Photoactivation of NH_2OH-treated leaves: reassembly of released extrinsic PS II polypeptides and religation of Mn into the polynuclear Mn catalyst of water oxidation

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Inactivation of the water-oxidizing enzyme by exposure of leaf segments to hydroxylamine results in a disassembly/perturbation of photosystem (PS) II extrinsic polypeptides (17>23 kDa) and solubilization of the tetra-Mn complex. Religation of the tetra-Mn complex, reassembly of these PS II extrinsic polypeptides with thylakoid membranes and reappearance of active water-oxidizing activity all require light, and all are unaffected by inhibitors of protein synthesis and photophosphorylation. The results indicate, however, that the reassembly of these PS II extrinsic polypeptides into the multimeric water oxidizing enzyme occurs more rapidly than the photoligation of Mn as the tetra-Mn complex.

Photoactivation *O_2 evolution* *Manganese* *Photosynthesis* *Photosystem II polypeptide*

1. INTRODUCTION

Much evidence indicates that the water-oxidizing enzyme of photosynthesis comprises a tetra-Mn polynuclear complex ([2–5] but see [6]) ligated principally with an intrinsic 34 kDa and a 33 kDa extrinsic polypeptide [7,8] and possibly secondarily with the 17,23 kDa extrinsic polypeptides [5,9]. Though it is clear that the catalytic activity of the tetra-Mn polynuclear complex does not demonstrate absolute requirements for the 17,23 kDa polypeptides [10], studies of the Cl^- ([11]; unpublished) and Ca^{2+} [12,13] requirements for O_2 evolution as well as studies of inactivation of the enzyme by NH_2OH and related derivatives [9] indicate that these polypeptides are important determinants of the microenvironment of the water oxidizing enzyme.

Previous studies with diverse species of oxygenic photosynthetic organisms have shown that the formation of catalytically active S-state water-oxidizing enzyme complexes requires photoactivation [2,14–18]. This multi-quantum process [2,15] is driven by PS II traps, is independent of photophosphorylation and protein synthesis [2,16–18] and results in ligation of Mn (possibly at valency states $> +2$) [2] by the apo-S-state enzyme. In vitro inactivation of the S-state water-oxidizing enzyme of isolated PS II membranes by NH_2OH results in solubilization of its ligated Mn and solubilization ($17 \sim 23 > 33$ kDa) of the PS II extrinsic polypeptides [5,9]. NH_2OH also causes solubilization of Mn and inactivation of the S-state enzyme in isolated chloroplasts [2], algae [2,19] and leaves [18], however, the effect(s) of NH_2OH on solubilization/perturbation of the PS II extrinsic polypeptides in these systems is unknown. Although photoactivation of the water-oxidizing

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Abbreviations: DTT, dithiothreitol; LHCP, light-harvesting chlorophyll protein; PS, photosystem; V_{O_2}, rate of O_2 evolution

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enzyme has been described for NH_2OH extracted algae [20] and leaves [18], it is unknown what effect photoactivation plays in reassembly of any PS II polypeptides that may undergo disassembly during inactivation by NH_2OH. Here we present evidence which demonstrates that NH_2OH inactivation of the water-oxidizing enzyme in leaves causes some disassembly of the PS II extrinsic polypeptides as well as Mn solubilization from the water-oxidizing enzyme. Religation of the Mn and reassembly of the PS II extrinsic polypeptide with thylakoids both specifically require light but occur at different rates.

2. MATERIALS AND METHODS

2.1. *NH_2OH treatment of leaf segments and dark preincubation*

Wheat (*Triticum aestivum* var. Oasis) leaf segments (1–2 mm) were prepared and treated with NH_2OH as in [18]. Unless otherwise indicated controls and NH_2OH-treated segments were then dark preincubated at 20°C for 6–8 h before illumination to avoid photoinhibition of the oxidizing side of PS II traps which occurs during weak light illumination of non-dark-preincubated, NH_2OH-extracted leaf segments [18].

2.2. *Photoactivation regimes; chloroplast and PS II membrane isolation and assays*

NH_2OH-treated, dark-preincubated segments were illuminated as described [18]. Following illumination segments were drained, resuspended in chloroplast isolation medium at 4°C and chloroplasts were isolated [18]. PS II membranes were prepared essentially in [5]. Procedures for rate measurements of O_2 evolution capacity of chloroplasts [18] and PS II membranes [5] have been described.

2.3. *SDS-polyacrylamide gel electrophoresis*

PS II membranes (5 mg protein/mg Chl) were solubilized (15:1, SDS/protein) at 70°C for 5 min in the presence of 50 mM DTT, 50 mM Na_2CO_3 and 10% (w/v) sucrose just prior to electrophoresis [21] on a 12–20% continuous acrylamide gradient stabilized with a 4–12% sucrose gradient. Protein bands were stained with Coomassie brilliant blue and scanned with a Gelman ACD-15 densitometer.

