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TOPOLOGY OF NH_2OH-INDUCED Mn(II) RELEASE FROM CHLOROPLAST THYLAKOID MEMBRANES

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Permeant and impermeant metal ion chelators have been used in conjunction with NMR relaxation time measurements (T_1) of solvent protons to probe the membrane topology of the Mn(II) released from the water-oxidizing center of chloroplast thylakoid membranes by NH_2OH. Chelex, a tightly binding divalent metal ion exchanger, quantitatively removes Mn^{2+} (added as $MnCl_2$) from the external thylakoid membrane without significantly affecting oxygen evolution activity or photophosphorylation efficiency. Because of its obvious impermeance (the resin is supplied as 0.2 mm beads), chelex selectively removes only manganese that is in equilibrium with the external aqueous phase. Both internal and external manganese pools are removed by chelex in the presence of A23187, a divalent cation-specific ionophore. Topological experiments using these reagents have shown that NH_2OH releases Mn(II) predominantly to the loculus in freshly prepared, dark-adapted thylakoid membranes at 0–3°C. This topology changes radically as a result of three pretreatments: (1) incubation of thylakoid membranes in the dark at 25°C, which redirects Mn(II) release toward the external medium with a half-time of 10–15 min; (2) illumination with saturating white light, which decreases the half-time of reorientation to about 1 min; (3) freeze-thawing in 0.4 M sucrose, which results in the appearance of 40–60% of the NH_2OH-liberated Mn(II) in the external medium. None of these treatments substantially degrades O_2 evolution activity or osmotic integrity as judged from measurements of photophosphorylation efficiency. It is concluded that the topology of the manganese site associated with photosystem II is not static but changes dramatically in response to external stimuli, possibly reflecting a regulatory mechanism of photophosphorylation.

Introduction

A singularly productive influence of the chemiosmotic coupling hypothesis of Mitchell [1–3] has been the stimulation of interest in assessing the topology of energy transducing membrane systems. The results of such studies have served to substantiate the prediction by Mitchell that the redox chains of organelles and bacteria are imbedded within membranes in a vectorial arrangement to promote the generation of proton and/or ion gradients. For the thylakoid membranes of chloroplasts, evidence exists to support a surface exposed topology of the chloroplast coupling factor (CF_1), ferredoxin, ferredoxin : NADP oxidoreductase, and the reducing side of PS II [4]. Similarly, components of the electron transfer chain such as cytochrome *f* [5] and plastocyanin [6] appear to be shielded from the external medium (but see also Ref. 7).

The water-oxidizing complex of PS II should, by chemiosmotic reasoning, also be internally oriented, by virtue of the fact that protons liberated from this reaction are required for maintenance of energy coupling at site II. Evidence for the internal orientation of this reaction is, however, indirect. Fowler and

Abbreviations: PS, Photosystem; Tris, tris(hydroxymethyl)-aminomethane; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine; Chl, chlorophyll.

Kok [8] showed that protons liberated from water by short flashes showed a delayed appearance in the external medium. It has also been shown (Junge and coworkers [9,10], Saphon and Crofts [11], Hope and Moreland [12]) that proton release, monitored by neutral red absorbance changes in externally buffered media, is consistent with deposition of protons originating from water into the thylakoid loculus. Inactivation of water oxidation in uncoupled thylakoid membranes by elevated pH has been cited as evidence for the internal topology of water oxidation [13,14], and assessments of the topology of the oxidizing side of PS II using lipophilic and hydrophilic electron donors suggest that electron donation to PS II occurs at a site shielded from the external medium [15]. Other data exist to suggest that water oxidation may not occur in the thylakoid loculus, however. Experiments with proteases [16], antisera against PS II membranes [17–22] and the inactivating effects of hydrophilic protein modifying reagents [23] have been interpreted to indicate that some part of the oxygen-evolving system may be exposed to the external surface of the membrane (but see Ref. 24).

