances where light requirements have been demonstrated for the appearance of V_{O_2} capacity.

For example, the multiquantum process leading to Mn ligation into the inactive apo-S-State complex (photoactivation) has been shown in algae to have minimum requirements (42): (a) functional PSII trapping center; (b) Mn^{2+}; and (c) an electron transport chain independent of PSI and energy coupling via phosphorylation. Moreover, in algae, photoactivation is independent of protein synthesis requirements. Similar minimum require-

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[3] Abbreviations: $DCIPH_2$, reduced 2,6-dichlorophenolindophenol; V_{O_2}, rate of O_2 evolution; TAP, Tris-acetate-phosphate medium; CAP, chloramphenicol; CH, cycloheximide; TN, 0.02 M Na-Tricine, 1 mM NaCl, pH 7.5; SHM, 0.35 M sucrose, 0.025 M Na-Hepes, 2 mM $MgCl_2$, pH 7.6; Z, secondary electron donor to P680 of PSII; P680, reaction center of PSII; SHN, 0.35 M sucrose, 0.025 M Na-Hepes, 0.1 M NaCl, pH 7.6.

ments have been noted for the photoactivation process in intact chloroplasts prepared from wheat leaves grown under intermittent flash illumination (39, 40). However, these studies yielded evidence suggesting a requirement of some stromal factor reduced by PSI photoreactions and a requirement of Ca^{2+} in addition to Mn^{2+} (39, 40).

On the other hand, the studies of a photoreactivation of V_{O_2} in broken chloroplasts (56–58) previously subjected to treatments rather specifically inactivating V_{O_2} have yielded evidence suggesting the following requirements: (a) prewashing with $DCIPH_2$; (b) Mn^{2+}, Ca^{2+}, DTT, and BSA or combinations thereof depending on methodology of inactivation of V_{O_2}; and (c) photophosphorylation.

Such contrasting requirements for the light-dependent recovery of V_{O_2} in the various systems, our inability to obtain significant yields of the photoactivation as reported by Yamashita and Tomita (57, 58), and the generally low yields of photoactivation in isolated chloroplasts (39, 40) compared to algal cells (9–11) prompted these studies in attempts to gain additional insights into the specifics of the photoactivation process itself and to identify factors that have limited our observation of the photoactivation process in *in vitro* systems over the years. Here, we report on studies made with NH_2OH-extracted wheat leaf segments and *Chlamydomonas*. Previous studies have shown that photoactivation occurs in several different NH_2OH extracted algae (10).

MATERIALS AND METHODS

Photoactivation of V_{O_2} and Measurements of Protein Synthesis in *Chlamydomonas*. *Chlamydomonas reinhardi* (UTEX 89) was grown on TAP medium (19), harvested by centrifugation after 48 h growth, and then resuspended in TAP medium to give about 1 mg Chl/ml. Cells (200 μg Chl/ml) were extracted with 10 mM NH_2OH for 1 min in darkness then diluted 27-fold with 20 mM Na-phosphate buffer (pH 6.0) before collection of cells by centrifugation. The cells then were washed (~60 μg Chl/ml) once with 20 mM Na-phosphate buffer (pH 6.0) before resuspension (~1 mg Chl/ml) in TAP medium. Preliminary experiments established that this NH_2OH extraction procedure sufficed to inactivate V_{O_2} by ≥90% but did not significantly affect rates of [^{3}H]acetate incorporation into protein by extracted cells compared to control unextracted cells. Longer exposures (10 min) to NH_2OH (5 to 10 mM) caused ~25% inhibition of protein synthesis but did not affect the time-course of photoactivation.

Unextracted or NH_2OH extracted cells (300 μg Chl in 3 ml TAP medium contained in 25-ml Erlenmeyer flasks) were incubated on a thermoregulated (19–20°C) shaker bath equipped with 40-W, 115-V tungsten lamps positioned directly below the samples. A yellow cinemoid filter (Kliegl No. 46) was used as a light filter. Light intensity was varied either with wire-screen neutral density filters or with a variable voltage regulator. Unless otherwise noted, we used a light intensity of 65 $\mu E/m^2 \cdot s$, measured at the base of the flasks. This intensity was sufficient to just saturate the photoactivation process.

Measurements of photoactivation and [^{3}H]acetate incorporation into protein were done in parallel. Cell suspensions were preequilibrated for 1 h in darkness in the absence or presence of CAP and/or CH at 500 μg/ml each; then 12.5 μCi (140 mCi/mmol) of Na[^{3}H]acetate were added followed by illumination for designated times. Following illumination, aliquots of the suspensions were assayed for V_{O_2} at conditions that excluded photoactivation during assay of V_{O_2} (9) and for radioactivity incorporated into protein.

[^{3}H]Acetate incorporation into protein was determined essentially by the procedure of Mans and Novelli (30). At times indicated, 1.5 ml of 30% (w/v) TCA were added to yield a final concentration of 10% (w/v) TCA. After incubation at 4°C for 10 min, the samples were vigorously mixed by vortexing, and 100-μl aliquots of the suspensions were applied to 2.3-cm discs of Whatman 3MM. The disks were dried and washed (30) before measurements of radioactivity. Less than 5% variance was encountered between replicates, and zero time controls showed ≤10 cpm over background.

Preparation of Leaf Segments and Extraction with Hydroxylamine. Wheat (*Triticum aestivum*, var Oasis) leaves were obtained from seedlings (7–9 d old from the time of planting) cultured in the greenhouse in vermiculite on half-strength nutrient solution (50). The tip and base (~15 mm each) of the leaves were discarded, then the remaining leaf tissue (~30 g) was cut transversely with a scalpel into 1- to 2-mm segments. The segments were placed in a 250-ml beaker containing 100 ml cold distilled H_2O, the water was drained with the aid of a wire screen, then following a similar wash, the drained segments were gently blotted between Whatman No. 1 filter paper and weighed.

The blotted segments (25 g/150 ml TN buffer containing 1.25 mM NH_2OH) were extracted with gentle stirring in a 400-ml beaker for 15 min at room temperature in total darkness. Following drainage of this buffer, the segments were washed 3 times with stirring (2 min) in 150 ml TN buffer then similarly for 10 min before again collecting and blotting of the segments. Deproteinized homogenates of the NH_2OH extracted segments showed less than 10 nmol NH_2OH/g segments as determined by a sensitive procedure (16). Since additional washings of the NH_2OH extracted segments altered neither the time courses nor extents of subsequent leaf segment responses to illumination regimes, we assumed that the low (~10 μM) residual NH_2OH concentration in the segments was not inhibitory. With dark adapted *Chlorella*, 10 μM NH_2OH suffices to delay $Y_{O_2}^{max}$ in a sequence of flashes by two flashes with <10% inactivation of the Mn-S-State water oxidizing complex (4). Preliminary experiments showed: (a) this extraction procedure yielded ≥90% inactivation of V_{O_2} of chloroplasts isolated from the NH_2OH extracted segments; (b) vacuum infiltration of segments with NH_2OH did not increase the extent of inactivation of V_{O_2}; (c) the V_{O_2} of chloroplasts from segments subjected to the extraction procedure but in the absence of NH_2OH was essentially equivalent to V_{O_2} of chloroplasts prepared directly from leaves, and; (d) the pH dependency of NH_2OH-induced inactivation of V_{O_2} in leaf segments was similar to isolated chloroplasts (8).

