Plant Physiol. (1986) 82, 261–269
0032-0889/86/82/0261/09/$01.00/0

Studies on the Photoactivation of the Water-Oxidizing Enzyme

II. CHARACTERIZATION OF WEAK LIGHT PHOTOINHIBITION OF PSII AND ITS LIGHT-INDUCED RECOVERY[1]

Received for publication March 10, 1986 and in revised form May 13, 1986

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ABSTRACT

Inactivation of the water splitting enzyme complex in leaves or isolated chloroplasts results in increased susceptibility of photosystem II (PSII) to damage by light. Photoinhibition under this condition occurs in very weak light. The site of damage is exclusive of the water splitting complex yet still on the oxidizing side of PSII, as the Q_B locus is unaffected while photoreduction of silicomolybdate is inhibited. The kinetics of loss in PSII activity are more complex than apparent first-order, and the quantum efficiency is low. We observe no evidence of deletion from thylakoid membranes of any PSII polypeptide as a consequence of photoinhibition, although recovery from the photoinhibition is dependent upon both light and 70S protein synthesis. Enhanced synthesis of two proteins occurs during recovery, only one of which (D2) appears to be causally related to the recovery. We present a model which describes the relationship of weak light photoinhibition and its recovery to photoactivation of the S-state water oxidizing complex.

Irradiation of oxygenic photosynthetic organisms or their isolated chloroplasts with intense visible light inhibits photosynthesis by poorly understood processes at specific loci in the electron transport sequence and reaction centers (see Refs. 33 and 38 for recent reviews). Such photoinhibitions of photosynthesis proceed via low quantum yield processes following light absorption by Chl (18) but without irreversible photooxidation of the pigments of the photosynthetic apparatus (20). The light dose required to attain a given extent of photoinhibition appears to be variable and dependent on various 'stress factors' (pCO_2, pO_2, temperature, and water) (33, 38).

Many data indicate that PSII is more susceptible to photoinhibition than PSI (8, 9, 23, 37, 49). Studies of the effects of photoinhibition on PSII associated reactions and PSII polypeptide components suggest that the reaction center or components closely associated with it, other than the water oxidizing complex, are adversely affected (8, 9, 12, 49).

The deleterious effects of PSII generally decrease F_{max} levels without changing F_o levels when these fluorescence parameters are measured at room temperature or at ~77°K in the presence and absence of DCMU, respectively (2, 23, 37, 43). Such loss of fluorescence cannot be restored by artificial electron donors to PSII (23, 44) or by reduction of Q_A/Q_B[3] by dithionite (41). However, Kyle *et al.* (25) have reported that despite significant loss of variable fluorescence measured in the absence or presence of DCMU, PSII of photoinhibited *Chlamydomonas* is nearly fully functional when SiMo, but not DCIP, is used to accept electrons from either the oxidation of H_2O or certain artificial electron donors to PSII. Loss of atrazine binding capacity and depletion of 32 kD protein abundance in thylakoid membranes of this photoinhibited alga point to the Q_B locus as the site affected by strong light (3000 $\mu E\ m^{-2}\ s^{-1}$). Other studies (12) on aging of illuminated, isolated chloroplasts indicate that photoinhibition of PSII function is due to loss of PSII reaction center polypeptides (48 and 50 kD) as well as a 36 kD polypeptide.

Previously we reported (4) that low concentrations of NH_2OH used to inactivate the S-state water oxidizing enzyme in leaves caused PSII traps to be immediately susceptible to photoinhibition at only weak photon flux densities (25–30 $\mu E\ m^{-2}\ s^{-1}$). The acceptor side of PSII was not affected since the rise time of the variable fluorescence measured in the presence or absence of DCMU was increased without altering F_{max} or F_o values. Dark preincubation even in the presence of concentrations of CAP and CH sufficient to inhibit protein synthesis abolished susceptibility to photoinhibition and permitted photoactivation of the S-state water oxidizing enzyme and reassembly of the PSII extrinsic polypeptides independent of protein synthesis (3).

In contrast, following photoinhibition of PSII traps, the recovery of the capacity of damaged PSII traps to photoactivate the water oxidizing enzyme was dependent on photoinduced 70S but not 80S protein synthesis. Here we report studies on (a) photon flux densities required for photoinhibition of PSII traps, (b) the site affected, (c) effects on PSII polypeptide components, and (d) the identity of the chloroplast encoded polypeptide which must be synthesized for the recovery of PSII traps from photoinhibition. The results indicate a photoinhibition and 'repair' of the oxidizing side of PSII traps without perturbation of the Q_B locus or loss of Chl *a* binding polypeptides of the PSII reaction center.

MATERIALS AND METHODS

Preparation of Leaf Segments; Extraction with NH_2OH; Photoinhibition; [^{3}H]Leucine Labeling. Procedures for growth of

[1] Supported by the Department of Energy DE-AS05-82ER13072 and United States Department of Agriculture-Science and Education Administration-82-CRCR-1-1062. This paper (86-3-49) is published with approval of the Director of the Kentucky Agricultural Experiment Station.

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[3] Abbreviations: Q_A, primary stable electron acceptor quinone of PSII; Q_B, secondary stable electron acceptor quinone of PSII; SiMo, silicomolybdic acid; DCIP, 2,6-dichlorophenolindophenol; CAP, D-*threo*-chloramphenicol; CH, cycloheximide; V_{O_2}, rate of oxygen evolution at saturating light intensity; SHM, 0.4 M sucrose, 25 mM Hepes/NaOH pH 7.5, 2 mM $MgCl_2$; PM, photomultiplier; DPC, 1,5-diphenyl-carbohydrazide; TPB, tetraphenyl boron; LDS-PAGE, lithium dodecyl sulfate-polyacrylamide gel electrophoresis; LSC, liquid scintillation counting; Pheo, pheophytin *a*; S-state, the multimeric, Mn-containing complex which oxidizes water to molecular O_2.

wheat (*Triticum aestivum*, var. Oasis), preparation of leaf segments and their extraction with NH_2OH have been described (4). [^{35}S]-labeled leaves were obtained from seedlings grown in aerated nutrient solution containing 5 mM $Ca(NO_3)_2$, 0.5 mM KH_2PO_4, 0.2 mM $MgSO_4$, 1.8 mM $Mg(NO_3)_2$, 2.5 mM K_2SO_4, and 2.5 mM KNO_3 supplemented with 5.0 mCi $Na_2[^{35}S]O_4$ (719 mCi/mmol) and a micronutrient solution (46). The [^{35}S]-labeled leaves appeared normal and yielded chloroplasts with V_{O_2} values equivalent to chloroplasts from wheat grown standardly (4).

For studies with leaf segments the washed and blotted NH_2OH extracted leaf segments were suspended in preincubation buffer (4), vacuum infiltrated, then incubated at 19 to 20°C without dark preincubation on an illuminated shaker bath (4). Unless otherwise noted the incident light intensity was 30 μE m^{-2} s^{-1} PAR, of which ~80% was absorbed by the leaf segment suspension. In experiments which included recovery from photoinhibition, the photoinhibited leaf segments were further incubated similarly in darkness or light (30 μE m^{-2} s^{-1}) before isolation of chloroplasts. For radiolabeling with [^{3}H]leucine during recovery from photoinhibition, the preincubation buffer during the photoinhibition treatment was drained from the segments and replaced with preincubation buffer containing 10 μCi (1.0 mCi/mmol) of Na[^{3}H]leucine, and where noted CH (500 μg/ml) and/or CAP (200 μg/ml). After vacuum infiltration, the leaf segment suspension was further incubated as described above.