A relatively unambiguous marker for the location of the site of water oxidation is Mn(II) released by inactivation treatments such as Tris. Blankenship and Sauer [25] exploited this approach, utilizing the characteristic 6-line ESR spectrum of hexaquomanganese produced by Tris treatment to show that this Mn(II) is apparently sequestered by thylakoid membranes at an EDTA-accessible site. An alternative method for monitoring Mn(II) topology is provided by the enhancement of the nuclear magnetic relaxation rate of water protons observed after NH_2OH inactivation of water oxidation [26,27]. In constrast to the ESR experiment, which monitors only the soluble hexaquomanganese species, NMR measurements of spin lattice relaxation rates ($T_1^{-1} \equiv R_1$) are sensitive primarily to the presence of membrane-bound Mn(II) (or strictly, to Mn(II) bound in sites of long reorientational correlation time [26]).

We have utilized the NMR experiment in extensive investigations to analyze the origins of R_1 in thylakoid suspensions [26–31] and to identify the causes of light-induced changes in R_1 [28,30]. We have in addition utilized this technique to monitor the effects of NH_2OH on water oxidation and to explore the mechanism by which NH_2OH inactivates this reaction [31]. Throughout these studies we have observed that NH_2OH produces two pools of NMR-observable Mn(II); one of these pools is accessible to EDTA, whereas the other is not [26]. In the present communication we describe the results of experiments utilizing permeant and inpermeant chelating probes to assess Mn(II) topology, after NH_2OH treatment of thylakoid membranes. Our results indicate that this Mn(II) is exposed to the solvent predominantly in the lumen of the thylakoid membrane in freshly isolated dark-adapted membranes. This topology is not static, however, and our present data show that substantial reorientation of Mn(II) topology is observed under mild conditions which cause little, if any, perturbation of physiological activity or osmotic integrity.

Materials and Methods

Thylakoid membranes were isolated from market spinach using the procedure of Robinson and Yocum [32] with the modifications that Hepes (pH 7.7) replaced Tricine in all media and that 1 mM EDTA was present during homogenization of the leaves. Freshly isolated thylakoids were resuspended in 50 mM Hepes, pH 7.5, containing 66 mM NaCl at 3°C for immediate use in experiments, or alternatively the membranes were resuspended in sucrose (0.4)/Hepes (20 mM, pH 7.5)/NaCl (15 mM) medium and stored frozen at −70°C until used. Thylakoids prepared by this method yielded uncoupled rates of oxygen evolution of 320–380 μmol O_2/h per mg Chl; P/e_2 values of about 1.2 were routinely observed.

Apparatus for the NMR measurements (all at 3°C, 20.7 MHz) has been described previously [26,27]. Preillumination of thylakoids was carried out at 25 ± 1°C using a coil of clear-walled catheter tubing as previously described [31]. A stream of air thermostatically maintained at 25 ± 1°C was directed over the coil during illumination to maintain the desired temperature. NH_2OH inactivation was conducted by addition of a 100 mM stock solution of NH_2OH, neutralized to pH 7.5 just before use, directly to the chloroplast suspension in the NMR tube to give a final concentration of 5 mM NH_2OH. Chelex-100 was obtained from Bio-Rad and was preequilibrated with an excess of 30 mM $MgCl_2$ in Hepes buffer (pH 7.7). Immediately before addition of Chelex to the chloroplast suspension, excess solvent was removed from

the resin with the aid of a fine hypodermic needle and then by tamping the resin with tissue. A23187 was obtained from Calbiochem.

Results

In order to determine the topology of a component of the photosynthetic electron transport apparatus, one must utilize probes that are well-defined with respect to permeance of the thylakoid membrane. For Mn(II), a convenient probe is Chelex, which is a very tightly binding chelator of divalent metal ions and which exhibits a high selectivity for transition metals, including Mn^{2+}, over Mg^{2+}. The resin is supplied as 0.2 mm diameter beads, which are clearly impermeant toward the thylakoid membrane. Chelex can remove, with high affinity, free Mn^{2+} in the external solvent as well as surface-exposed Mn(II) that equilibrates between sites on the thylakoid membrane and the external medium. It is very unlikely that Chelex can remove very tightly bound, surface-exposed Mn(II) that is not in equilibrium with the external medium, since this would require intimate surface contact between the resin, for which the externally exposed surface area is minute, and the thylakoid surface on which Mn(II) is exposed.