Photoactivation of NH_2OH Extracted Leaf Segments. Blotted leaf segments (1.5 g) were suspended routinely in 10 ml Preincubation Buffer (TN buffer containing 0.5% [w/v] glucose, 50 μM $MnCl_2$, and 200 μM $CaCl_2$) in 50-ml beakers. Other additions are noted in the figure legends. The segments were vacuum infiltrated (12 p.s.i.) twice at room temperature before incubation at 19° to 20°C on the illuminated shaker bath. The light intensity (30 $\mu E/m^2 \cdot s$, ~80% absorption by the leaf segment suspension) was determined to be optimal for photoactivation of extracted, dark incubated leaf segments. Light was excluded from dark incubated samples with aluminum foil. Following incubations, the leaf segments were recovered by draining for subsequent isolation of chloroplasts.

Chloroplast Preparation. Drained leaf segments (1.5 g) were suspended in 15 ml SHM buffer containing 0.1% BSA and 2 mM Na-ascorbate, homogenized for 6 s at full voltage with a Polytron homogenizer (Brinkmann Co.), then filtered through eight layers of cheesecloth before centrifugation at 2900*g* for 4.5 min. The resulting pellet was suspended in 15 ml SHM buffer and centrifuged at 1200*g* for 20 s before recovery of chloroplasts at 2900*g* for 4.5 min. The chloroplasts were resuspended in 0.2 ml SHM to yield 500 to 1000 μg Chl/ml. All operations were made at 0° to 4°C.

When Mn and Ca chloroplast abundances were to be determined, chloroplasts were isolated in SHN buffer containing 1 mM EDTA then finally resuspended in SH buffer (SHM with

omission of $MgCl_2$). This modification did not affect V_{O_2} values but eliminated high and wildly fluctuating Mn and Ca abundances frequently observed with SHM isolated chloroplasts.

Rate Measurements of V_{O_2} and Donor Photooxidations. Rate measurements were made polarographically (8) in saturating light unless otherwise noted. Neutral density filters were used to vary light intensities.

The reaction mixture for assay of V_{O_2} of *Chlamydomonas* has been described (10): the reaction mixture for assay of chloroplasts contained 1 mM FeCN, 30 mM methylamine in 0.4 M sucrose, 40 mM Tricine-NaOH (pH 7.5). PSI donor photooxidations were made in a reaction mixture containing 30 mM methylamine, 100 μM methylviologen, 400 μM KCN, 50 μM DCIP, 10 mM Na-ascorbate, and 10 μm DCMU in 0.4 M sucrose, 40 mM Tricine-NaOH (pH 7.5). PSII donor photooxidations were made similarly except for omissions of Na-ascorbate, DCIP, and DCMU and where indicated the addition of 10 mM $MnCl_2$ or 20 mM NH_2OH.

Fluorescence Measurements. Fluorescence measurements were made essentially as described previously (29). The chloroplasts were suspended in 0.4 M sucrose, 40 mM Tricine-NaOH (pH 8.0) containing 6 mM $MgCl_2$ and 10 μM DCMU, and where indicated, 10 mM NH_2OH.

Other Determinations. All glassware used in Mn and Ca determinations was washed in a 1/1 (v/v) mixture of 1 N HNO_3 and 1 N HCl and rinsed with glass distilled H_2O. Samples (standards and chloroplasts equivalent to 50 to 500 μg Chl, depending on the metal analyzed and its abundance) were digested in test tubes covered with watch glasses with 0.3 ml of a mixture of 9 volumes of concentrated HNO_3 per 1 volume of 70% (v/v) $HClO_4$ for 2 h at 90°C then 3 h at 198°C, and finally 2 h at 220°C. Watch glasses were removed after 2 h digestion at 90°C. After complete digestion and cooling, 2.5 ml 4 mM HNO_3 containing 10 mM HCl were added to the tubes: the tube-tops were covered with parafilm; then the tubes were heated for 12 h at 88°C. The sample and water rinses of each tube were combined, and the volume was adjusted to 5 ml with water then centrifuged to remove any precipitate before analyses. Flameless and flame atomic absorption were used for analysis of Mn and Ca, respectively. Chl determinations were made following extraction with 80% acetone. Light measurements were made with a LI-185B Quantum/Radiometer/Photometer (LI-COR, Inc./LI-COR, Ltd.) and the LI-1905B quantum sensor (400–700 nm light).

RESULTS

Time Courses of Light-Dependent Recovery of V_{O_2} of NH_2OH Extracted Wheat Leaf Segments: Effect(s) of Dark Preincubation. The effects of NH_2OH extraction and subsequent illumination regimes on PSII parameters of leaf segments were determined by assaying chloroplasts isolated from the variously incubated segments. Preliminary experiments showed that the NH_2OH extraction abolished V_{O_2} (≥90%) but did not affect either PSI or PSII artificial donor photooxidation activity and that long (≤12 h) dark preincubation of extracted segments even in the presence of 50 μM Mn^{2+} and 200 μM Ca^{2+} did not result in any increase of residual V_{O_2}. Moreover, V_{O_2} of unextracted segments remained essentially unchanged (<10% decrease) during such prolonged dark incubations. Such results are entirely analogous to previous results obtained with algae (8, 10, 11).

Figure 1 shows results of the effects of illumination on increase in V_{O_2} (ΔO_2/Chl·h) of NH_2OH extracted leaf segments either illuminated in the absence (curve 2) or presence of CAP (50 μg/ml) (curve 3) or after 8 h dark preincubation (curve 1). The data are a compilation of results obtained from a number of different experiments in which only small variation of ΔV_{O_2} was observed from experiment to experiment.

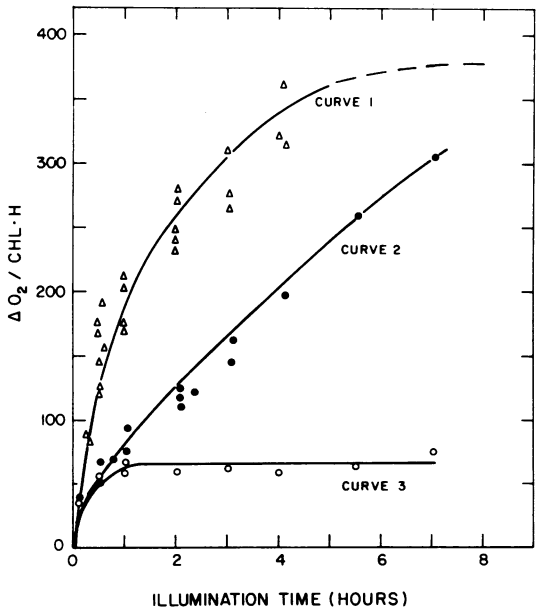

FIG. 1. Time courses of light-dependent recovery of V_{O_2} in NH_2OH extracted wheat leaf segments. Curve 1, dark preincubated (8 h); curve 2, non-dark preincubated; curve 3, non-dark preincubated illuminated in the presence of CAP (50 μg/ml). Typical initial V_{O_2} values for chloroplasts from nonilluminated NH_2OH extracted leaf segments were 30 to 50 O_2/Chl·h. The initial values did not change significantly during dark preincubations of segments in the presence or absence of CAP and CH. See text for other details.

