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Hydrogen peroxide oxidation catalyzed by chloride-depleted thylakoid membranes

P.O. Sandusky * and C.F. Yocum

Departments of Biology and Chemistry, University of Michigan, Ann Arbor, MI (U.S.A.)

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Chloride-depleted thylakoid membranes which are unable to catalyze the oxidation of water can nonetheless catalyze the oxidation of hydrogen peroxide (Kelly, P. and Izawa, S. (1978) Biochim. Biophys. Acta 502, 198–210). Using steady-state kinetic analyses, the inhibition of hydrogen peroxide oxidation by Tris, other amines, and F^- has been further examined in chloride-depleted thylakoid membranes. Based on these investigations, we conclude that inhibitions of H_2O_2 oxidation produced by such reagents are unrelated to their inhibitory activities on the photosynthetic oxygen-evolving complex which are observed when water is the substrate (Sandusky, P.O. and Yocum, C.F. (1984) Biochim. Biophys. Acta 766, 603–611; Sandusky, P.O. and Yocum, C.F. (1986) Biochim. Biophys. Acta 849, 85–93). Experiments using the ionophore A23187 along with exogenously added Mn^{2+} indicate that hydrogen peroxide oxidation by chloride-depleted thylakoids is catalyzed by a pool of free or loosely bound Mn. Further, EPR measurements indicate that the presence of hydrogen peroxide, under the assay conditions employed in our experiments, release Mn from the oxygen-evolving complex in chloride-depleted membranes. It is therefore this amine-sensitive pool of loosely bound Mn that is responsible for catalysis of H_2O_2 photooxidation in chloride-depleted thylakoids.

Introduction

Hydrogen peroxide is an obvious candidate for a putative intermediate between water and molecular oxygen in the process of photosynthetic water oxidation by Photosystem II (PS II), since it is two electrons more oxidized than a pair of water molecules and two electrons more reduced than oxygen. A number of investigations have indicated that hydrogen peroxide can, in fact, interact with the oxidizing side of PS II. For example, Kelly and Izawa [1] have shown that H_2O_2 acts as a donor to the oxidizing side of PS II in chloride-depleted membranes in a DCMU-sensitive reaction, while Velthuys and Kok [2] have reported results showing that H_2O_2 reduces the S-states in a reversible manner. Similar results, reported by Mano et al. [3] and Frasch and Mei [4], support the conclusion that under the appropriate conditions, hydrogen peroxide can interact with the oxidizing side of PS II, specifically with the manganese cluster that catalyzes the oxidation of water. It is therefore reasonable, in lieu of evidence to the contrary, to

* Present address: Chemical Biodynamics Laboratory, Lawrence Berkeley Laboratory, University of California, Berkeley, CA 94720, U.S.A.

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; AEPD, 2-amino-2-ethylpropanediol; DCBQ, 2,5-dichloro-*p*-benzoquinone; DMQ, 2,5-dimethyl-*p*-benzoquinone; PS II, Photosystem II; BSA, bovine serum albumin; Chl, chlorophyll.

Correspondence: C.F. Yocum, Departments of Biology and Chemistry, University of Michigan, Ann Arbor, MI 48109, U.S.A.

suppose that after chloride depletion, hydrogen peroxide would react with the disabled oxygen-evolving complex.