2.4. *Other determinations*

Mn content of acid-digested thylakoids was determined by flameless atomic absorption [18]. Chl determinations were made in 80% acetone.

3. RESULTS AND DISCUSSION

Fig.1 shows a time course for the photoactivation of the water-oxidizing enzyme and the photoligation of Mn into this enzyme in NH_2OH-extracted, dark-preincubated leaf segments. The inset to fig.1 records a plot of O_2 evolution capacity vs Mn ligated during the course of photoactivation shown in the main figure. Upon illumination both measured parameters increased in parallel over the entire time course of photoactivation until ultimately O_2 evolution capacity and ligated Mn

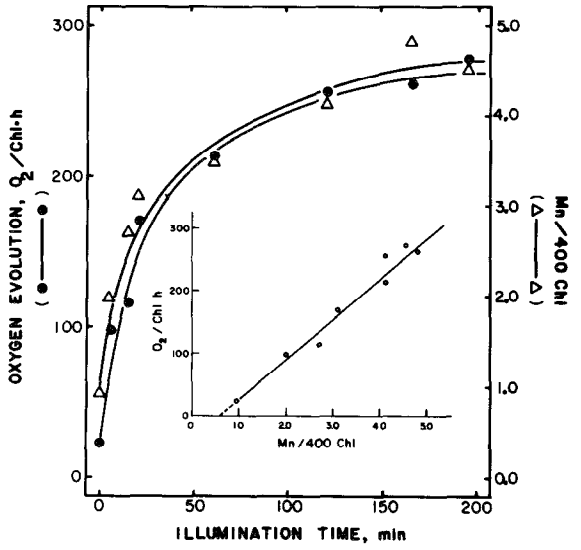

Fig.1. Time course of photoactivation of V_{O_2} and ligation of Mn into the water-oxidizing enzyme. (Inset) Activity of the water-oxidizing enzyme vs Mn ligated into the enzyme. Chloroplasts were isolated in the presence of EDTA then washed in the presence of EDTA and ionophore A23187 to extract adventitiously bound Mn before determinations of V_{O_2} and Mn [18]. Chloroplasts from non-NH_2OH-treated, dark-preincubated leaf segments yielded 285 O_2/Chl per h and 4.8 Mn/400 Chl. During the long (6 h) dark preincubation (see section 2) there was no change in O_2 evolution capacity or Mn abundance. We note that for reasons unknown the time course for photoactivation in this experiment was particularly rapid.

reached the levels in chloroplasts from control (non-NH_2OH-treated) leaf segments (285 O_2/Chl per h and ~4.8 Mn/400 Chl, respectively). Between the limits of 0.6 and 4.8 Mn/400 Chl the increase in O_2 evolution capacity proved linearly related suggesting again [2,5] that the tetranuclear Mn complex is required for catalytic activity of the water-oxidizing enzyme.

Photoactivation of the water-oxidizing enzyme in NH_2OH-extracted, dark-preincubated leaf segments requires neither 70 S nor 80 S protein synthesis. Table 1 shows that addition of D-threo-chloramphenicol (CAP) and cycloheximide (CH) prior to dark preincubation inhibited [^{3}H]leucine incorporation into thylakoid polypeptides by 95% during both the 6 h dark preincubation and the subsequent 2 h photoactivation incubation. This inhibition did not, however, diminish the extent of increase in V_{O_2} capacity (ΔV_{O_2} ~200 O_2/Chl per h) by the 2 h photoactivation regime. Note also that despite significant protein synthesis during the 6 h dark preincubation in the absence of inhibitors, no conversion of inactive to active water-oxidizing enzyme occurred. Similar experiments, but with addition of an uncoupler of photophosphorylation (50–300 mM methylamine) prior to the dark preincubation, showed that this uncoupler had no effect on photoactivation in contrast to its inhibitory effect upon the light-dependent recovery of V_{O_2} in photoinhibited (NH_2OH-extracted, non-dark-preincubated) leaf segments [18]. We conclude that photoactivation requires neither protein synthesis nor photophosphorylation, a conclusion reached previously from indirect arguments [2].

In the experiments of fig.2 we investigated whether NH_2OH extraction of leaves results in disassembly of PS II extrinsic polypeptides similar to that reported for NH_2OH extraction of isolated PS II membranes [5,9] which have everted membrane orientation. We also checked if any such disassembled polypeptides reassemble with thylakoid membranes during dark preincubation or whether reassembly occurs only during the photoactivation process.