Inspection of the data shows that dark preincubation (curve 1) resulted in a more rapid light-dependent increase of V_{O_2} relative to curves 2 and 3, except possibly during the initial times (10 min) of illumination, and also a more complete recovery of V_{O_2} during the course of illumination. The final levels of recovery of V_{O_2} (Δ~350 O_2/Chl·h plus initial V_{O_2} at zero time illumination) obtained with illumination of the dark preincubated segments were equivalent to V_{O_2} values obtained with unextracted, control leaf segments. The presence of high concentrations of CAP (500 μg/ml) and/or CH (1000 μg/ml) throughout the dark and light incubation regime affected neither the final attainable ΔV_{O_2} nor the time-course of recovery of V_{O_2} for the dark preincubated leaf segments. The monophasic exponential decrease of inactive S-State enzyme centers obtained on illumination of this process to translational inhibitors are characteristic of photoactivation (42).

Without dark preincubation of the segments, the recovery of V_{O_2} was still observed but with contrasting behaviors. As shown by curve 2, obtained with non-dark preincubated segments in the absence of CAP, the time course for recovery of V_{O_2} was distinctly biphasic showing a rapid phase followed by a slow nearly linear phase. Moreover, CAP addition to non-dark preincubated segments (curve 3) abolished the slow nearly linear phase of recovery of V_{O_2} but had no discernible effect on the rapid phase of recovery.

Figure 2 shows results of experiments obtained with non-dark preincubated segments that were illuminated for 4 h in the presence of several different translational inhibitors. This duration of illumination was sufficient to permit meaningful measurements of the CAP-sensitive phase of recovery of V_{O_2} of non-dark preincubated segments. The data show: (a) CAP concentrations of 5 and 50 μg/ml sufficed to yield half-maximal and maximal inhibition (85%), respectively, with no additional inhibition by a high CAP concentration (250 μg/ml); (b) an entirely similar maximum extent of inhibition was obtained with puromycin (500 μg/ml); and (c) CH was noninhibitory at the concen-

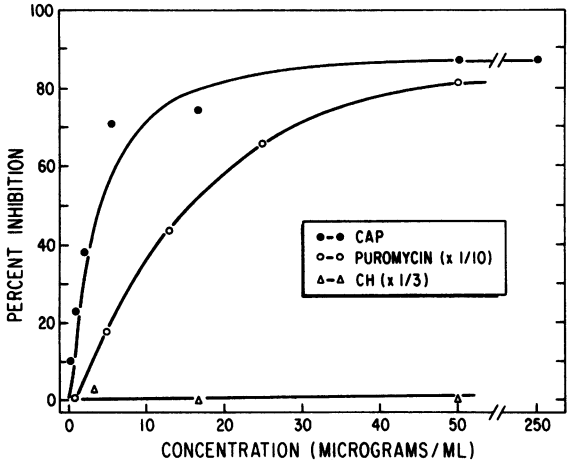

FIG. 2. Effects of CAP, PM, and CH on the light-dependent recovery of ΔV_{O_2} in NH_2OH extracted wheat leaf segments. The inhibitors were infiltrated just prior to a 4-h light incubation which, in the absence of inhibitors, yielded a V_{O_2} of 195 O_2/Chl·h.

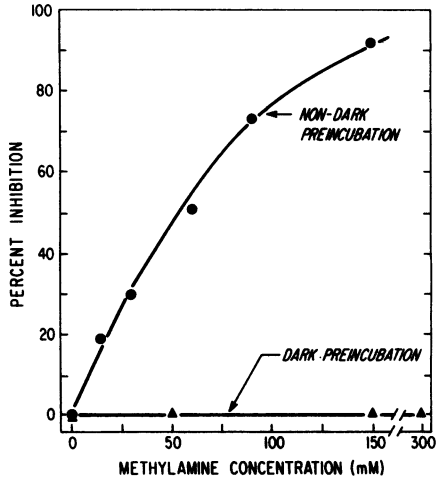

FIG. 3. Effect of methylamine on light dependent recovery of V_{O_2} in non-dark (direct illumination) and dark preincubated (8 h) leaf segments. Methylamine was infiltrated just prior to illumination. The illumination times for the non-dark and dark preincubated segments were 120 and 95 min, respectively. In the absence of methylamine, these illumination times yielded ΔV_{O_2} values of 107 and 171 O_2/Chl·h for the non-dark and dark preincubated segments, respectively.

trations shown in Figure 2 or even at 1000 μg/ml. Moreover, L-threo-CAP, the inactive isomer (14) of the D-threo-CAP used in the experiments of Figures 1 and 2, had no effect on the recovery of V_{O_2} of non-dark preincubated segments even at concentrations up to 1000 μg/ml. Thus, the effects of D-threo-CAP on recovery of V_{O_2} of non-dark preincubated segments cannot be attributed to nonspecific effects sometimes observed with this compound (15).

In experiments such as those of Figure 2 but with shorter illumination times, the maximum extent of inhibition of recovery of V_{O_2} in non-dark preincubated segments diminished with diminishing times of illumination, thus reflecting the small change (≤75 O_2/Chl·h) in V_{O_2} that can occur in non-dark preincubated segments in the absence of 70S protein synthesis. Similar small increases in V_{O_2} have been observed with photoactivation of V_{O_2} in intact chloroplasts from wheat leaves greened under widely spaced light flashes (39, 40) and in the $DCIPH_2$-induced and light-induced reactivation of Tris-extracted chloroplasts (56–58).

The data of Figures 1 and 2 suggested that rapid and complete recovery of V_{O_2} in NH_2OH extracted leaf segments is obtainable in absence of protein synthesis requirements (photoactivation) only following long dark preincubation of segments, but that without dark preincubation only a small fraction of the abundance of the NH_2OH inactivated S-State enzyme can be photoactivated without resynthesis of some polypeptide(s) on chloroplast 70S ribosomes. These interpretations implied that the weak light irradiation of non-dark preincubated NH_2OH segments possibly caused some photoinhibition thereby imposing protein synthesis requirements for appreciable recovery of V_{O_2}. The corollary hypothesis, namely, that dark preincubation eliminates the susceptibility to photoinhibition and eliminates requirements for protein synthesis, suggested that differential effects of uncouplers of photophosphorylation would be observed as with CAP for the recovery of V_{O_2} in non-dark *versus* dark preincubated segments.