Isolation of Chloroplasts; Photoinhibition of Chloroplasts; V_{O_2} and Photoreduction Assays. Chloroplasts were isolated in SHM buffer and V_{O_2} was assayed as previously described (4). NH_2OH extraction of chloroplasts was carried out for 15 min in darkness at 0.5 mg Chl/ml in SHM buffer containing 2.5 mM NH_2OH. Following three washes (0.5 mg Chl/ml) with SHM buffer, the extracted chloroplasts were resuspended in SHM buffer. Tris extracted chloroplasts were prepared similarly with the exception that 0.8 M Tris-HCl, pH 8.0, was used during the 15 min dark incubation.

The chloroplasts (0.5 mg Chl/ml in 2 ml SHM buffer in 50 ml beakers) were illuminated at 4°C on a shaker bath for durations and photon flux densities specified in "Results." Incident light intensity was varied with a variable voltage regulator.

Rate measurements of NH_2OH photooxidation were made polarographically (6). DCIP and SiMo photoreduction assays were carried out with an Aminco-DW-2 spectrophotometer (dual beam mode) equipped with side-illumination. The light from the side illuminator was filtered through Schott 116, Corning 2-59 and neutral density filters, and a Corning 4-95 filter was employed in front of the PM tube. All assays were performed such that the rates were linear with respect to incident intensity.

The reaction mixture for DCIP photoreduction contained 0.4 M sucrose, 15 mM NaCl, 50 mM Tricine/NaOH, pH 7.5, 25 μM DCIP, chloroplasts equivalent to 10 μg Chl/ml and either 1 mM DPC or 10 μM TPB as indicated. Rates were calculated using 7.25 as the mM extinction coefficient (580 nm *versus* 540 nm). The reaction mixture for SiMo photoreduction contained 100 mM KCl, 10 mM $CaCl_2$, 50 mM Hepes/NaOH, pH 7.0, 0.5 mM DPC, 100 μM SiMo, 10 μM DCMU, and chloroplasts equivalent to 10 μg Chl/ml. Rates were calculated using 2.45 as the mM extinction coefficient (550 nm *versus* 470 nm).

In some instances where noted, the isolated chloroplasts were purified by the density gradient centrifugation procedure described by Jackson and Moore (17).

Gel Electrophoresis; Sample Solubilization; Gel Slicing. Chloroplasts (~8 mg protein/mg Chl) to be analyzed by LDS-PAGE (10) were solubilized unless noted otherwise with 4 mg LDS/mg protein at 4°C for 10 min in 50 mM DTT, 50 mM Na_2CO_3, and 10% (w/v) sucrose. For SDS-PAGE analyses, chloroplast samples were solubilized similarly except for use of 15 mg SDS/mg protein and 70°C for 5 min. In the experiment of Figure 8, 5 and 12% acrylamide were used in the stacking and running gels, respectively. In experiments of Figures 6, 9, and 10, the running gel contained a 12 to 18% continuous gradient of acrylamide stabilized with a 5 to 17% continuous sucrose gradient. Gels were stained with Coomassie brilliant blue R-250.

Individual lanes of the slab gels were frozen in Dry Ice powder and sliced (1 mm, unless otherwise noted) sequentially with a Mickle gel slicer. The slices were placed in scintillation vials, dried at 60°C for 1.5 h, then solubilized with 0.25 ml of 30% (w/v) H_2O_2 in capped vials by heating at 25°C for 2 h and 75°C for 2 h before addition of 0.25 ml H_2O and scintillation fluid and determinations of radioactivity by LSC.

Other Methods. Protein determinations (26) were made in the presence of 0.1% (w/v) Na deoxycholate. Azido-[^{14}C]atrazine (49.4 mCi/mmol) photoaffinity labeling of chloroplasts was performed as described by Pfister *et al.* (36). Atrazine binding analyses of chloroplasts were made essentially as outlined by others (47, 48). Light measurements were made with a LI-Cor Radiometer (model LI-185B) equipped with a LI 1905B quantum sensor.

RESULTS

Time Course and Intensity Dependence of the Photoinhibition of PSII Donor Reactions. Figure 1 shows the effect of weak light irradiation (30 μE m^{-2} s^{-1}) of NH_2OH extracted wheat leaf segments (×) and NH_2OH extracted isolated chloroplasts from spinach (□, △) or wheat (■, ●) on the relative quantum requirement for photooxidation of NH_2OH by PSII ($NH_2OH \rightarrow$ viologen). In these experiments, the concentration of the isolated chloroplasts employed in the weak but optimal photoinhibitory light was adjusted to yield approximately the same percentage light absorption (~80%) as the leaf segments.

Under these conditions, the data indicate a rather rapid ($t_{1/2}$ ~6 min) decrease in the capacity of PSII to photooxidize NH_2OH before leveling off at about 40% of the rates observed in nonilluminated controls consisting of either unextracted or NH_2OH

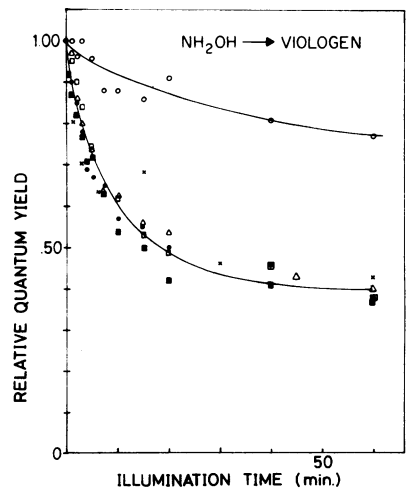

FIG. 1. Light-induced loss of PSII/NH_2OH photooxidation activity in NH_2OH extracted wheat leaf segments and chloroplasts. Wheat leaf segments (×) and chloroplasts from spinach (□, △, separate experiments) and wheat (■, ●, separate experiments) were extracted with NH_2OH and illuninated (see "Materials and Methods"). Aliquots were removed (entire leaf segment samples were removed and chloroplasts prepared immediately as in "Materials and Methods") at times indicated and assayed for $NH_2OH \rightarrow$ viologen activity. Rates are expressed relative to the zero illumination time value, which varied ≤5% from the rate for the zero illumination time sample of unextracted chloroplasts (○) (typically 100–120 μeq mg Chl^{-1} h^{-1}) or leaf segments (not shown).

extracted leaf segments or isolated chloroplasts. Moreover, illuminated unextracted chloroplasts (○, Fig. 1) experienced only ~5% and 20% decline in activity during the times of irradiation shown to give ~30% (6 min) and 60% (≥40 min) decrease in activity in NH_2OH extracted samples (Fig. 1). PSI donor photooxidation capacity was unaffected even during prolonged (≥60 min) illumination of NH_2OH extracted leaf segments or isolated chloroplasts (data not shown). Thus the weak light induced photoinhibition is specific for PSII and occurs over an entirely similar time course in NH_2OH extracted isolated chloroplasts and leaf segments. Equivalent results were obtained using Tris-inactivated chloroplasts (data not shown).