There are two plausible and testable alternative hypotheses for a mechanism by which hydrogen peroxide might be oxidized by a chloride-depleted oxygen evolving complex. Based on results described in previous publications [5–7] we have suggested that chloride, in its role as a cofactor of the oxygen-evolving complex, binds to a ligand site on manganese (although such binding, if it occurs, has not been detected by EXAFS spectroscopy [8]). If this were the case, hydrogen peroxide might be oxidized in chloride-depleted membranes at a vacated chloride site on manganese, and it should be possible to show that inhibitors of water oxidation which are competitive with respect to chloride (for example, Tris, ammonia and fluoride) are also competitive inhibitors of H_2O_2 oxidation. Alternatively, it is possible that hydrogen peroxide oxidation by chloride-depleted thylakoid membranes might be catalyzed by a loosely bound pool of Mn or by Z^+ (the primary donor to P-680$^+$), and that a disabled form of the oxygen-evolving complex is in no way involved in the peroxide-to-oxygen catalytic cycle. Some support for this second hypothesis exists; Pan and Izawa [9] demonstrated that hydroxylamine-treated membranes, in which the oxygen-evolving complex has been destroyed and functional Mn is released, are nonetheless capable of hydrogen peroxide oxidation. Likewise, experiments by Velthuys [10] and by Klimov et al. [11] have shown that H_2O_2 oxidation by PS II-enriched membranes is supported by exogenous added manganese. In addition, Schröder and Åkerlund [12,13] have demonstrated that the unusual flash-induced oxygen yield pattern observed in salt-washed inside-out thylakoid preparations arise from a catalytic process involving both H_2O_2 and an EDTA chelatable pool of Mn. Since Babcock and Sauer have documented the oxidation of Mn by Z^+ [14], and Mn is known to catalyze H_2O_2 redox chemistry, one can visualize a number of different, multistep mechanisms for light induced, DCMU-sensitive hydrogen peroxide oxidation in which a free or loosely bound pool of photooxidized Mn plays the role of a redox mediator.

The experiments described in this communication address the mechanism of H_2O_2 oxidation by PS II in chloride-depleted thylakoid membranes. We show that amines which inhibit water oxidation inhibit the oxidation of hydrogen peroxide, that this inhibition differs from that observed during water oxidation in chloride-sufficient systems, and that in the absence of chloride, hydrogen peroxide labilizes functional Mn to generate an altered form of the oxygen-evolving complex which is incapable of water oxidation, but which retains the capacity to use loosely bound manganese to catalyze the photooxidation of H_2O_2.

Materials and Methods

Chloride-depleted thylakoid membranes were prepared from market spinach. Depetiolated leaves (0.5 kg) were homogenized in a blender containing 500 ml of cold buffer (0.4 M NaCl, 50 mM Hepes (pH 7.5), 2 mM $MgCl_2$, 1 mM EDTA and 1 mg per ml BSA). The resulting homogenate was filtered through four layers of cheese cloth and centrifuged for 30 s at $300 \times g$ to remove cell debris. The membranes were then collected by centrifugation at $5000 \times g$ for 10 min. The pellets were resuspended and homogenized into 500 ml of a cold wash buffer containing 200 mM Hepes (pH 7.5). Pellets were collected at $6000 \times g$ (10 min). This washing procedure was then repeated. The pellets collected after the second washing step were resuspended and homogenized into cold buffer containing 200 mM Hepes (pH 8) and a 3 μg per ml gramicidin. The membranes were incubated in 1 liter of this medium at a Chl concentration of approx. 100 μg per ml in the dark, on ice, for one half hour. After incubation the membranes collected at $6000 \times g$ (10 min). The pellets were resuspended and homogenized in 0.4 M sucrose and 20 mM Hepes (pH 7.5). The Chl concentration was adjusted to between 2 and 3 mg per ml using this same buffer; the membranes were stored at −70 °C until use. Membranes prepared in this manner were essentially inactive when assayed as described in 'Results' in the absence of chloride, but evolved oxygen at rates of 250–300 μmol oxygen per mg Chl per h when assayed in the presence of 10 mM chloride in an

assay medium containing 50 mM Hepes (pH 7.8), 7 μg per ml gramicidin and 250 μM DCBQ.

Chlorophyll concentrations were determined as described in Ref. 15. Measurements of oxygen-evolving activity were carried out in a thermostatted (25°C) Clark-type electrode [16]. Assay conditions varied from experiment to experiment and are described in the figure legends.