Figure 3 records the effects of increasing concentrations of methylamine on the recovery of V_{O_2} of non-dark *versus* dark (8 h) preincubated NH_2OH extracted leaf segments. The data show that methylamine effectively inhibited (50 and 90% by 60 and 150 mM methylamine, respectively) the recovery of V_{O_2} of non-dark preincubated segments; however, no effect of methylamine, even at a 300 mM concentration, was observed on the light-dependent increase of V_{O_2} of the dark-preincubated NH_2OH extracted leaf segments. Such differential effects of an uncoupler

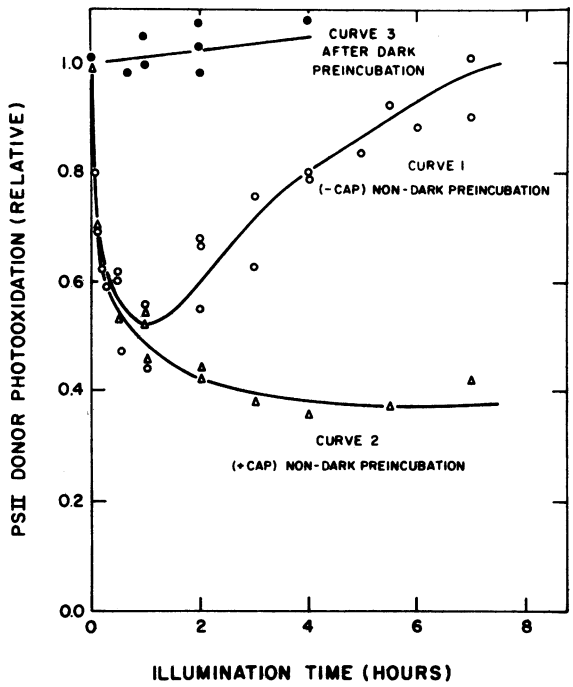

FIG. 4. Effects of illumination on relative PSII/NH_2OH photooxidation capacity of non-dark (curves 1 and 2) and dark (7 h) preincubated extracted leaf segments. Where added, CAP (50 μg/ml) was infiltrated just prior to illumination. The PSII/NH_2OH donor photooxidation rates, measured at quantum yield intensities, ranged only from 90 to 115 μeq/mg Chl·h for zero time controls in several different experiments and was unaffected by dark preincubations.

of photophosphorylation reinforce suppositions reached from data of Figures 1 and 2. We therefore sought direct evidence for the occurrence of photoinhibition processes in the non-dark preincubated leaf segments.

Figure 4 shows the effect(s) of the weak light illumination regime on PSII/NH_2OH donor photooxidation (5, 12) of chloroplasts from extracted leaf segments receiving either 7 h dark preincubation (curve 3) or no dark preincubation (curves 1 and

2) before illumination for times given on the abscissa. Curve 2 was obtained similar to curve 1 but in the presence of CAP. The extracted segments which had been preincubated in darkness showed no loss of the DCMU-sensitive PSII donor photooxidation capacity over 4 h illumination of the segments (curve 3, Fig. 4). In contrast, this activity decreased rather rapidly on illumination of the non-dark preincubated segments (curves 1 and 2, Fig. 4). These data suggest an exponential decay ($t_{½}$ ~2.5 min) but only to ~50% of the initial activity in the presence of CAP.

Neither higher light intensities (1200 $\mu E/m^2 \cdot s$) nor attempts to increase the uniformity of light absorption of the leaf segment suspensions (or use of Mn^{2+} as the donor in the assays) modified the rate or extent of decay of the PSII donor photooxidation activity. Apparently, the weak light intensity, optimal for photoactivation, of dark preincubated leaf segments also was optimal for photoinhibition of non-dark preincubated segments. Such results contrast to the high light photoinhibition effects obtained in the isolated chloroplasts in the presence of NH_2OH (8, 21). In these studies, total loss of PSII donor was reported (8, 21): Cyt b_{559} was converted to the very low redox species; and the Mn abundance of chloroplasts was depleted to less than the level obtained with dark NH_2OH extractions of the chloroplasts (21). The relevance of the direct inhibition of the Z-P680 locus by NH_2OH (12, 17) to the high light photoinhibition obtained in the presence of NH_2OH is not obvious.

As shown by curve 1 of Figure 4, continued weak light illumination of the photoinhibited non-dark preincubated leaf segments in the absence of CAP results in a slow but virtually complete recovery from the photoinhibited state. This recovery from the photoinhibited state proved light dependent. In many different experiments, less than 10% recovery of PSII donor photooxidation occurred during prolonged (≤8 h) dark incubation of previously photoinhibited segments.

We argue that the light-dependent, CAP-sensitive increase in V_{O_2} obtained with non-dark preincubated NH_2OH extracted segments (curve 2, Fig. 1) reflects initial recovery from the photoinhibited state followed by subsequent reactions of the photoactivation process since: (a) the time course for recovery from the photoinhibited state (curve 1, Fig. 4) is very similar to the recovery of V_{O_2} (curve 2, Fig. 1); (b) such time courses are appreciably slower than photoactivation (curve 1, Fig. 1) and; (c) the light dependent recovery from the photoinhibited state, but not photoactivation, is CAP sensitive. Apparently, the observed ~50% extent of photoinhibition is sufficient to completely abolish photoactivation.

For technical reasons, we routinely assayed photoinhibition by measurements of PSII donor photooxidations via PSI with viologen as the electron acceptor. Though none of the photoinhibition regimes used in these studies affected PSI donor photooxidations, conceivably the photoinhibitory effects limiting photoactivation might be a consequence of impairment of electron flow between PSII and I as has been described (27) for a high light photoinhibition at the secondary electron acceptor in PSII (Q_B) locus in photosynthetically competent *Chlamydomonas*.

However, as shown in Table I these deleterious effects on PSII donor photooxidation from weak light illumination of non-dark preincubated segments are manifested in measurements of variable fluorescence in the presence of DCMU. Table I records the half-times of the fluorescence rise of chloroplasts from extracted segments that had been subjected to the various incubation conditions yielding effects on PSII donor photooxidations. As indicated, the fluorescence rises were measured in the absence and presence of NH_2OH (10 mM) serving to act as an artificial electron donor to PSII and to inhibit charge recombination in PSII (5).

First, line 1 of Table I shows that the half-time of the fluorescence rise of chloroplasts from NH_2OH extracted, non-dark preincubated and nonilluminated segments (control) was not significantly diminished by addition of NH_2OH in the measurements. Second, lines 2 to 4, obtained with non-dark preincubated segments, show: (a) photoinhibition (45 min illumination) results in an ~15-fold and an ~4-fold increase in half-times measured in the absence and presence of NH_2OH, respectively; and (b) continued illumination (6 h) of the photoinhibited segments in the absence (line 3) but not in the presence of CAP (line 4) results in the recovery from photoinhibition as evidenced by the decrease of half-times to control values independently of NH_2OH addition to the assays. Third, lines 5 and 6 show that dark preincubation (6 h) preceding the 45-min illumination causing photoinhibition in non-dark preincubated segments essentially eliminated the susceptibility of NH_2OH extracted segments to photoinhibition. The data of Table I therefore delineate one locus of photoinhibition limiting photoactivation to the oxidizing side of PSII traps.

Table I. *Effects of Different Incubation Conditions of NH_2OH Extracted Leaf Segments on the Chl a Variable Fluorescence of DCMU-Inhibited Chloroplasts*

Where indicated, the concentrations of CAP and NH_2OH were 250 μg/ml and 10 mM, respectively. The control values were obtained with chloroplasts isolated directly from NH_2OH extracted leaf segments. None of the various incubations significantly affected the initial or maximal variable fluorescence levels. Routinely, F_{max}/F_i (maximum/invariable Chl *a* fluorescence yield) values of ~4.0 were obtained.