Such susceptibility of PSII to photoinhibition following inactivation of the water oxidizing enzyme is apparent also in measurements of DCMU sensitive photoreduction of DCIP by PSII with either DPC or TPB serving as artificial electron donors to PSII traps (Fig. 2). Significantly, the quantum yield of the DCMU insensitive photoreduction of SiMo (●, Fig. 2) also decreased entirely similarly as a result of illumination. This result corroborates conclusions reached from measurements of the inhibitory effect of photoinhibition on the rise time of Chl *a* variable fluorescence in the presence of DCMU (plus or minus a PSII electron donor) (4). Since the photoinhibition affects neither F_o nor F_{max} (4), we exclude from consideration possible decoupling of Pheo from Q_A (21) as an explanation for the photoinhibitory effects on PSII traps. Such decoupling results in high invariant F_o (21). As noted in Figure 2, dark incubation of NH_2OH extracted chloroplasts resulted in no decline in capacity of PSII to photooxidize DPC or TPB when DCIP or SiMo was used as the electron acceptor. We thus attribute the small decline in the quantum yield of the $NH_2OH \rightarrow$ viologen reaction observed in unextracted, illuminated chloroplasts (Fig. 1) to some disconnection of PSII/PSI trapping centers.

Figure 3A demonstrates that weak light photoinhibition in NH_2OH extracted chloroplasts follows first-order kinetics only over the initial 30 to 40% decline in PSII donor photooxidation capacity. In contrast, Jones and Kok (18) showed that intense irradiation of unextracted chloroplasts induced photoinhibition which followed first-order kinetics over ≥90% loss of both PSII and PSI activities. For purposes of comparison with data in Ref. 18, we plotted the apparent first-order rate constants for the initial rates of photoinhibition of PSII traps in NH_2OH extracted chloroplasts *versus* rate of quantum absorption (Fig. 3B). These data show that the initial rates of photoinhibition are linear with rates of quantum absorption only up to ~0.15 μE mg Chl^{-1} s^{-1}. This differs considerably from the data of (18) which demonstrate linearity of rate of photoinhibition with 10- to 2000-fold greater than photosynthesis-saturating absorption rates.

Figure 4 shows an analysis of data obtained similarly to those in Figure 3; however, here we plot the extent of observed photoinhibition *versus* the total quanta absorbed over a 15-fold range of photon flux densities. We note that the reciprocity law is obeyed over the 15-fold range of photon flux densities, but that the half-maximal extent of photoinhibition per total number of quanta absorbed occurs at ≤50 μE mg Chl^{-1} and is independent of photon flux density.

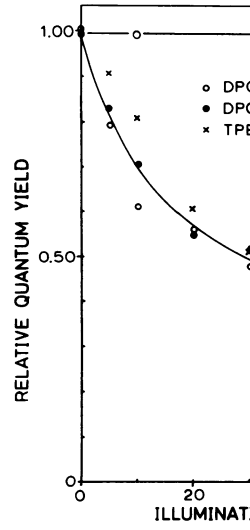

FIG. 2. Light-induced loss of specific PSII reaction activities in NH_2OH extracted chloroplasts. NH_2OH extracted wheat chloroplasts were illuminated as in "Materials and Methods." At the times indicated samples were assayed for the indicated PSII activities (see "Materials and Methods"). Rates for each reaction are expressed relative to the zero illumination time rate. Control represents NH_2OH extracted, dark incubated chloroplasts (56 μeq mg Chl^{-1} h^{-1}).

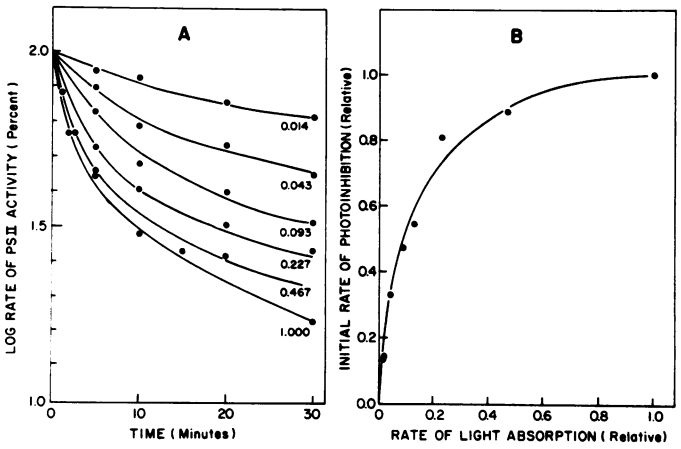

FIG. 3. Effect of light absorption rate on rate of photoinhibition. A, NH_2OH extracted chloroplasts were illuminated at the different relative light absorption rates indicated (1.000 = 1500 nE s^{-1} mg Chl^{-1}) and assayed for DPC → DCIP activity (see "Materials and Methods") at the times indicated. Rates are expressed as percent of the zero illumination time sample for each experiment. B, First-order rate constants were determined for the initial rates of photoinhibition shown in A, normalized to the maximum rate obtained and plotted as a function of relative light absorption rate.

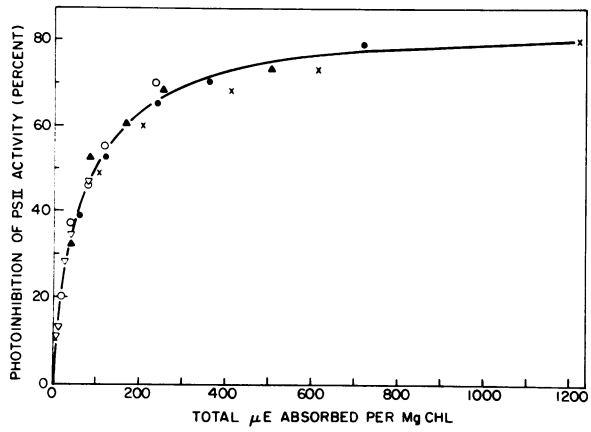

FIG. 4. Extent of photoinhibition as a function of total quanta absorbed. NH_2OH extracted chloroplasts were illuminated at five different light absorption rates for various lengths of time, assayed for DPC → DCIP activity (see "Materials and Methods") and rates compared to those for nonilluminated, extracted chloroplasts. Total quanta absorbed for each sample were calculated as the product of illumination time and quantum absorption rates (nE s^{-1} mg Chl^{-1}) used: ▽, 21; ○, 65; ▲, 140; ●, 200; ×, 340.

Assuming an equal abundance of PSII and PSI traps (1/400 Chl) and equal distribution between traps of the quanta absorbed, we calculate from the data of Figure 3B that ~18 hits PSII trap^{-1} s^{-1} is sufficient to yield half-maximal initial rate of photoinhibition. Similarly, from the initial slope of the curve of Figure 4 we calculate that 50% photoinhibition of PSII traps in NH_2OH (or Tris) extracted chloroplasts requires ~9000 quantum events within PSII traps. Thus, the data of Figures 3 and 4 show that the photoinhibition of PSII traps occurs via reaction(s) having low but finite probability and a slow rate-limiting step with a half-time of ~50 ms. Weak light photoinhibition of chloroplasts incapable of O_2 evolution occurs with low quantum efficiency (5×10^{-3}%/quantum), albeit a 50-fold higher efficiency than that in the high light photoinhibition of O_2-evolving chloroplasts found in (18).