Chloride-free amine stock solutions were adjusted to the desired pH using H_2SO_4, NaOH or Hepes. Contaminating chloride in buffer and amine solutions was tested qualitatively using the $AgNO_3$ test described by Kelly and Izawa [1]. (In order to prevent reconstitution of activity by chloride, sulfate was used as the counter ion in all the reagent solutions.)

ESR spectra of Mn^{2+} were taken at room temperature on a Bruker ER 200E-SRC X-Band (9.7 GHz) spectrometer fitted with a TM cavity. The aqueous samples were held in a Scanco model s-813 quartz flat cell which accommodated a volume of 0.5 ml. The cavity was swept with a flow of N_2 to prevent heating of the samples. The console modulation amplitude readings were corrected to give the true modulation amplitudes using a calibration curve based on the modulation amplitude broadening of the DPPH spectrum [17,18]. Estimations of loosely bound Mn^{2+} in intact thylakoid membranes and total Mn^{2+} in acidified samples were made as described by Yocum et al. [19]. More details are given in Table IV. Instrument settings were the same for all spectra; 115 mW microwave power, 3495 G center filed, 1000 G sweep width and 12.5 G peak to peak modulation amplitude.

Results

Inhibition of hydrogen peroxide photooxidation by amines and fluoride

Hydrogen peroxide oxidation by chloride-depleted thylakoid membranes can be studied from the rate of hydrogen peroxide-supported oxygen evolution measured on a Clark-type oxygen electrode (see Fig. 1). In several respects the assay conditions differ from those which would be used in measuring rates of water oxidation. Firstly, the 'chloride-depleted thylakoid membranes' are not completely chloride depleted as stored after the chloride depletion procedure described in Materials and Methods. Assays employing these membranes, carried out at pH 7.5, can give rates as high as 70 μmol oxygen per mg Chl per h in the absence of added chloride. Therefore, in order to complete the chloride-depleting process, the 'chloride-depleted thylakoid membranes' were incubated in the oxygen-electrode cuvette for 30 s prior to assay in 10 μM gramicidin at pH 7.8. Under these conditions, oxygen evolution in the absence of either H_2O_2 or chloride is too low to quantitate. Secondly, for reasons not completely understood at this time, DCBQ is ineffective in supporting hydrogen peroxide photooxidation. It is probable that the hydrogen peroxide itself, present at mM concentrations, reacts with and consumes the DCBQ; DMQ was effective as an alternative, stable, electron acceptor in our assay system. Lastly, addition of H_2O_2 to a reaction medium containing thylakoid membranes causes very high rates of oxygen evolution prior to application of the actinic light. This light-independent oxygen evolution is probably due to a catalase contaminant present in the membrane preparations, and is completely suppressed by the inclusion in the reaction medium of 4 mM KCN.

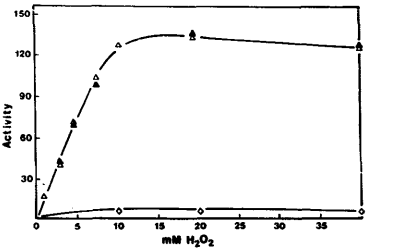

Fig. 1. H_2O_2-supported oxygen evolution by chloride-depleted thylakoid membranes. Membranes were assayed at chlorophyll concentrations of 30 μg/ml in a reaction medium containing 100 mM Hepes (pH 7.8), 7 μg/ml gramicidin and 4 mM KCN. The thylakoid membranes were incubated in the reaction medium for 1 min prior to assay. H_2O_2 and 250 μM DMQ were added 15 s before assay. Activity is given as μmoles oxygen per mg Chl per h. The results are the average of three experiments (six assays per point). Open triangles, control; solid triangles, 100 mM Na_2SO_4 in assay medium; diamonds, 3 μM DCMU in assay medium.