Incubation	Half-Time of Fluorescence Rise	
	Minus NH_2OH	Plus NH_2OH
	ms	
Control	115	100
Non-dark preincubated, illuminated 45 min	1720	395
Non-dark preincubated, illuminated 6 h	113	100
Non-dark preincubated, illuminated 6 h plus CAP	1195	380
Dark preincubated, 6 h	100	93
Dark preincubated, then illuminated 45 min	128	106

Dark Incubation Effects on PSII Donor Photooxidation and Photoactivation of V_{O_2}. In the experiments of Figure 5, we asked if decreased susceptibility to photoinhibition kinetically correlated with increased extents of photoactivation. Here, we varied the dark preincubation time of the NH_2OH extracted segments prior to illumination for a fixed time (60 min). This fixed illumination time was predicated on the data of Figure 4 showing maximum loss of PSII donor photooxidation capacity within this time and the data of Figure 1 showing a ~2.5-fold difference of ΔV_{O_2} between dark and non-dark preincubated segments. The obtained rates of PSII donor photooxidations were normalized to rates obtained with chloroplasts from non-dark preincubated, nonilluminated leaf segments. Thus, the ascending curve for PSII donor photooxidation capacity measures the time course of escape of PSII susceptibility to photoinhibition. The ΔV_{O_2} values obtained in such experiments were normalized to values obtained with segments given no dark preincubation; accordingly, we plot the relative increase of ΔV_{O_2} (photoactivation) resulting from the various intervals of dark preincubation. The results obtained show a slow sigmoidal escape of PSII traps from photoinhibition (triangles) with increasing dark preincubation times that is closely paralleled by increased photoactivation of the water oxidizing enzyme (circles). Such results were obtained routinely even when high concentrations of CAP (500 μg/ml) and CH (1000 μg/ml) were present throughout the dark and light incubations.

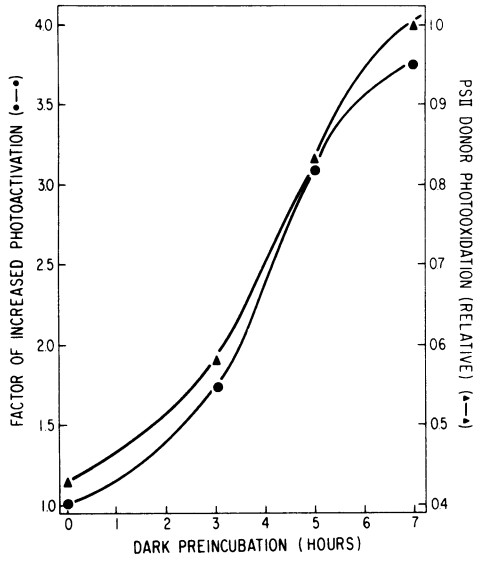

FIG. 5. Decrease of PSII susceptibility to photoinhibition and increase of yield of photoactivation of V_{O_2} with increasing duration of dark preincubation of NH_2OH extracted leaf segments. Following dark preincubations indicated on the abscissa, segments were illuminated for 60 min, then chloroplasts were isolated ("Materials and Methods") and assayed for both PSII/NH_2OH photooxidation and V_{O_2}. The obtained rates were normalized to a ΔV_{O_2} value (50 O_2/Chl·h) obtained with 60 min illumination of non-dark preincubated segments and to a PSII/NH_2OH photooxidation rate of 116 μeq/mg Chl·h obtained with non-dark preincubated nonilluminated segments. V_{O_2} measurements were made in saturating light; PSII/NH_2OH photooxidation was measured at a quantum yield intensity.

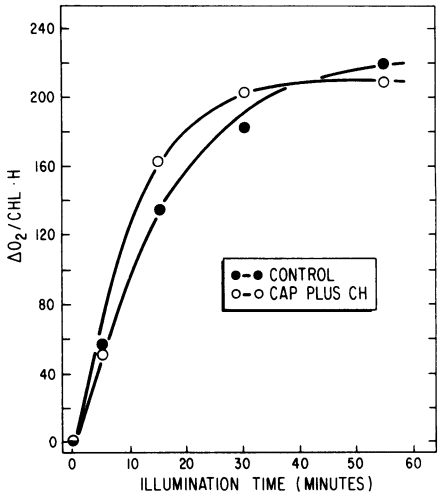

FIG. 6. Time-course of photoactivation of V_{O_2} with non-dark preincubated NH_2OH extracted *Chlamydomonas* in the absence and presence of CAP and CH. CAP (500 μg/ml) and CH (500 μg/ml) were added immediately after NH_2OH extraction and washing of cells to remove NH_2OH. The minimum elapsed time between NH_2OH extraction and the onset of the photoactivation regime was 75 min. The V_{O_2} values for unextracted and nonilluminated NH_2OH extracted cells were 217 and 6 O_2/Chl·h, respectively.

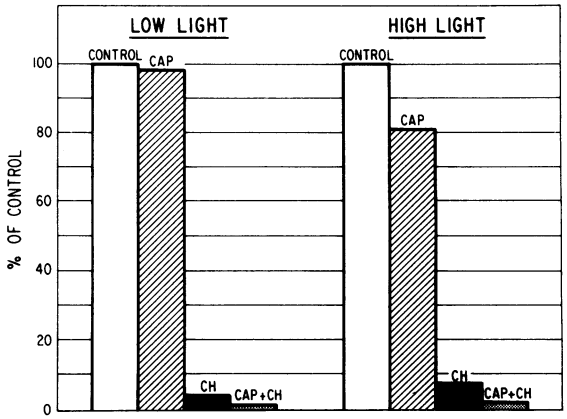

FIG. 7. Effects of CAP and CH on [^{3}H]acetate incorporation into protein by *Chlamydomonas* cells. The low and high light intensities were just saturating for photoactivation of V_{O_2} (extracted cells) and photosynthesis (unextracted cells), respectively. The data were obtained with unextracted cells illuminated for 60 min; however, extracted cells gave equivalent rates of protein synthesis in the absence of inhibitors. The uninhibited rates of protein synthesis (dpm/mg Chl·h) at low and high light were 4.88×10^5 and 4.58×10^5, respectively.

We conclude that PSII traps of NH_2OH extracted wheat leaf segments are highly susceptible to partial photoinhibition in very weak light, and that photoactivation of the water oxidizing enzyme does not occur when PSII traps are only partially (~50%) photoinhibited. However, dark preincubation even in the absence of protein synthesis, eliminates the susceptibility to photoinhibition thereby permitting photoactivation.

NH_2OH extracted algae presumably require neither dark preincubation nor protein synthesis for photoactivation (10). The results reported here prompted reexamination of the conclusions reached with NH_2OH extracted algae. Despite the absence of long dark preincubation comparable to that required with leaf segments, the data of Figure 6 show that rather rapid and complete photoactivation was obtained. Moreover, neither the time-course nor the final extent of recovery of V_{O_2} was diminished by additions of CAP and CH to the cells immediately after NH_2OH removal.