Absence of Modification of the Q_B Locus by Photoinhibition. Data in the preceding section gave evidence that the donor side of PSII traps becomes highly susceptible to photoinhibition following inactivation of the water-oxidizing enzyme. Moreover, this photoinhibition occurs at quantum fluxes far less (~10-fold) than the quantum fluxes used routinely for growth of *Chlamydomonas* (15) or *Chlorella* (30) cells. In *Chlamydomonas*, the locus of damage by high quantum fluxes has been ascribed to the 32 kD Q_B protein, as evidenced by a diminished number of binding sites for atrazine and decreased thylakoid abundance of 32 kD Q_B protein (25, 32).

Figure 5 shows a comparison of the 32 kD Q_B atrazine binding characteristics of chloroplasts from control, unextracted leaves (●) *versus* NH_2OH extracted leaves subjected either to a weak but saturating photoinhibitory intensity (×) or to dark incubation (○). First, we note that the atrazine binding constant (K_b) was increased slightly (~40%) by NH_2OH extraction and dark incubation relative to controls. Inclusion of 2 mM NH_2OH in the binding assays made with chloroplasts from NH_2OH extracted leaves caused no significant changes in the values shown for K_b. Apparently, NH_2OH extraction of leaves alone slightly modifies the Q_B protein atrazine binding similar to that reported in NH_2OH extracted isolated chloroplasts (19).

Second, we note that photoinhibition causes no additional increase in the atrazine/Q_B binding constant; moreover, the number of atrazine specific receptor sites, reflecting the abundance of 32 kD Q_B protein (47, 48), does not diminish. Irrespective of the treatments, the specific atrazine receptor site abundance was ~1/400 Chl, a value consistent with those for normal chloroplasts (19, 34, 35, 47) but far greater than observed (~1/2000 Chl) for thylakoids from *Chlamydomonas* cells subjected to intense light and diminished CO_2 (25).

In the experiments of Figure 6 we asked if photoinhibition leads to diminished abundance(s) of any of the polypeptides comprising the PSII trap/water oxidizing complex via processes of solubilization and/or proteolysis. Polypeptides identified with the PSII core complex are the intrinsic ~9 kD (b_{559} apoprotein), the LHCP complex, the 32 kD Q_B, the 34 kD and the 43 and 47 kD proteins (45), while the 17, 23, and 33 kD proteins constitute

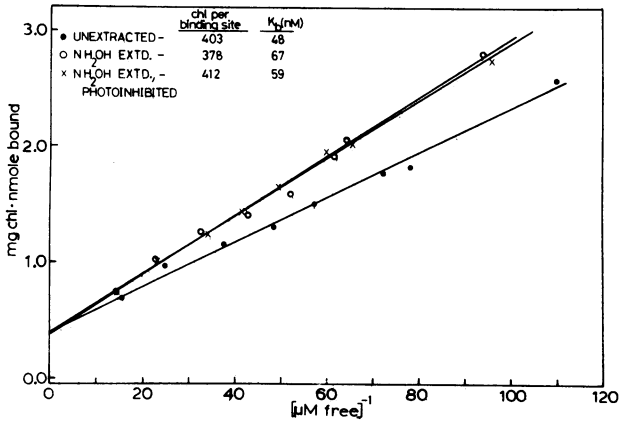

FIG. 5. [^{14}C]Atrazine binding properties of chloroplasts from non-NH_2OH extracted, NH_2OH extracted, and extracted then photoinhibited leaf segments. Chloroplasts from treated segments were assayed for PSII/NH_2OH photooxidation activity then immediately assayed for atrazine binding properties (see "Materials and Methods"). Photoinhibited segments yielded chloroplasts which demonstrated 50% PSII/NH_2OH photooxidation rates compared to unextracted or extracted, nonilluminated controls. Values for atrazine binding constants (1/abscissa intercept) and numbers of binding sites (derived from ordinate intercept) were obtained by linear regression analyses of the data.

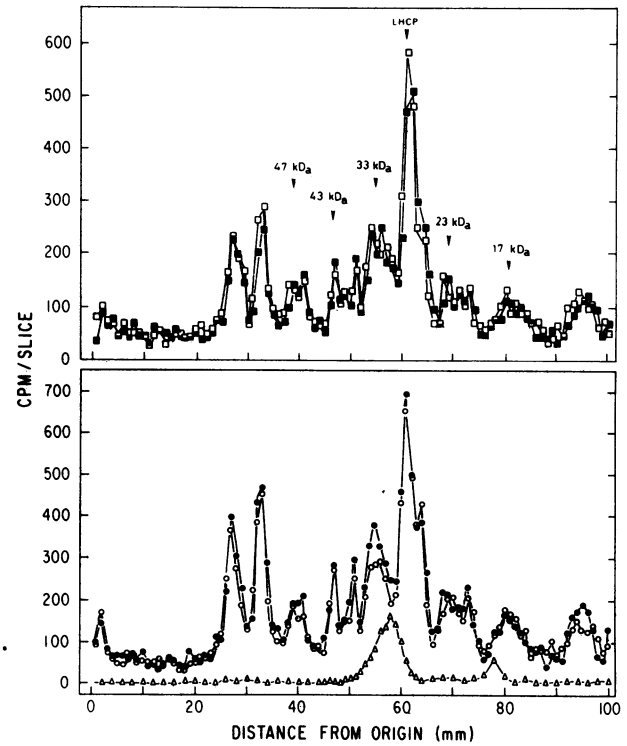

FIG. 6. Effect of photoinhibition of NH_2OH extracted leaf segments with weak and strong light on the distribution of [^{35}S]-containing thylakoid polypeptides. Extracted segments were prepared from leaves of seedlings grown hydroponically in [$^{35}SO_4^{2-}$]-containing nutrient medium (see "Materials and Methods") and were illuminated for 60 min with either weak light (see "Materials and Methods") (upper panel) or strong light (1200 μ E m^{-2} s^{-1}) (lower panel). Control extracted segments were maintained in darkness. CAP (200 μg ml^{-1}) and CH (500 μg ml^{-1}) were present throughout all incubations. Rates of PSII/NH_2OH photooxidation by chloroplasts isolated from segments declined from 110 to 61 μeq mg Chl^{-1} h^{-1} following either weak or strong light incubations, indicating saturation of photoinhibition at the weak light intensity. Following PSII assays, chloroplasts were washed twice by resuspension (~50 μg Chl ml^{-1}) in 2.5 mM Tricine/NaOH pH 8.5 and centrifugation at 38,000*g* for 5 min before final resuspension (500 μg Chl ml^{-1}) in SHM. Chloroplasts were analyzed by SDS-PAGE (10 cm gel) as in "Materials and Methods." ■ (upper panel), ● (lower panel), chloroplasts from extracted, dark incubated segments; □ (upper panel), ○ (lower panel), chloroplasts from extracted, weak and strong light photoinhibited segments, respectively; Δ, chloroplasts with photoaffinity (azido-[^{14}C]atrazine) labeled Q_B protein (see "Materials and Methods"). Markers in the upper panel indicate locations of gel band corresponding to the following PSII polypeptides: LHCP complex, 43 and 47 kD 'reaction center' proteins, and the 17, 23, and 33 kD extrinsic proteins. The location of 32 kD Q_B protein is marked by the peak in the azido-[^{14}C]atrazine plot (Δ, lower panel).

the PSII extrinsic proteins (1, 5, 29). The approximate mobilities of various PSII polypeptides are noted in the upper panel, and the mobility of the protein photoaffinity labeled with azido-[^{14}C]atrazine (36) is shown (Δ) in the lower panel of Figure 6. These experiments were performed with NH_2OH extracted leaf segments from wheat seedlings which had been cultured in [$^{35}SO_4^{2-}$]containing nutrient medium (see "Materials and Methods"). Leaf segments were incubated in the presence of concentrations of CAP and CH sufficient to inhibit protein synthesis >90% (3). Thus analyses of the effect of photoinhibition on polypeptide abundance (Fig. 6) were not complicated by synthesis of new protein.