The application of Chelex in demonstrating the topology on thylakoid membranes of exogenously added Mn(II) is shown in Fig. 1, where the exchanger removes the ion quantitatively. Also present in Fig. 1 are data showing that Chelex produces no deleterious effects on physiological activity (photophosphorylation, oxygen evolution) and that the ion exchange resin does not adsorb thylakoid membranes, removing them from the suspension.

Since the data in Fig. 1 indicate that Chelex is a physiologically benign agent for the removal of Mn(II) from the exterior surfaces of thylakoid membranes, we examined its use for assessing the topology of Mn(II) exposed to solvent by NH_2OH treatment. We have previously shown that the R_1 enhancement resulting from NH_2OH treatment correlates closely with the kinetics of inactivation of water oxidation and reflects the appearance of Mn(II) from the water-oxidizing complex [27]. Fig. 2A presents the results of a typical experiment where freshly isolated thylakoids were first exposed to 5 mM NH_2OH at 3°C to permit the development of the R_1 enhancement.

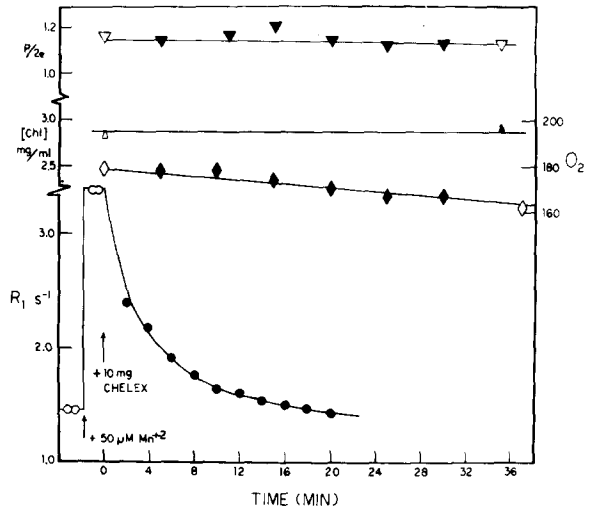

Fig. 1. The time course of removal of exogenous Mn(II) (50 μM, added as $MnCl_2$) from thylakoid membranes (equivalent to 2.5 mg Chl/ml) by gentle stirring in the presence of Chelex (50 mg damp resin/ml). the effect of this procedure on the $P/2e^-$ ratio (△,▲), on the chlorophyll concentration (△,▲), and on O_2 activity (μmol O_2/mg Chl per h, in the presence of ADP + P_i) (◇,◆) are shown. Open symbols correspond to measurements on control samples, unexposed to Chelex.

Next, Chelex was introduced to the suspension with gentle stirring, and, as seen in the figure, a small fraction of the NH_2OH-induced relaxivity is removed from the thylakoid membranes. The effect of A23187 on the Chelex-inaccessible portion of the NMR-visible Mn(II) is also shown in Fig. 2A. Addition of this divalent cation-specific ionophore effectively removes the (previously) Chelex-inaccessible pool of Mn(II) returning R_1 to its baseline value. The order of addition is reversed in the experiment of Fig. 2B showing that A23187 alone is ineffective in suppressing the R_1 enhancement and that the total suppression observed in Fig. 2A reflects an increased accessibility of liberated Mn(II) to Chelex. Although findings such as those shown in Fig. 2 were typical of most experiments involving freshly isolated thylakoids at 0–5°C, in some cases the Chelex-sensitive fraction of the R_1 enhancement was much greater (approx. 40%). This suggested that the topology of Mn(II) exposed to the solvent by NH_2OH may not be static, a point considered more fully below.