Lanes 1 and 2 of fig.2 are polypeptide profiles of PS II membranes isolated from control (non-NH_2OH-extracted) and NH_2OH-extracted leaf segments, respectively. Markers identify the 33, 23 and 17 kDa extrinsic PS II polypeptides of wheat PS II membranes [22]. Comparison of lane 2 vs 1 reveals an absence and diminished abundance of the 17 and 23 kDa polypeptides, respectively, in PS II membranes from the NH_2OH-extracted leaf segments but no apparent decrease in abundance of the 33 kDa polypeptide. This differs from NH_2OH extraction of isolated PS II membranes

Tabe 1

Absence of protein synthesis requirement for photoactivation of water-oxidizing enzyme

Incubation conditions		Additions	ΔV_{O_2}/Chl per h	[^{3}H]Leucine incorporation into thylakoid protein (dpm/nmol Chl)
Dark (h)	Light (h)			
6	0	–	0	433
6	0	+CAP, +CH	0	20
6	2	–	218	670
6	2	+CAP, +CH	195	33

Na [^{3}H]leucine (10 μCi; 1 mCi/mmol) and where indicated CH (1000 μg/ml) and CAP (200 μg/ml) were vacuum infiltrated immediately after NH_2OH extraction of leaf segments. Following incubation of samples, chloroplasts were isolated in the presence of 15 mM leucine, then V_{O_2} was determined. Chloroplasts were washed [18], digested (65°C) with 30% (w/v) H_2O_2 and the [^{3}H]leucine incorporation was determined. V_{O_2} of non-dark-incubated samples was 20 O_2/Chl per h

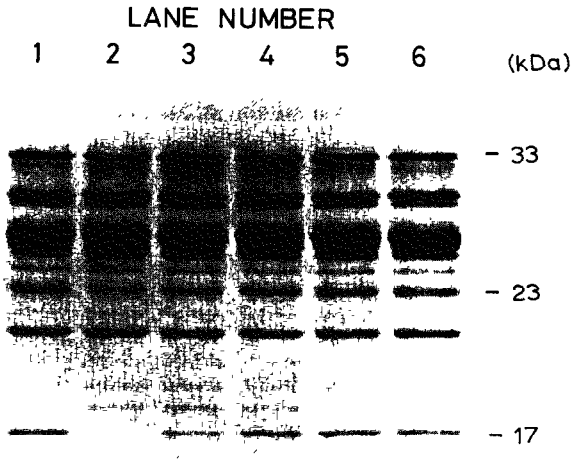

Fig.2. Disassembly of the 17,23 kDa extrinsic PS II polypeptides by NH_2OH treatment of leaves; reassembly during photoactivation of the water-oxidizing enzyme. PS II membranes from non-NH_2OH-treated (lane 1), NH_2OH-treated, dark-preincubated (lane 2) and NH_2OH-treated, dark-preincubated then photoactivated leaf segments (lanes 3–6) were analyzed by SDS-polyacrylamide gel electrophoresis. Lanes 3,4,5 and 6 are polypeptide profiles of PS II membranes isolated following photoactivation for 30, 90, 180 and 360 min, respectively. NH_2OH-treated, non-dark-preincubated samples show polypeptide profiles indistinguishable from that of lane 2. See fig.3 for V_{O_2} values of the various PS II preparations.

[5,9], which causes a significant diminution in amount of 33 kDa polypeptide in addition to extensive depletion of 17,23 kDa polypeptides. The diminished abundances of the 17,23 kDa polypeptides shown in lane 2 did not change during the course of 6 h dark preincubation after NH_2OH extraction of leaf segments. Thus we conclude that: (i) NH_2OH inactivation of the water-oxidizing enzyme in leaves causes disassembly of PS II extrinsic polypeptides (17 > 23 kDa); and (ii) these polypeptides do not reassemble with PS II membranes during prolonged dark incubation of NH_2OH-extracted leaf segments.

In contrast, however, lanes 3–6 of fig.2 provide evidence that these polypeptides do reassemble during the course of photoactivation. Inspection of lanes 2–6 reveals that increasing duration of photoactivation leads to both increasing recovery of V_{O_2} capacity (see fig.3) and increasing reassembly of the 2 extrinsic PS II polypeptides until complete reassembly has occurred (cf. lanes 1–6).