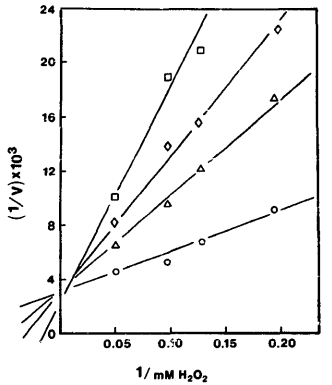

Fig. 2. Lineweaver-Burk plot demonstrating that Tris is a competitive inhibitor of H_2O_2-supported oxygen evolution by chloride-depleted membranes. Assays were performed as described in the legend of Fig. 1. The results are the average of four experiments (eight assays per point). Tris-free base concentrations: circles, 0; triangles, 1.6; diamonds, 3.1; and squares, 6.3 mM.

The dependence of light-induced hydrogen-peroxide-supported oxygen evolution catalyzed by chloride-depleted thylakoid membranes on H_2O_2 concentration is shown in Fig. 1. This reaction has a K_m for H_2O_2 of about 4 mM and a V_{max} of 130 μmol oxygen per mg Chl per h. The reaction is unaffected by 100 mM sulfate but is completely suppressed by 3 μM DCMU. Light-induced hydrogen peroxide oxidation by chloride-depleted thylakoid membranes is competitively inhibited by Tris (Figs. 2 and 3). Similar results were obtained using 2-ethyl-2-amino propanediol (data not shown). However, ammonia is an uncompetitive inhibitor of the reaction (Figs. 4 and 5) while fluoride is a partial noncompetitive inhibitor (data not shown). These differences between the inhibitory effects of ammonia and fluoride on chloride-supported water oxidation and hydrogen peroxide oxidation by chloride-depleted thylakoid membranes are summarized in Table I. The differences are obvious, and indicate that hydrogen peroxide photooxidation is not a simple reaction involving

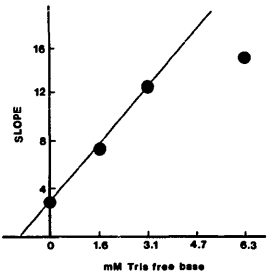

Fig. 3. Replot from experiment of Fig. 2; the Tris K_i is approximately 0.8 mM.

only the binding of the substrate to the vacated chloride binding site in the oxygen-evolving complex. This conclusion is supported by the experiments described below.

Inhibition of H_2O_2 photooxidation by the ionophore A23187

Irreversible inhibitions which destroy oxygen evolution also release some or all of the PS II-associated Mn into the thylakoid lumen [20]. If free or loosely bound Mn acts as a redox mediator between H_2O_2 and some component on the

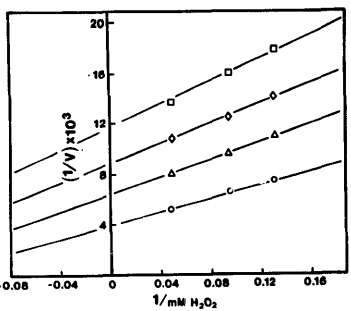

Fig. 4. Lineweaver-Burk plot demonstrating that ammonia is an uncompetitive inhibitor of H_2O_2 oxidation by chloride-depleted thylakoid membranes. These results are the average of four experiments (eight assays per point). Ammonia circles, 0; triangles, 2.6; diamonds, 5.2; and squares. 7.8 mM.

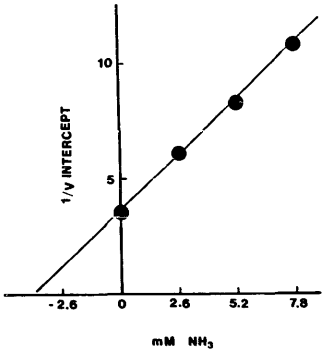

Fig. 5. Replot of Fig. 4 experiment. Ammonia has a K_i of approximately 3.6 mM.

oxidizing side of PS II, then it is likely that the local lumen concentration of Mn is critical to the rate of hydrogen peroxide oxidation. The thylakoid lumen volume has been estimated as 25 μl per mg chlorophyll [21]. Therefore, at a chlorophyll concentration of 30 μg per ml, a thylakoid suspension assayed under our conditions for H_2O_2 oxidation activity would contain about 1 μl of thylakoid lumen volume in an external volume of 1.6 ml.