The data in Fig. 2 show that two topologically

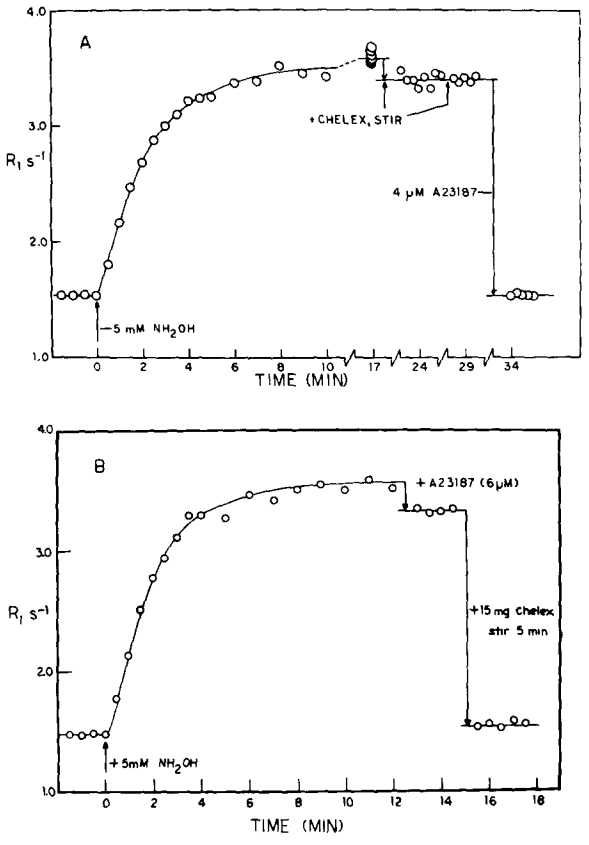

Fig. 2. (A) Effect of Chelex and A23187 on NH_2OH-induced R_1 enhancement following addition of NH_2OH (5 mM final concentration) at $t = 0$ is shown. After 17 min, Chelex (0.1 g damp resin/ml) was added and the sample was stirred for 6 min, R_1 was measured and the sample was stirred again for 3 min and R_1 was remeasured. 4 μm A23187 was then added and the sample was stirred for 10 min prior to the final set of R_1 measurements. All operations and measurements were carried out at 0–3°C. (B) Similar to (A), except that the order of addition of A23187 and Chelex was reversed. Final concentrations were 6 μm A23187, 0.1 g/ml Chelex with 5 min stirring. Samples contained 3 mg Chl/ml.

distinct pools of Mn(II) are exposed to the solvent by NH_2OH inactivation; one of these pools (the smaller) is accessible to Chelex, and therefore in equilibrium with the external medium, whereas the second pool is shielded from Chelex but is reached by the ionophore A23187. Blankenship and Sauer [25] utilized EDTA chelation of hexaquomanganese, a treatment which suppresses the characteristic 6-line hyperfine ESR spectrum of the ion, to explore the topology of Mn(II) released to the solvent by Tris-inactivation of water oxidation. These results were used to support the assertion that Mn(II) is liberated to the thylakoid loculus, where it is rapidly chelated by added EDTA; they concluded that the chelator rapidly crosses the thylakoid membrane with a $t_{1/2}$ of about 750 ms. Fig. 3 shows the results of an experiment similar to that of Fig. 2, except that EDTA replaced Chelex as the added, strong chelator of Mn(II). The results shown are typical of many similar experiments and are somewhat surprising; EDTA removes the same pool of Mn(II) that is accessible to Chelex and leaves behind a large population of the ion; only when A23187 is added does the R_1 enhancement return to the baseline value. These and other experiments, which suggest that EDTA, like Chelex, is not membrane-permeant, will be described more fully elsewhere (Sharp, R.R. and Yocum, C.F., unpublished results).

Freshly isolated thylakoid membranes show a manganese topology that is predominantly Chelex inaccessible. Since these membranes may be stored frozen at −70°C with little, if any loss of physiological function, we next examined the topology of Mn(II) in such preparations (Fig. 4). Note that approx. 50% of the R_1 enhancement observed after inactivation of water oxidation by NH_2OH is sensitive to Chelex in these preparations. This result has been