The upper panel of Figure 6 shows the profiles of [^{35}S]-labeled chloroplast polypeptides from dark incubated (■) *versus* weak light photoinhibited (□) NH_2OH extracted leaf segments. The lower panel shows profiles of the radiolabeled chloroplast polypeptides from NH_2OH extracted leaf segments subjected to photoinhibition at 40-fold higher quantum flux density (○) *versus* dark controls (●). Both illumination regimes resulted in maximal extent of photoinhibition. No significant differences are apparent in the [^{35}S]-labeled chloroplast polypeptides from dark *versus* photoinhibited leaves irradiated at either quantum flux density, except possibly in the 34 kD and 10 kD regions in the lower panel. In neither case, however, did we observe a gross depletion of polypeptide(s) in the 32 kD region as was observed during the very high light photoinhibition of photosynthetically competent *Chlamydomonas* cells (25); nor did we observe diminished abundances of PSII reaction center Chl *a* binding proteins, as reported for illuminated isolated chloroplasts (12). We recognize that small extents of protein depletion may not be detectable by this method; however, we believe that a 45% loss (to match the extent of photoinhibition) would be evident.

Events Required for Recovery of PSII Trap Function(s) following Photoinhibition. Evidence has been presented previously showing that the return to normal PSII trap function (PSII donor photooxidation, variable fluorescence kinetics) following weak light photoinhibition of NH_2OH extracted leaves is light dependent and requires 70S but not 80S protein synthesis (4) (see also Ref. 43). Prolonged incubation (>6 h) in darkness of photoinhibited leaves in the absence of translational inhibitors yielded only ≤20% and often no recovery from the photoinhibited condition. Figure 7 presents time courses of [^{3}H]leucine incorporation into thylakoid proteins by NH_2OH extracted, photoinhibited (45 min), light incubated leaf segments. As noted in the figure legend, the weak light employed was sufficient to restore the photoinhibited PSII/NH_2OH photooxidation capacity by 50% after 280 min illumination. As shown previously (4), continued illumination results in complete recovery. In contrast, no recovery occurred during 280 min dark incubation even though the rate of [^{3}H]leucine incorporation into thylakoid polypeptides was 65 to 75% of the rate in light over the entire time-course (Fig. 7).

These results coupled with previous observations (4) imply that quantitative/qualitative differences exist in thylakoid polypeptides synthesized in light *versus* dark; moreover, they suggest that such differences relate to the complete recovery of photoinhibited PSII traps in light but not in dark (4). CAP, but not CH, abolishes the photoinduced recovery (4). We therefore surmised that any quantitative/qualitative differences in thylakoid polypeptides synthesized in light *versus* dark are causally related to recovery from photoinhibition and that these polypeptides are encoded by chloroplast DNA. This supposition excludes from consideration the 17, 23, and 33 kD PSII extrinsic polypeptides, since these are nuclear encoded (50).

Figure 8 shows the distribution of [^{3}H]leucine labeled thylakoid polypeptides as determined by LDS-PAGE. NH_2OH extracted leaf segments were incubated with [^{3}H]leucine and the radioactivity incorporated into thylakoid polypeptides in the light

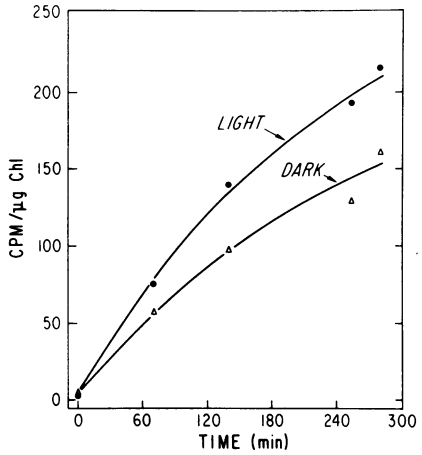

FIG. 7. Comparison of [^{3}H]leucine incorporation into thylakoid proteins by NH_2OH extracted, photoinhibited, light incubated leaf segments *versus* NH_2OH extracted, nonphotoinhibited, dark incubated leaf segments. LIGHT = Extracted segments were subjected to photoinhibitory preillumination (45 min) prior to addition of [^{3}H]leucine and subsequent illuminated incubation (see "Materials and Methods"). DARK = Extracted segments were incubated in darkness for 45 min prior to addition of [^{3}H]leucine and further dark incubation. Extracted segments which were photoinhibited, then incubated in darkness gave entirely similar values to the DARK values shown. At times shown, chloroplasts were isolated and incorporated radioactivity determined. The values shown on the ordinate represent radiolabeling of thylakoid polypeptides specifically, since the specific radioactivity (cpm μg Chl^{-1}) did not change following density gradient purification of chloroplasts (see "Materials and Methods").

(●) and dark (Δ) was measured. The upper panel data were obtained with the 280 min incubated samples of Figure 7 (~50% recovery from photoinhibition) and the lower panel data were obtained following 420 min incubation in light (~90% recovery). We note: (a) in both cases the light incubated samples showed markedly greater labeling of thylakoid polypeptide(s) relative to the dark incubated samples in only two regions (peaks 1 and 2) of the gel; and (b) the extended incubation leading to virtually complete recovery from photoinhibition of PSII traps (lower panel) increased [^{3}H]leucine incorporation into all peaks (light and dark); however, the qualitative differences in polypeptides synthesized in light *versus* dark were similar to those which occurred with the shorter incubation (upper panel). Comparison of integrated areas (total counts) of peak 1 in light and in darkness for the upper and lower panels revealed a 2.9- and 2.1-fold greater [^{3}H]leucine incorporation, respectively, in the light. For peak 2, this same comparison of integrated areas gave 3.7- and 3.2-fold differences in the upper and lower panels, respectively.

In contrast, nonextracted, control leaf segments which experienced no photoinhibition showed only weak radiolabeling of these bands during similar light incubation (data not shown). Such results coupled with the total inhibition by CAP of both [^{3}H]leucine incorporation into protein and photorecovery from photoinhibition (4) (but the absence of any effect of CH on either process) suggest that 70S synthesis of polypeptide(s) in peak 1 and/or peak 2 is causally related to the recovery of photoinhibited PSII traps (see "Discussion").