The various samples of fig.2 were assayed also for V_{O_2} capacity in the presence and absence of Cl^- (fig.3). The rationale for this comparison was: (i) neither of the 17,23 kDa polypeptides is directly required for V_{O_2} ([10], unpublished) since their complete depletion from wheat PS II membranes diminishes V_{O_2} in any light regime only ~50%; but (ii) their depletion confers on V_{O_2} a high dependency upon Cl^- in the assay: without Cl^- V_{O_2} is essentially abolished with such wheat membranes; however, membranes with fully assembled 17,23 kDa polypeptides show only ~30% enhancement of V_{O_2} by added Cl^- (unpublished). The PS II membranes of fig.3, photoactivated to various extents, show minimal dependency of V_{O_2} to Cl^- addition to assays irrespective of V_{O_2} capacity (0–700 O_2/Chl per h). We conclude from these data that reassembly of the 17,23 kDa polypeptides occurs at least as fast as ligation of Mn into the tetra-Mn complex catalyst of water oxidation.

In fig.4 we record results from 2 separate experiments (including that of figs 2 and 3) in which we measured ΔV_{O_2} and relative abundances of the

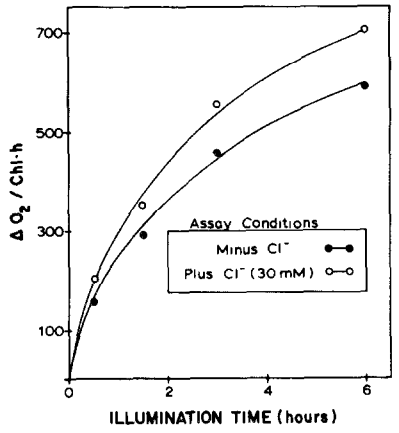

Fig.3. Response to chloride of the water-oxidizing enzyme in PS II membranes from photoactivated leaf segments. Following 8 h dark preincubation leaf segments were photoactivated for the times indicated. PS II membranes were then prepared and assayed in the presence and absence of 30 mM NaCl. PS II membranes isolated from non-NH_2OH-extracted leaves and depleted of the 17,23 kDa polypeptides [23] typically yield 50 and 300 O_2/Chl per h when assayed in the absence and presence of 30 mM NaCl, respectively.

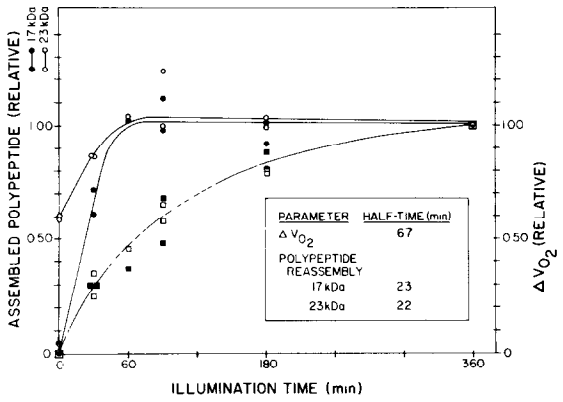

Fig.4. Time courses of reassembly of 17,23 kDa polypeptides and recovery of V_{O_2} during photoactivation. Densitometer analyses were made of gels of PS II membranes isolated from leaf segments photoactivated for the times indicated, and the integrated areas of the 17 and 23 kDa bands were determined and normalized to the LHCP bands. Abundances relative to fully reassembled 360 min samples are plotted for 2 separate experiments. V_{O_2} recovered fully to control (non-NH_2OH-treated) levels (720, 615 O_2/Chl per h and 380, 420 O_2/Chl per h, respectively, for PS II membranes and chloroplasts) in both experiments, and ΔV_{O_2} (relative to fully recovered 360 min samples) is plotted for both chloroplasts (□) and PS II membranes (■).

17,23 kDa polypeptides during photoactivation. This comparison indicates that the light-dependent reassembly of both the 17 and 23 kDa polypeptides is more rapid than the light-dependent increase in V_{O_2} (and ligation of Mn, fig.1). This conclusion is clearly shown in the tabulation of half-times for reassembly and ΔV_{O_2} (fig.4, inset) for these data. Note that the reassembly of both the 17 and 23 kDa polypeptides is ~3-fold faster than the recovery of V_{O_2}.

We conclude that during photoactivation of the water-oxidizing enzyme in NH_2OH-extracted leaves there occur reassembly of certain extrinsic polypeptides and religation of Mn, both of which are strictly light dependent but independent of protein synthesis and photophosphorylation. The photoinduced reassembly of the 17,23 kDa polypeptides precedes photoligation of Mn as a tetra-Mn cluster. The possibility that photoligation of Mn as an intermediate (<4 Mn/PS II trap) in the photoactivation of V_{O_2} is involved in the polypeptide reassembly is not excluded. We also do not exclude some function for Ca in the reassembly process: we note that (i) NH_2OH effects partial solubilization of the specifically bound ~2 Ca/PS II trap [9], but (ii) complete photoactivation of the water-oxidizing enzyme is accompanied by a return to the full complement of specifically bound Ca.

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