TABLE I

INHIBITORY PROPERTIES OF AMINES AND FLUORIDE ON THE OXIDATION OF WATER, OR OF HYDROGEN PEROXIDE IN CHLORIDE-DEPLETED THYLAKOID MEMBRANES

Activity was assayed as described in Materials and Methods. K_i values in mM are given in parentheses.

Inhibitor	Activity assayed	
	Water oxidation; inhibition with respect to Cl^-	Hydrogen peroxide oxidation; inhibition with respect to hydrogen peroxide
Tris	competitive (14)	competitive (0.8)
AEPD	competitive (0.8)	competitive (0.2)
Ammonia	mixed (0.39)	uncompetitive (3.6) [a]
Fluoride	competitive (2.5)	noncompetitive (150) [b]

[a] K_i'.
[b] $K_i = K_i'$.

TABLE II

A23187 INHIBITION OF HYDROGEN PEROXIDE OXIDATION IN CHLORIDE-DEPLETED THYLAKOID MEMBRANES: EFFECTS OF ADDED DIVALENT CATIONS

Assays were carried out as described in Materials and Methods; when present the concentration of A23197 was 1.25 μM.

Addition	Activity (μmol O_2/h per mg Chl)	Control (%)
None	124	100
3 μM DCMU	5	4
1 mM EDTA	81	65
A23187	35	28
A23187 + 5 mM $MgSO_4$	25	20
A23187 + 5 mM $CaSO_4$	25	20
A23186 + 0.5 mM $MnSO_4$	113	90

The ionophore A23187 mediates the passage of Mn^{2+} and other divalent cations through biological membranes [22]. Thus, if hydrogen peroxide oxidation is catalyzed by a population of Mn which has been dissociated from its native binding site in the oxygen-evolving complex, A23187 might be expected to suppress the photooxidation reaction by affecting a 1 : 1600 dilution of the lumen Mn concentration.

Low concentrations of A23187 (1.25 μM) do, in fact, suppress hydrogen peroxide oxidation as shown in Table II. This inhibition is reversed by 500 μM $MnSO_4$; $CaSO_4$ and $MgSO_4$ have little effect. Further, in the presence of A23187 and exogenously added Mn^{2+}, Tris inhibition of H_2O_2

TABLE III

TRIS INHIBITION OF HYDROGEN PEROXIDE OXIDATION BY CHLORIDE-DEPLETED THYLAKOID MEMBRANES: REVERSAL BY A23187 AND ADDED MANGANESE

Assays were carried out as described in Materials and Methods; when present, the concentration of A23187 was 1.25 μM.

Addition	Activity (μmoles O_2/h per mg Chl)		Inhibition (%)
	−Tris	+10 mM Tris-SO_4	
None	120	65	46
A23187 + 20 μM $MnSO_4$	128	75	41
A23187 + 5 mM $MnSO_4$	128	126	0

oxidation is attenuated when the external Mn^{2+} concentration is increased (see Table III). This suggests that Tris induces an inhibition of H_2O_2 oxidation by complexing with free or loosely bound Mn, which is consistent with a Lewis acid–Lewis base interaction between the metal and the amine-free-base [7,23,24].

It is important to note that A23187 does not inhibit chloride-supported water oxidation (data not shown). This is the case even when 'chloride depleted' thylakoid membranes are incubated with A23187 prior to addition of chloride, a result which suggests that it is the presence of hydrogen peroxide which releases PS II-associated Mn into the thylakoid lumen. This point is addressed below.