Figure 7 shows that the concentrations of CAP and CH used in the experiments of Figure 6 were sufficient to inhibit protein synthesis by ~98% in nonextracted cells at either high light intensity, assumed to saturate photosynthesis, or the low light intensity determined to be just saturating for photoactivation of V_{O_2}. Additionally, the observed absence of any significant effect of CAP on protein synthesis at the low light condition which yielded complete photoactivation of V_{O_2} again indicates that no 70S protein synthesis is required for photoactivation of V_{O_2} in the NH_2OH extracted, non-dark preincubated *Chlamydomonas* cells.

The results of Figures 6 and 7 therefore support previous conclusions (9–11) based only on indirect arguments. The contrasting dark preincubation requirements for observings photoactivation in NH_2OH extracted algae *versus* wheat leaf segments implies: (a) any necessary dark preincubation for *Chlamydomonas* is <75 min; or (b) notable differences exist in effects of NH_2OH on PSII in wheat leaf segments *versus Chlamydomonas* despite our attempts to minimize these differences.

In data not shown, we observed no recovery of V_{O_2} during prolonged (8 h) incubation of NH_2OH extracted *Chlamydomonas* cells in Mn-sufficient TAP medium. Such results and others (9–11) contradict the claim (51) that dark incubation of Mn-deficient *Chlamydomonas* cells made Mn-sufficient results in recovery of V_{O_2}.

Effects of NH_2OH Extraction and Dark Incubations on Microcompartmentation of Chloroplast Mn. Apparently, we could not duplicate with leaf segments the extraction condition used for *Chlamydomonas* permitting photoactivation without dark preincubation and without photoinhibition of PSII traps. This contrasting behavior was observed despite use of a wide range of

NH_2OH concentrations with both leaf segments and *Chlamydomonas*.

We sought explanations for the dark preincubation requirements for leaf segments and determined Mn and Ca abundances of chloroplasts from extracted leaf segments as affected by dark preincubation. Additionally, the effects of EDTA and/or A23187 on the light induced recovery of V_{O_2} in dark *versus* non-dark incubated NH_2OH extracted leaf segments were determined. These measurements were based on a number of observations (3, 35, 36, 45, 49, 53, 59) which can be interpreted to indicate that Mn^{2+} released from the S-State can be microcompartmentalized within an aqueous domain of the S-State enzyme. This microcompartment has been postulated (35, 36) to be a more restrictive diffusional barrier to Mn^{2+} than the thylakoid, the magnitude of the barrier perhaps dependent on the extent of perturbation from the thylakoids of specific polypeptide components. We surmised that such microcompartmentation related to observations (45) showing reactivity of Mn^{2+} derived from the S-State enzyme with lipophilic divalent cation ionophore A23187 (44) but not to EDTA. The reactivity of such derived Mn^{2+} with EDTA alone (3) can be interpreted to indicate a loss of microcompartmentation around the S-State apoenzyme. Since Mn^{2+} (9) and possibly Ca^{2+} (40) are specifically required for photoactivation of the S-State apoenzyme, their occurrence specifically within the microcompartment of the S-State apoenzyme *versus* within the thylakoid lumen might be an important determinant in the photoactivation process.

Figure 8A shows time-courses of depletion of S-State enzyme derived Mn^{2+} from carefully prepared wheat chloroplasts that were subjected to NH_2OH extraction then incubated in darkness in SHN buffer alone or containing either EDTA or EDTA plus A23187. These data show a slow depletion of the Mn^{2+} in the absence or presence of EDTA but a rapid depletion of the initial ~6.5 Mn/400 Chl to a base level of only ~0.5 Mn/400 Chl in the presence of A23187 and EDTA. On the other hand, either freezing (−80°C) or mild temperature (35°C) shock of such NH_2OH extracted SHN resuspended chloroplasts resulted in extensive depletion of Mn (to 1–2 Mn/400 Chl) on washing of the chloroplasts in SHN buffer containing only 1 mM EDTA.

Similar slow diffusion of Mn^{2+} from NH_2OH extracted chloroplasts even in the presence of EDTA has been reported previously (45, 48); additionally, mild procedures, *e.g.* shearing forces from resuspension and subsequent dilution of the NH_2OH extracted chloroplasts, were observed, however, to release the NH_2OH released Mn^{2+}. Similarly, Miller and Cox (36) have reported that heat (45°C) but not Tris or Zn^{2+} solubilization of Mn^{2+} from the S-State enzyme yields immediate release of Mn^{2+} into the suspension medium. Thus, the microcompartmentation around the S-State apoenzyme was not drastically perturbed in the NH_2OH extracted isolated chloroplasts. In contrast, the data of Figure 8B give evidence that NH_2OH extraction of leaf segments modified the microcompartmentation.

Note that the Mn^{2+} derived from the S-State enzyme decreased from an initial value of 5.3 Mn/400 Chl to ~3.0 and 1.0 Mn/400 Chl after 8 and 12 h incubation, respectively, in the absence of EDTA despite the presence of 50 μM Mn^{2+} in the preincubation buffer. Addition of EDTA to the NH_2OH extracted leaf segments resulted in more rapid and more extensive depletion of the Mn^{2+}. Only ~1.6 and ~0.5 Mn/500 Chl remained in the thylakoids after incubation of leaf segments with EDTA for 3.5 and 8 h, respectively. The dashed line of Figure 8B is a conservative estimate of the early time course of EDTA-enhanced depletion of Mn^{2+} from chloroplasts; however, we suspect even more rapid depletion based on results shown in Figure 9.

Here we show the effects of EDTA on the light-induced recovery of V_{O_2} of NH_2OH extracted segments which were infiltrated with EDTA then either illuminated immediately (curve 1) or following 8 h preincubation in darkness (curve 2). Curve 3 shows that V_{O_2} of control unextracted leaf segments was not appreciably diminished by incubation with EDTA, thus the inhibitory effects of EDTA observed in curves 1 and 2 reflect effects on the recovery of V_{O_2}. In both cases, we obtained ~75% maximum inhibition with half-maximal inhibition by ~1.5 mM EDTA. If such inhibition by EDTA is due to complexing the Mn^{2+} required in photoactivation, then we conclude that the Mn^{2+} derived from the S-State complex is readily complexed by EDTA and that the microcompartmentation of such Mn^{2+} observed in Figure 8A with isolated chloroplasts did not exist in the NH_2OH extracted leaf segments. This loss of microcompartmentation of Mn^{2+} derived from the S-State complex has been observed with Tris-extracted isolated chloroplasts (3, 7).