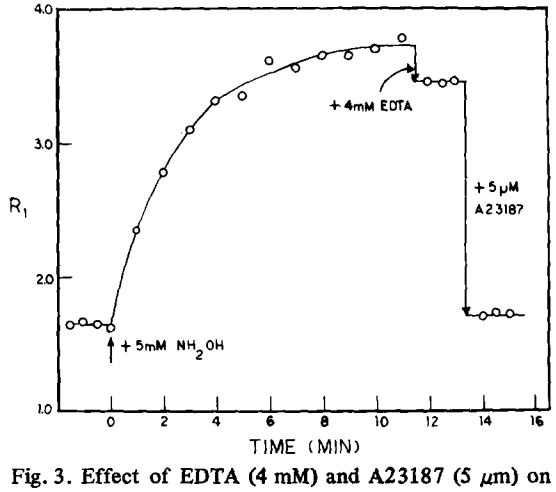

Fig. 3. Effect of EDTA (4 mM) and A23187 (5 μm) on the NH_2OH-induced R_1 enhancement is suspensions of freshly prepared chloroplast thylakoid membranes (3.0 mg Chl/ml).

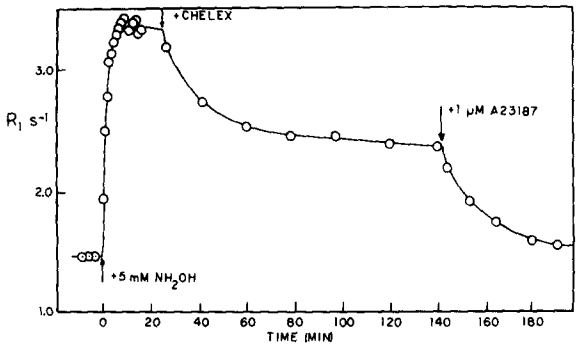

Fig. 4. Effect of Chelex (50 mg/ml) and A23187 (1 μm) on the NH_2OH-induced R_1 enhancement in frozen thylakoid suspensions.

repeatedly observed with a large number of samples of frozen thylakoid preparations, and indicates that the predominantly internal topology of Mn(II) seen in freshly isolated thylakoid membranes is not fixed.

We have taken advantage of the orientation of the Mn(II) in frozen thylakoid membranes to further explore the properties of the internal and external Mn pools. For example, if the two pools of Mn(II) do exhibit different membrane accessibilities as the Chelex/A23187 data indicate, then they ought also to be differentially accessible to membrane-impermeant buffers such as Mes, Hepes or Tricine. Of these buffers, only Tricine is a significant chelator of Mn(II) [33]. This is confirmed by the data of Fig. 5, where the effects of Hepes and Tricine (both at pH 8.0), on R_1, produced by exogenously added Mn(II), are shown. Tricine at increasing concentrations effectively lowers R_1, whereas Hepes shows only a very weak binding capacity. The effect of Tricine (but not of Hepes or Mes) is strongly pH-dependent (data not shown). In this buffer the R_1 enhancement drops sharply near pH 8.1 as the concentration of the dianionic form of the buffer increases.

We next examined the pH dependence of the NH_2OH-induced R_1 enhancement of thylakoid membranes that had been stored frozen at −70°C to preserve physiologically activity (Fig. 6). Before addition of EDTA to remove Mn(II) from the external surface

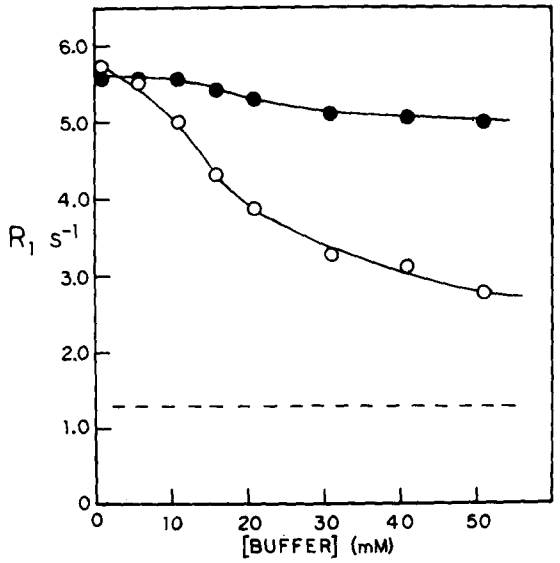

Fig. 5. Effect of two buffers, Hepes (•) and Tricine (○) on the relaxation enhancement produced by 70 μM added $MnCl_2$. Chloroplasts were pelleted and resuspended in buffer containing 0.4 M sucrose, 15 mM NaCl and 1 mM Tricine (pH 8.0). $MnCl_2$ was added to a final concentration of 70 μM, and subsequently aliquots of 1.0 M buffer (pH 8.0) were added to achieve the indicated final concentration. R_1 values were corrected for dilution.