H_2O_2-induced release of manganese from chloride-depleted thylakoid membranes

The preceding experiments suggest that hydrogen peroxide oxidation in chloride-depleted thylakoid membranes is catalyzed by a pool of free or loosely bound Mn localized in the thylakoid lumen, which is derived from the functional Mn cluster of the oxygen-evolving complex. However, EPR spectra of these chloride-depleted membranes show very little loosely bound Mn prior to exposure to H_2O_2 (data not shown). Ghanotakis et al. [25] have reported that a variety of reductants, including H_2O_2, are capable of inducing the release of Mn from PS II-enriched membranes depleted of the 17 and 23 kDa extrinsic polypeptides. It thus seems quite likely that during the H_2O_2 oxidation assays, hydrogen peroxide itself induces the release of Mn into the thylakoid lumen where the metal subsequently acts as a redox mediator in the catalysis of H_2O_2 photooxidation. The hydrogen-peroxide-induced release of Mn is documented by the results of EPR studies summarized by the data of Table IV. In these experiments, chloride-depleted thylakoids were incubated at chlorophyll concentrations of 0.2 mg per ml in the presence of 1.25 μM A23187 and 1 mM EDTA. In other respects, the conditions of the incubations duplicated exactly those of the H_2O_2 oxidation assays carried out in the oxygen electrode cuvette. After incubation plus or minus 20 mM H_2O_2 the membranes were collected by centrifugation, resuspended to a chlorophyll concentration of about 2 mg per ml, acidified and transferred to an EPR flat cell. The Mn which remained bound to the membranes during the incubation was then observed by EPR according to the procedure described in Materials and Methods.

TABLE IV

MANGANESE EXTRACTION FROM CHLORIDE-DEPLETED THYLAKOID MEMBRANES BY HYDROGEN PEROXIDE AND TRIS

Chloride-depleted thylakoid membranes were incubated at 25 °C plus or minus hydrogen peroxide at Chl concentrations of 0.2 mg/ml for 30 s in the dark, and then for another 30 s in actinic light. The reaction mixture also contained Hepes (50 mM, pH 7.8), DMQ (0.25 mM), 4 mM KCN, 7 μg/ml gramicidin, 1.25 μM A23187 and 1 mM EDTA. Following the incubation, the membranes were collected by centrifugation and resuspended to a final concentration of 2 mg Chl/ml in 0.4 M sucrose containing 50 mM Hepes (pH 7.5) and then acidified by addition of HCl to a final concentration of 0.5 M. Samples were transferred to an EPR flatcell, 6-line spectra from hexaquo manganese were recorded, and relative concentrations of manganese retained by the membranes were estimated from the amplitudes of the third line from the low-field side of the 6-line spectrum.

Treatment	Manganese loss (%)
None	0
20 mM H_2O_2	82
20 mM H_2O_2 + 10 mM NaCl	40
0.8 M Tris	62

The data of Table IV show that hydrogen peroxide induces a release of Mn, as evidenced by the increased loss of manganese from the chloride-depleted thylakoid membranes. The extent of this loss is roughly equivalent to that induced by 0.8 M Tris (Table IV). Hydrogen peroxide added after acidification of control membranes had no effect on the Mn^{2+} signal, and the results of other experiments (not shown) indicated that the presence or absence of DMQ, KCN and actinic light had no significant effect on the H_2O_2-induced Mn release. Chloride, however, does attenuate the amount of Mn released, as shown by the data of Table IV.

Discussion

Fluoride and amines such as ammonia and Tris inhibit oxygen evolution activity by binding in

competition with chloride. Ammonia also has a secondary mode of inhibition which is noncompetitive with respect to chloride [6]. Although these reagents also inhibit hydrogen peroxide oxidation by chloride-depleted thylakoid membranes, their modes of inhibition vary widely. Tris and AEPD competitively inhibit hydrogen peroxide oxidation, but ammonia is an uncompetitive inhibitor of the reaction. The effects of fluoride we report here are particularly interesting because, as an inhibitor of water oxidation, this anion competes both with chloride binding and with the binding of inhibitory amines (K_i = 2.5 mM [7]). It is therefore curious that in the case of the hydrogen peroxide photooxidation reaction fluoride produces only a partial (40%) noncompetitive inhibition, and only at very high concentrations (K_i = 150 mM), suggesting that substantial changes to native halide ligation sites have been induced by exposure of the manganese cluster to hydrogen peroxide.