The data of Table II show, however, that EDTA alone is only

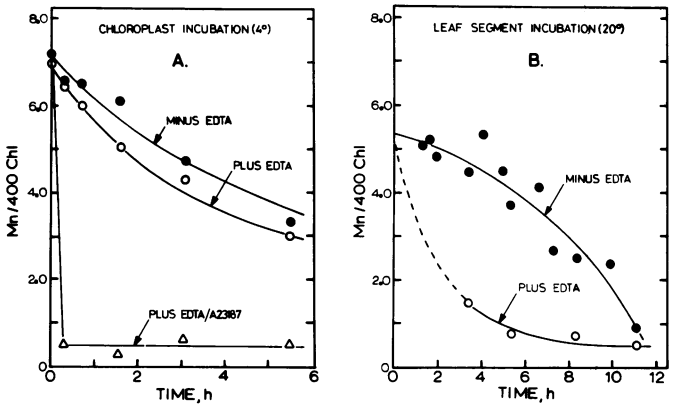

FIG. 8. Rates of depletion of Mn from chloroplasts *in vitro* (A) and *in vivo* (B). A, Isolated wheat chloroplasts (200 μg Chl/ml in SHN) were extracted in darkness with 5 mM NH_2OH for 10 min then diluted 2-fold with SHN before pelleting. The extracted chloroplasts then were incubated (200 μg Chl/ml) at 4°C in darkness for designated times in SHN alone or supplemented either with 1 mM EDTA or 1 mM EDTA and 20 μM A23187 before pelleting and Mn analyses. B, NH_2OH extracted leaf segments were infiltrated and incubated for designated times in darkness at 20°C in preincubation buffer either with or without 4 mM EDTA. Chloroplasts then were isolated and Mn determined.

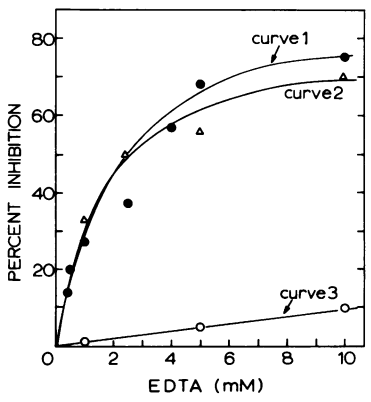

FIG. 9. Effects of EDTA on the light-dependent recovery of V_{O_2} in NH_2OH extracted leaf segments. Curve 1, NH_2OH extracted, non-dark preincubated segments were illuminated for 2 h following infiltration of the EDTA concentrations; curve 2, the EDTA concentrations were infiltrated after NH_2OH extraction of the segments then the segments were preincubated in darkness for 8 h before a 1-h illumination; curve 3, control unextracted segments infiltrated with EDTA concentrations and incubated for 1 h in darkness. The illumination regimes used for Curves 1 and 2 yielded ~50% maximum recovery of V_{O_2} in the absence of EDTA.

Table II. *Effects of EDTA and/or A23187 on Photoactivation of V_{O_2} ($\Delta O_2/Chl \cdot h$) in NH_2OH Extracted, Dark Preincubated Leaf Segments*

The extracted leaf segments were preincubated in darkness for 7 h then EDTA and/or A23187 were infiltrated just prior to a 1-h photoactivation regime. See text for other details.

Addition		ΔV_{O_2}	Inhibition
EDTA	A23187		
mM	*μM*	$\Delta O_2/Chl \cdot h$	%
0	0	283	
5	0	266	6
0	45	202	29
5	6	180	36
5	15	137	52
5	30	124	56
4	45	95	66

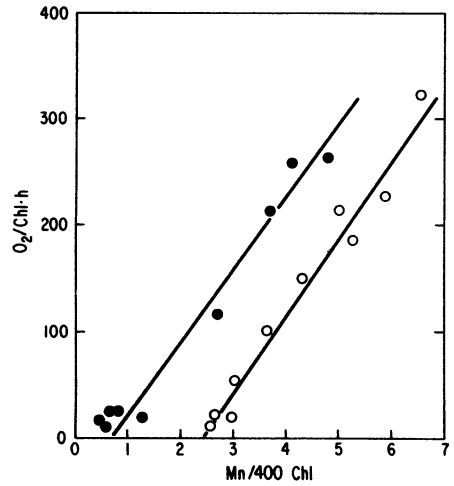

FIG. 10. V_{O_2} *versus* functional Mn abundance of chloroplasts. NH_2OH extracted segments were preincubated (6 h) in darkness then photoactivated to various extents (0- to 180-min illumination) before chloroplast isolation. The isolated chloroplasts then were washed with SHN buffer containing either 1 mM EDTA and 20 μM A23187 (●) or 0.75 μM A23187 and Chelex resin (10 mg/ml) (○) before determinations of V_{O_2} and Mn.

weakly inhibitory to the photoactivation process following long dark preincubation. A 5 mM concentration yielding near-maximal inhibition of recovery of V_{O_2} without dark preincubation gave only ~6% inhibition following the 7-h dark preincubation. On the other hand, the lipophilic divalent cation ionophore, A23187, did give significant inhibition when used by itself or in the presence of EDTA. The absence of significant inhibition by EDTA following dark preincubation confirms previous results from other studies of photoactivation (8, 40) while the inhibition by A23187 is consistent with results of studies of this process using chloroplasts from leaves greened under widely spaced flashes (40). Since photoactivation is not inhibited by uncouplers of photophosphorylation (Fig. 3; 40, 42), the inhibitory effect of A23187 is assigned to its ionophoric properties.

The contrasting effectivity of EDTA for inhibiting the recovery of V_{O_2} in dark *versus* non-dark preincubated leaf segments suggests that dark preincubation prohibits accessibility of EDTA to divalent cations required in the photoactivation process. We attribute the dark preincubation effect to reformation of the microcompartment of the apo-S-State enzyme buried in the thylakoid and the sequestering of Mn^{2+} and possibly Ca^{2+} (40) in the microcompartment for religation into the apo-S-State complex via the photoactivation process.

Relationship between V_{O_2} and Functional Mn and Ca Abundances. In the experiments of Figure 10, we photoactivated dark preincubated (6 h) NH_2OH extracted segments for various times then determined V_{O_2} and functional Mn thylakoid abundances of isolated chloroplasts following washings with SHN containing either A23187 and EDTA (closed circles) or A23187 and Chelex resin (open circles) (45). Without photoactivation, V_{O_2} of the chloroplasts used in either experiment was only 10 to 15 O_2/Chl·h. The Mn abundance of SHN-washed chloroplasts before photoactivation was 4.8 and 6.8 Mn/400 Chl (closed and open circles, respectively) which was diminished to ~0.5 and 2.4 Mn/400 Chl by EDTA/A23187 and Chelex/A23187 washing, respectively. From preceding arguments, we suggest that most, if not all, of the Mn abundance of SHN washed chloroplasts from dark preincubated NH_2OH extracted segments is within the microcompartment of the apo-S-State complex and available for the photoactivation process. Accordingly, we conclude that sufficient Mn^{2+} 'substrate' was available for photoactivation of either a bi- or tetranuclear Mn-S-State water-oxidizing complex without invoking diffusion/transport of Mn^{2+} as sometimes supposed (3) for explaining the light requirement in formation of the Mn-S-State complex.

The data of Figure 10 show that increasing extents of photoactivation resulted in increasing ligation of A23187 extractable Mn^{2+} into the Mn-S-State enzyme; moreover, they show that V_{O_2} between the limits of complete and ~0 V_{O_2} capacity is linearly correlated with a tetra-Mn-polynuclear complex. This latter conclusion rests on the linear correlation between V_{O_2} and A23187 nonextractable, ligated functional Mn abundances between the limits of 4.6 and 0.5 Mn/400 Chl (closed circles) and 6.5 and 2.4 Mn/400 Chl (open circles) and an assumed PSII unit of 400 Chl (see, however, Refs. 32 and 54).