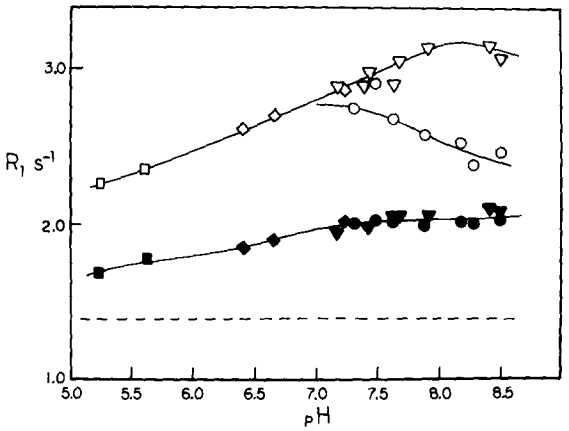

Fig. 6. pH dependence of the R_1 enhancement produced by NH_2OH in the presence of various buffers. Frozen chloroplasts were pelleted and resuspended in 0.4 M sucrose, 15 mM NaCl, 1 mM Tricine (pH 8.0), 5 μg/ml gramicidin-D and 1 mM NH_4Cl. Samples were incubated in 5 mM NH_2OH 0°C) for 15 min. Concentrated buffer at various pH values was then added to a final concentration of 50 mM, and R_1 was measured (open symbols). EDTA (1 mM final concentration) was then added, R_1 was redetermined (closed symbols), and the pH value was measured. The dashed line is the R_1 value before NH_2OH addition. (△,▲) Hepes; (◇,◆) Mes; (○,●) Tricine; (□,■) acetate.

of the membrane, the R_1 enhancement shows a gradual increase with increasing pH, presumably the net result of the titration of surface binding groups over a range of pK values. Anomalous behavior is observed in the presence of Tricine, which in the dianionic form ($pK_{a_2} = 8.1$) binds external Mn^{2+}. Also shown in Fig. 6 is the effect by buffers of varying pH on the R_1 of the EDTA-inaccessible fraction of the Mn(II) produced by NH_2OH inactivation of water oxidation. Two results are apparent in these data. First, the internally bound Mn(II) does not show as substantial an effect of pH as does the externally bound ion; second, added Tricine does not reduce the R_1, a result confirming the findings presented earlier, namely, that a pool of Mn(II) exists which is inaccessible to membrane-impermeant chelators.

Although the topological orientation of Mn(II) exposed to the solvent by NH_2OH inactivation of water oxidation is predominantly toward the loculus of the thylakoid membrane in our freshly isolated preparations that have been prepared and stored at 0–5°C (Figs. 2–3), simple dark incubation at 25°C changes this topology radically. In these experiments, freshly isolated dark-adapted thylakoid suspensions prepared and stored at 0–5°C were incubated at 25°C; at the various times shown, aliquots were withdrawn and cooled to 3°C, 5 mM NH_2OH was added to produce the relaxivity enhancement, and R_1 was measured after full development of the enhancement (circles). Then Chelex was used to assess the extent to which the Mn(II) was surface-exposed (diamonds). The experiment was conducted in the presence of 5 μg/ml gramicidin-D plus 1 mM NH_4Cl to suppress ion and/or pH gradients that could provide a driving force for transmembranal Mn transport subsequent to release of the ion by NH_2OH. In the time period covered by this experiment (60 min) there is a time-dependent reorientation of Mn(II) topology as revealed by the NH_2OH incubation/Chelex exposure technique. Samples maintained at 0°C exhibited an external Mn(II) pool corresponding typically to 15% of the R_1 enhancement, but they did not show the time-dependent reorientation observed at 25°C (Fig. 7). The reorientation observed at 25°C appears to be biphasic; the rapid phase ($t_{1/2} \approx 10$ min) occurs on a timescale during which deterioration of O_2 evolution activity is minimal, indicating that a subtle process unassociated with denaturation is involved. Similar