Identity of Polypeptides in Peaks 1 and 2. Figure 9 shows Coomassie blue visualized polypeptide profiles of chloroplasts from NH_2OH extracted leaves subjected to photoinhibition followed by [^{3}H]leucine labeling during the photorecovery of PSII trap function. Following photorecovery, the chloroplasts were solubilized at either mild (lanes 1 and 3 of LDS-PAGE), or stringent conditions (lane 2 of LDS-PAGE and lanes 1 and 2 of

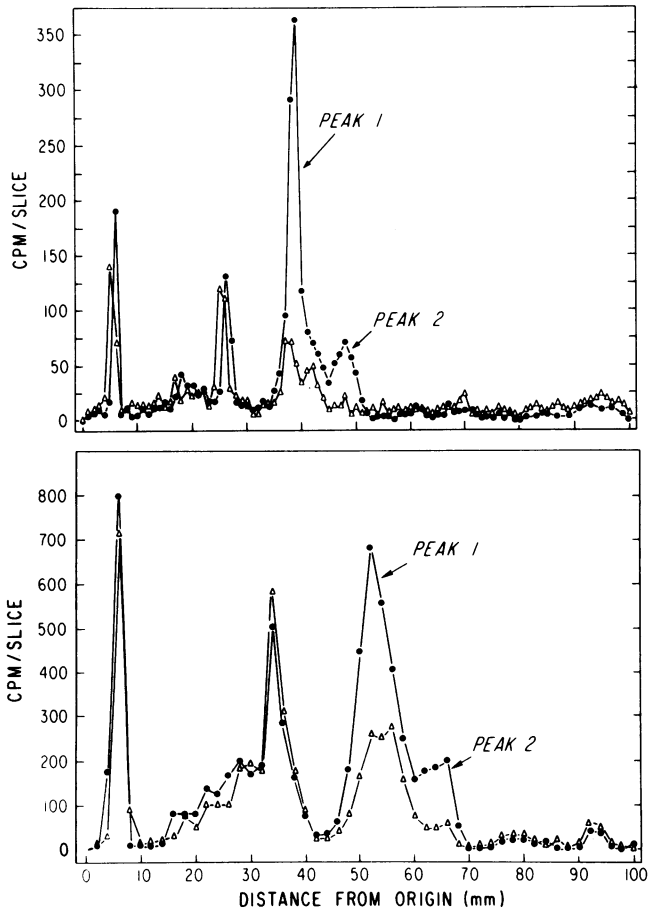

FIG. 8. Distribution of [^{3}H]leucine in thylakoid polypeptides from leaf segments which were photoinhibited, then incubated further in light or dark. Radioactivity was determined in 1 mm (upper panel) or 2 mm slices (lower panel) of LDS-PAGE lanes (see "Materials and Methods") from samples incubated in light (●) or dark (△) for 280 min (upper panel) or 420 min (lower panel). Illuminated samples demonstrated 50% (upper) and 90% (lower) recovery from photoinhibition of PSII donor photooxidation activity.

SDS-PAGE), electrophoresed under the two conditions shown and then stained. Subsequently, the gels were sliced, solubilized and counted to determine the location of the [^{3}H]leucine labeled polypeptides in peaks 1 and 2. The outer lanes of Figure 9 show the mobilities of the nuclear encoded PSII extrinsic 33, 23, and 17 kD polypeptides.

Inspection of Figure 9 reveals: (a) radiolabeled peak 1 exhibits a mobility corresponding to ~37 kD in the LDS gel but only ~29 to 30 kD in the SDS gel; and (b) radiolabeled peak 2 shows a mobility of ~34 kD in LDS-PAGE regardless of the solubilization condition (lanes 2 and 3) and a mobility of ~32 kD in the SDS-PAGE system. The contrasting mobility behaviors of peaks 1 and 2 in the two gel systems argues against the supposition that the two peaks represent processed and nonprocessed species of the same polypeptide. Additionally, the data argue against identifying either peak 1 or 2 with the 33 kD extrinsic polypeptide since neither comigrates with this polypeptide in either gel system. Moreover, the synthesis of peaks 1 and 2 is abolished by CAP but not CH, whereas the synthesis of the nuclear encoded 33 kD polypeptide (50) would be abolished by CH but not CAP. Clearly, neither peak 1 nor 2 can be identified with the 33 kD extrinsic polypeptide.

In the analyses shown in Figure 10 we employed azido-[^{14}C]atrazine photoaffinity labeling of chloroplast polypeptide(s) (36) in attempts to obtain additional insights into the identity of polypeptides of peaks 1 and 2. Chloroplasts were [^{3}H]leucine labeled under conditions for photoinduced recovery from photoinhibition (Fig. 7) then photoaffinity labeled with azido-[^{14}C]-atrazine at a low concentration before SDS- and LDS-PAGE (as in Fig. 9) and measurements of radiolabel in the separated polypeptides.

We note: (a) the [^{3}H]leucine peak 1 comigrates with the azido-[^{14}C]atrazine-labeled polypeptide in both the LDS- and SDS-PAGE systems despite the rather large changes in the mobility of peak 1 (Fig. 9); and (b) the [^{3}H]leucine peak 2, which runs nearly coincident with the extrinsic 33 kD PSII polypeptide in LDS- but not in SDS-PAGE (Fig. 9) was essentially unlabeled by azido-[^{14}C]atrazine. Accordingly, we identify peak 1 with the 32 kD Q_B protein (see, however, Ref. 28) on the basis of [^{3}H]-leucine/azido-[^{14}C]atrazine comobility, its poor staining by Coomassie and its variable mobility dependent on solubilization and electrophoresis conditions (45).

It is clear that peak 2 polypeptide cannot be equated with the 33 kD extrinsic polypeptide; in addition, the contrasting mobilities of peaks 1 and 2 in the different gel systems, and the lack of azido-[^{14}C]atrazine labeling of peak 2 argue against equating the polypeptide of peak 2 as the nonprocessed species (40) of the 32 kD Q_B protein.

DISCUSSION

Previous studies on photoinhibition of photosynthetic reactions suggest a multiplicity of deleterious effects dependent upon the experimental conditions employed (*e.g.* photon flux density, intact algal cells *versus* leaves *versus* isolated chloroplasts and their histories before exposure to intense visible light) (33, 38). The primary effects from photoinhibition are expressed as loss of PSII and/or PSI trap function (33, 38) or as loss of the 32 kD Q_B protein function in PSII (25, 32). In these previous studies photon flux densities of several to 2500 fold greater than required for light saturation of photosynthesis or the Hill reaction have been employed. Generally, these studies have been made with cells/chloroplasts possessing active S-state water oxidizing complexes.

We have used leaf segments and chloroplasts in which we have inactivated the S-state complex by NH_2OH or Tris extraction prior to photoinhibition. The principal results and conclusions which emerge from these studies are: (a) the inactivation of the water oxidizing complex specifically increases the susceptibility of PSII trap function to photoinhibition at photon flux densities ($\leq$30 μE $m^{-2}s^{-1}$) far less than generally employed ($\geq$2000 μE $m^{-2}s^{-1}$) with O_2 evolving cells/chloroplasts; (b) this photoinhibition of PSII trap function obeys the 'reciprocity law,' proceeds at least initially at rates directly proportional to intensity (18), not to the square of the intensity (22), and tends to saturate at intensities much less than required to saturate photosynthesis or the Hill reaction; (c) in contrast to previous results showing first-order character of photoinhibition of PSII over essentially >90% loss of PSII trap function (18), the loss of PSII trap function does not occur via simple, apparent first-order kinetics; however, a rate limitation of ~50 ms is imposed early in the course of photoinhibition, during which the kinetics appear to be first-order; and (d) the extent of loss of PSII trap function, with assays of chloroplasts judged by loss of PSII photooxidation of artificial electron donor(s), tends to level off after ~60% loss of activity.