We note the contrasting abscissa intercept values in Figure 10 obtained with A23187/EDTA (closed circles) *versus* A23187/Chelex (open circles) washed chloroplasts. The curve described by the open circles has been reported previously (18, 42) in other studies on the relationship between V_{O_2} and Mn-ligated to the S-State enzyme. Such data were interpreted (42) to indicate two Mn pools in PSII, one associated with the S-State enzyme and the smaller pool (~2 Mn/400 Chl) with unknown function. The data of Figure 10 (closed *versus* open circles) suggest, however, that the smaller Mn pool may simply reflect incomplete depletion of Mn^{2+} in the S-State enzyme microcompartment.

In experiments similar to those shown in Figure 10 but where we measured A23187 nonextractable Ca^{2+} abundances, we observed a rather invariant Ca abundance (~19–23 Ca/400 Chl) independent of NH_2OH extraction, subsequent dark incubations, or photoactivation regimes. If some fraction of the 19 to 23 Ca/400 Chl represents the Ca^{2+} functioning in the PSII/S-State complex (40, 57, 58), our results do not show any changes accompanying the NH_2OH-induced disassembly and the photoactivation-induced reassembly of the water-oxidizing S-State complex.

DISCUSSION

The results reported here are an outgrowth of our past failures over the last 10 years to obtain a highly reproducible *in vitro* system showing high yields of photoactivation with general requirements similar to those established for algae (9–11) or wheat leaves greened with widely spaced flashes (39). Previous studies (42), indicating lack of requirements of protein synthesis, photophosphorylation, and PSI in the photoactivation process, do not reflect the complexity of events necessary for formation of an active Mn-S-State water-oxidizing enzyme. This conclusion is based on our past failures with *in vitro* systems, and apparently those of other workers, despite the facts that NH_2OH does not grossly affect electron transport (24) and associated events (41)

but inactivates the Mn-S-State enzyme with high specificity and at low NH_2OH concentrations (6, 8).

Though others (56–58) have observed light-dependent increases of V_{O_2} (photoreactivation) with Tris and NH_2OH extracted broken chloroplasts, generally the reported increases in V_{O_2} have been small (≤60–80 O_2/mg Chl·h). Additionally, photoreactivation demonstrated requirements for photophosphorylation and/or a high energy state (ΔpH) which are not observed for the photoactivation process either with algae or with intact chloroplasts from wheat leaves greened under widely spaced flashes. The small V_{O_2} observed by photoreactivation, with requirements dissimilar to those of photoactivation, suggests that the yields of V_{O_2} from photoreactivation are limited by unknown processes, and that at least some reactions in the photoreactivation process are dissimilar to those involved in the photoactivation of V_{O_2}.

In the *in vivo* studies with NH_2OH-extracted leaf segments reported here, recovery of V_{O_2} with large increases of V_{O_2} (~300–350 ΔO_2/Chl·h) could be obtained by two distinct processes: (a) a slow process requiring light, 70S protein synthesis, and photophosphorylation which was inhibited by EDTA; this inhibition reflected in part the depletion by the chelator of Mn^{2+} required for photoactivation; and (b) a more rapid process (photoactivation) requiring neither photophosphorylation nor protein synthesis and which was not inhibited by EDTA but was inhibited by A23187. The system showing the slow, more complex process of V_{O_2} recovery could be transformed to the more rapid 'simple' photoactivation process by dark preincubation in the absence of protein synthesis. On the other hand, photoactivation of NH_2OH-extracted *Chlamydomonas* occurred independent of prior dark preincubation. Apparently, despite intensive efforts to duplicate the NH_2OH extraction conditions used for *Chlamydomonas* in extractions of leaf segments (or isolated chloroplasts), this was not achieved.

The precise mechanism of NH_2OH-induced inactivation of the Mn-S-State water-oxidizing complex is unknown; however, the mechanism appears to proceed via the S_0-State (49) with solubilization of its Mn and perturbation/solubilization (6) of at least the PSII extrinsic 17-, 23-, and 33-kD polypeptides.

The scheme (Fig. 11) is offered to explain the two distinct processes observed here for the light-dependent complete recovery of V_{O_2} in NH_2OH extracted leaf segments. Reaction 1 shows NH_2OH induced, *in vivo* solubilization/perturbation of the PSII extrinsic polypeptides and Mn^{2+} from the S-State complex (data not shown) which is similar to that observed on NH_2OH extraction of PSII membranes *in vitro* (6). Reaction 2 represents reformation in darkness of the microcompartment of the apo-S-State complex which is essential for the subsequent photoactivation process (reaction 3). We suspect reaction 2 involves refolding/reassembly of the 33-kD extrinsic polypeptide (1, 6, 26) and the 34-kD intrinsic polypeptide (33) suffering perturbation

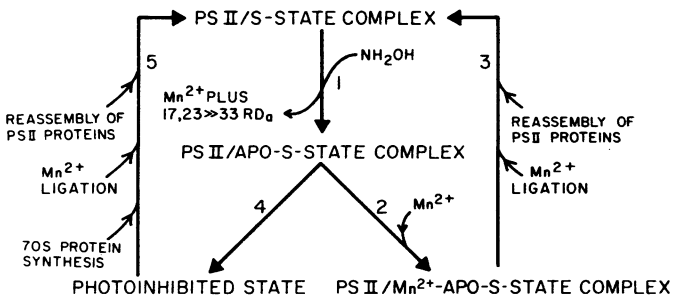

FIG. 11. Scheme illustrating the two observed mechanisms for recovery of V_{O_2} in NH_2OH extracted leaf segments. The PSII/S-State complex represents the fully functional water oxidizing complex coupled to a nonphotoinhibited PSII trapping center. See text for details.

from NH_2OH extraction and that such refolding/reassembly diminishes the extractability of Mn^{2+} from the microcompartment by EDTA.

During photoactivation (reaction 3), Mn^{2+} is ligated into the S-State complex possibly at valency states >+2 (11, 55) and becomes nonextractable even by A23187. The data presented here support previous conclusions (9–11, 39, 40) indicating that neither protein synthesis nor phosphorylation events are required. We indicate, however, that certain PSII extrinsic polypeptides are reassembled in parallel only with Mn ligation (D. Becker, F. E. Callahan, G. M. Cheniae, unpublished data).

Reaction 4 represents the photoinhibition of the PSII/apo-S-State complex resulting in partial loss of PSII donor photooxidation, modification of the kinetics of the variable fluorescence rise in the presence of DCMU, complete inhibition of photoactivation, and the imposition of 70S protein synthesis requirements for the recovery of V_{O_2} via reaction 5. This recovery, like photoactivation, also is dependent on light; however, in contrast to photoactivation, it requires 70S protein synthesis. It is therefore not surprising that the two processes for recovery of V_{O_2} show different kinetics and requirements. We suspect the recovery of V_{O_2} following photoinhibition requires the photoactivation mechanism, but this mechanism becomes possible only after the light-dependent resynthesis of specific PSII polypeptide(s).

The photoinhibition of the oxidant side of PSII traps reported here with NH_2OH extracted leaf segments also is observed with NH_2OH/Tris-extracted chloroplasts washed free of these reagents before weak light illumination. This may explain our previous failures to obtain photoactivation of the water-oxidizing enzyme in such extracted chloroplasts.

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