The results of our experiments using A23187 and exogenously added Mn^{2+} demonstrate that light-induced hydrogen peroxide oxidation by chloride-depleted thylakoid membranes depends on the presence of a pool free of loosely bound Mn (which can originate from the destruction of oxygen evolution activity by the labilizing action of H_2O_2). In this regard, our data reinforce the findings reported by Velthuys [10] and by Klimov et al. [11]. It seems likely, therefore, that the mechanism of the Tris and AEPD inhibitions of hydrogen peroxide oxidation involve ligation of the free, catalytically cycling Mn by the amine following a mechanism in which the amine competes with H_2O_2 for a ligand site on the metal. This suggestion is consistent with the observation that the inhibition can be relieved by adding excess Mn, and reinforces the earlier conclusion that amines bind to manganese by means of a Lewis base–Lewis acid interaction [23]. Interestingly, the data of Table II provide a direct confirmation of amine ligation to manganese; note the decreased sensitivity to Tris of the hydrogen peroxide photooxidation reaction when the external concentration of Mn is increased in the presence of A23187. On the other hand, we have no facile explanation for the uncompetitive mode of inhibition observed in the presence of ammonia (Table I). Uncompetitive inhibition will arise in the simplest case from the formation of a tertiary inhibitor-catalyst-substrat complex. While an unreactive ammonia-Mn-peroxide complex would not be an unreasonable explanation for the observation of uncompetitive inhibition, there are other possibilities which cannot be discarded based on the results of steady-state kinetics experiments alone [26]. Regardless of the specific mechanisms involved, it is clear that the effects of Tris, AEPD, ammonia and fluoride on the light-induced oxidation of hydrogen peroxide are substantially different from the inhibitions of photosynthetic oxygen evolution produced by these reagents.

Previously, Ghanotakis et al. [25] have shown that a wide variety of reductants including NH_2OH, hydroquinone and H_2O_2 release Mn and destroy oxygen-evolving activity in salt-washed PS II-enriched membranes. Schröder and Åkerlund [13] have described the H_2O_2-induced destruction of the S-states in salt-washed inside-out thylakoids. It is therefore not surprising that PS II-associated Mn in chloride-depleted thylakoids is also released by the action of H_2O_2. This peroxide-induced effect in chloride-depleted membranes does not require light (as Tris-induced Mn release does [27,28]), and probably involves the reduction of Mn to a labile oxidation level as proposed by Ghanotakis et al. [25]. A similar mechanism for NH_2OH inhibition (dark extraction of manganese) has been described in Ref. 29, and Beck and Brudvig [30] have more recently shown an interaction of chloride with the site of NH_2OH attack.

In light of the results presented here and those of Schröder and Åkerlund [13], it is clear that hydrogen peroxide oxidation does not proceed through a modified form of the oxygen-evolving complex; rather, the Mn complex of PS II, in the absence of chloride, is labilized by the presence of hydrogen peroxide. Clearly, the latent, chloride-depleted oxygen-evolving complex cannot be viewed as an enzymatic system capable of catalyzing the oxidation of hydrogen peroxide in place of the natural substrate water. There is thus no evidence that oxidizing equivalents can travel between P-680$^+$ and the site of water oxidation in chloride-depleted membranes. This conclusion agrees with the results of Itoh et al. [31] and Theg et al. [32], which show that chloride depletion

creates a lesion in electron transport between P-680 and the site of water oxidation, and with the results of Ono et al. [33] showing that chloride depletion alters the EPR properties of the manganese cluster of PS II.

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