The site affected by photoinhibition of NH_2OH or Tris extracted chloroplasts or NH_2OH extracted leaves is very close to the secondary donor, Z, to $P680^+$ of PSII traps. The rise-times of the Chl *a* variable fluorescence (plus DCMU and even in the presence of NH_2OH) become longer following photoinhibition but without alteration of F_o and F_{max} values. This result contrasts with the generally observed quenching of variable fluorescence

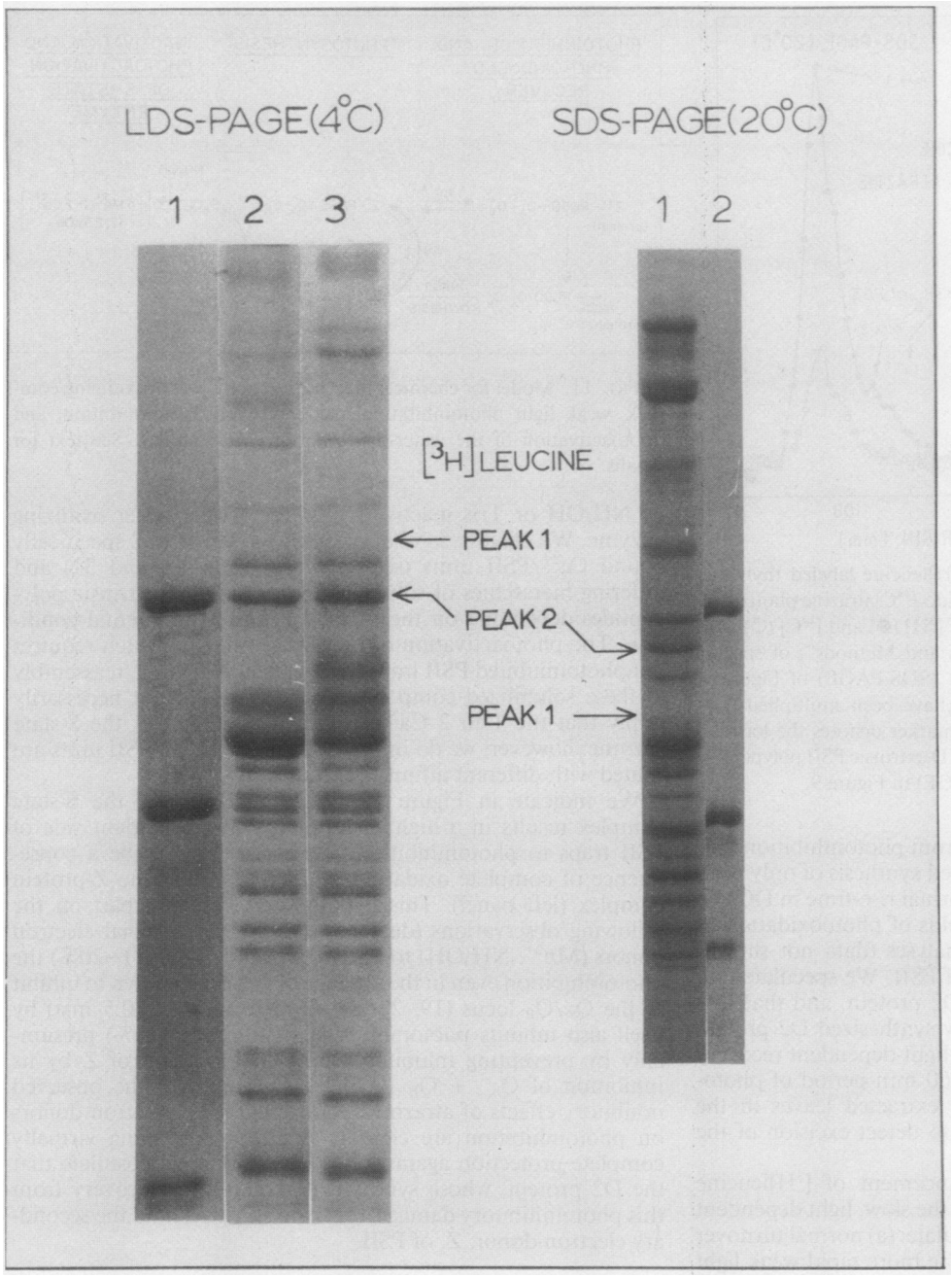

FIG. 9. LDS- *versus* SDS-PAGE polypeptide profiles of chloroplasts prepared from leaf segments which were photoinhibited, then [^{3}H]leucine labeled during recovery. The Coomassie stained polypeptide profiles shown represent pertinent regions cut from 30 cm resolving gels. [^{3}H]Leucine labeled polypeptides of peaks 1 and 2 (see Fig. 8) shown by markers were indentified by gel slicing (see "Materials and Methods"). Lane 1 (LDS-PAGE) and lane 2 (SDS-PAGE): 33, 23, and 17 kD Tris extractable, extrinsic polypeptides of the water-oxidizing enzyme complex; lane 3 (LDS-PAGE) and lane 1 (SDS-PAGE): chloroplasts isolated from photoinhibited, then light incubated (240 min) leaf segments (60% recovery from photoinhibition) which were photoaffinity labeled with azido [^{14}C]atrazine (see Fig. 10) prior to solubilization for electrophoresis: lane 2 (LDS-PAGE): same as lane 3 except thylakoids were solubilized at 70°C for 5 min prior to electrophoresis. No Chl-containing bands were observable in lane 2, in contrast to lane 3 (LDS-PAGE).

following photoinhibition of PSII by high photon flux densities (2, 23, 37, 44).

Additionally, the photoinhibition results in increased quantum requirements for the photooxidation of electron donors (NH_2OH, DPC, TPB) to PSII via Z^+ irrespective of electron withdrawal from Q_A (SiMo plus DCMU) (14, 51) or Q_B (DCIP) (24). The kinetics of loss of PSII donor photooxidation likewise are independent of the site of electron withdrawal from PSII or the type chemical serving as electron donor. Thus these results (Fig. 2) and the results of Figure 5 showing the absence of modification of the 32 kD Q_B protein contrast with those obtained by photoinhibition of *Chlamydomonas* cells (25) and isolated pea thylakoids (32) by moderately high photon flux densities (~2000 μE $m^{-2}s^{-1}$).

Four separate lines of evidence indicate that weak light photoinhibition of NH_2OH extracted leaves and NH_2OH or Tris extracted chloroplasts affects principally the secondary donor, Z, to $P680^+$ and not the 32 kD Q_B protein. First, the DCMU variable fluorescence (F_v/F_o) is unchanged even though the variable fluorescence rise-time is increased ~14-fold (4). Second, a PSII artificial electron donor diminishes the slow rise-time by ~4-fold (4). Third, the relative quantum yield of photoreduction of SiMo by DPC is diminished (Fig. 2) even in the presence of DCMU which inhibits electron transfer between Q_A and Q_B. Fourth, neither the atrazine binding constant nor the number of atrazine specific binding sites for the Q_B protein (Fig. 5) is affected by weak light photoinhibition.

Our data show that recovery from weak light photoinhibition of NH_2OH extracted leaves is dependent upon slow, light-induced 70S protein synthesis (4). Enhanced incorporation of [^{3}H]leucine during light dependent recovery from photoinhibition is apparent in only two polypeptides (Fig. 8, peaks 1 and 2). Peak 1 is identified as the 32 kD Q_B protein; however, based on the preceding arguments, we dismiss the synthesis of this protein

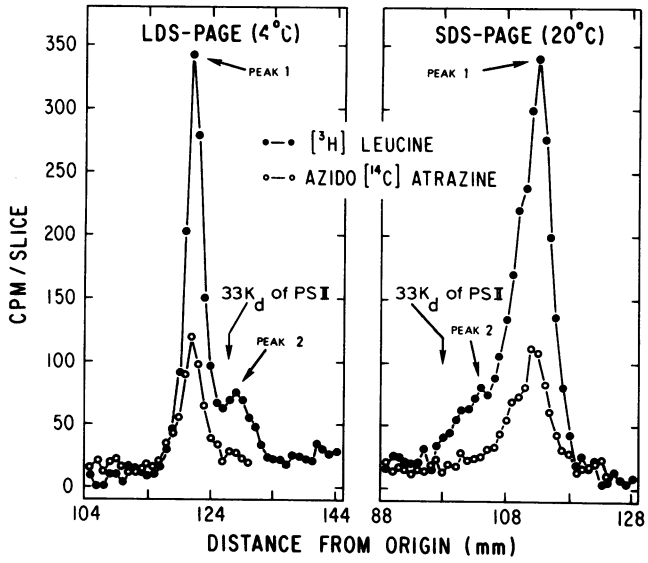

FIG. 10. Electrophoretic mobilities of [^{3}H]leucine labeled thylakoid polypeptides of peaks 1 and 2 (Fig. 9) and azido-[^{14}C]atrazine photoaffinity labeled Q_B protein. The radioactivities of [^{3}H] (●) and [^{14}C] (○) were determined in 1 mm slices (see "Materials and Methods") of specific regions of lane 3 (LDS-PAGE) and lane 1 (SDS-PAGE) of Figure 9. Values of cpm/slice (SDS-PAGE analysis) have been multiplied by a factor of 2.0 for illustrative purposes. The marker denotes the location in each gel system of the Tris-extractable 33 kD extrinsic PSII polypeptide of lane 1 (LDS-PAGE) and lane 2 (SDS-PAGE) in Figure 9.

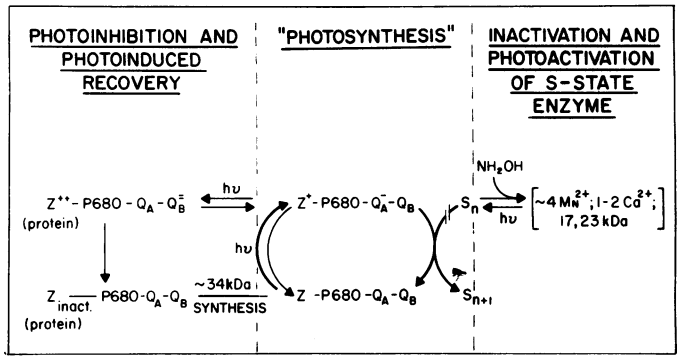

FIG. 11. Model for chemical inactivation of the water-oxidizing complex, weak light photoinhibition, recovery from photoinhibition, and photoactivation of the water-oxidizing enzyme complex. See text for details.

from being related to recovery of PSII from photoinhibition. We therefore conclude that the photoinduced synthesis of only peak 2 (Fig. 8) is necessary for recovery to normal rise-time in DCMU variable fluorescence and quantum yields of photooxidation of PSII electron donors. Western blot analyses (data not shown) identify peak 2 as the D2 protein (7) of PSII. We speculate that photoinhibition rapidly modifies the D2 protein, and that it is slowly excised and replaced with newly synthesized D2 protein (11, 16) *in vivo* during slow ($t_{1/2}$ ~3 h), light-dependent recovery from photoinhibition. Apparently the 60 min period of photoinhibition of [^{35}S]-prelabeled NH_2OH extracted leaves in the experiment of Figure 6 was too short to detect excision of the D2 protein.

To account for the observed enhancement of [^{3}H]leucine labeling of the 32 kD Q_B protein during the slow, light dependent recovery from photoinhibition, we postulate: (a) normal turnover of the 32 kD Q_B protein, unrelated to the more rapid weak light photoinhibition process, also is too slow to be detected in the experiment of Figure 6, but is apparent over the longer recovery period; and/or (b) modification of the D2 protein by photoinhibition leads to increased turnover of unmodified 32 kD Q_B protein as a consequence of its excision along with the D2 protein. We note the considerable amino acid sequence homology between D2 and the 32 kD Q_B protein (39, 42) and the likelihood of their close proximity. Thus, both proteins may be susceptible to attack by a single protease.

We offer the scheme of Figure 11 to explain our conclusions from studies on the disassembly of the S-state complex, the photoinhibition of the oxidant side of PSII traps, the photoinduced recovery of PSII trap function via processes requiring 70S synthesis of a ~34 kD (D2) polypeptide, and the reassembly of the S-state complex via photoactivation (3, 4, 6). The center panel shows PSII charge separation and S-state advancement normally occurring within functional S-state complexes. Also shown is charge recombination ($Z^+ + Q_A^- \rightarrow Z + Q_A$).

The right panel displays the known components solubilized by NH_2OH or Tris inactivation of the S-state water oxidizing enzyme. We observe solubilization of ~4 Mn, 1 to 2 specifically bound Ca^{2+}/PSII units (see, however, Refs. 13 and 31) and differing hierarchies of solubilization of the PSII extrinsic polypeptides depending on the extractant and experimental condition. The photoactivation of the S-state enzyme which requires nonphotoinhibited PSII traps (4) results in religation/reassembly of these solubilized components (3, 4). We do not necessarily imply that the 1 to 2 Ca^{2+} are an integral part of the S-state enzyme; however, we do imply that the 2 to 3 Ca/PSII units are ligated with different affinity constants.

We indicate in Figure 11 that disassembly of the S-state complex results in a high susceptibility of the oxidant side of PSII traps to photoinhibition. We suggest this to be a consequence of complete oxidation of Z (Z^{++}) within the Z-protein complex (left panel). This hypothesis is based in part on the following observations (data not shown): (a) artificial electron donors (Mn^{2+}, NH_2OH) to PSII significantly inhibit (~20%) the photoinhibition even in the absence of atrazine, known to inhibit at the Q_A/Q_B locus (19, 27, 34, 36); (b) atrazine (0.5 mM) by itself also inhibits photoinhibition significantly (70%) presumably by preventing inhibitory complete oxidation of Z by its inhibition of $Q_A^- + Q_B \rightarrow Q_A + Q_B^-$; and (c) the observed inhibitory effects of atrazine and artificial PSII electron donors on photoinhibition are close to additive, conferring virtually complete protection against photoinhibition. We postulate that the D2 protein, whose synthesis is required for recovery from this photoinhibitory damage, serves to stabilize/bind the secondary electron donor, Z, of PSII.

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