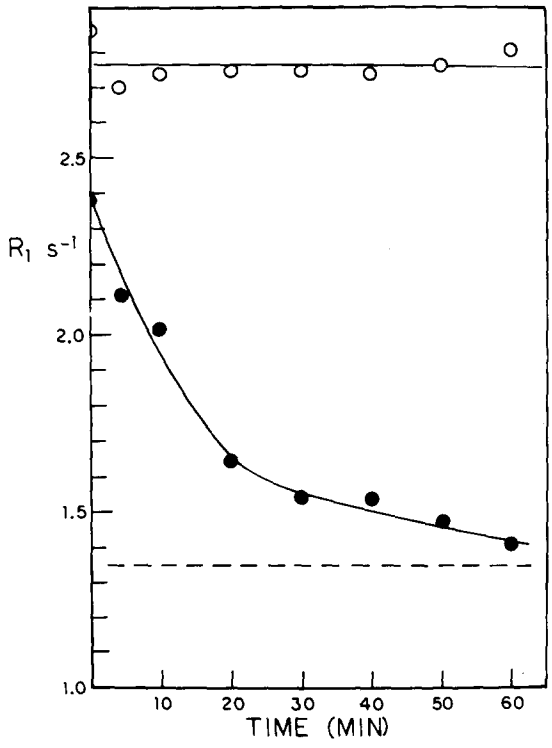

Fig. 7. Effect of dark preincubation at 25°C on the topology of Mn release by NH_2OH. A 2 ml sample of freshly prepared thylakoid membranes (2.8 mg Chl/ml containing 5 μg/ml gramicidin-D) was incubated at 25°C. 200 μl aliquots were removed after the indicated time intervals and placed in sample tubes on ice. Topological changes resulting from this treatment were then probed by adding NH_2OH (5 mM, 0°C) and measuring R_1 (○) after full development of the enhancement (15 min). External Mn was then removed by Chelex (0.2 g/ml stirred for 2 min), and R_1 was remeasured (●). Dashed line is the background R_1 before addition of NH_2OH.

results were obtained in the absence of gramicidin.

Lastly, we have examined the effects of saturating illumination at 25°C on the topology of NH_2OH-induced Mn release. In these experiments (Fig. 8), the thylakoids were incubated at 25°C for a fixed period of 3 min and were exposed to saturating white light for a variable interval during the latter portion of this incubation period. The sample was then cooled to 0°C and incubated with 5 mM NH_2OH in the presence of gramicidin-D for 15 min. Then the topology of manganese release was probed by measuring R_1 before and after addition of 1 mM EDTA. Fig. 8

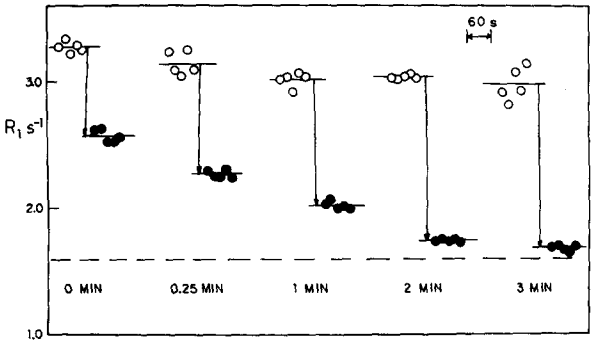

Fig. 8. Effect of preillumination on the topology of manganese release. Chloroplasts (equivalent to 3.0 mg Chl/ml contained 100 μg/ml catalase, 50 μM methyl viologen) were incubated for 3 min at 25°C and were illuminated in saturating white light for the indicated interval at the end of the incubation period. Then the samples were cooled to 3°C in the dark, gramicidin (10 μg/ml) was added plus 5 mM NH_2OH. After full development of the NH_2OH-induced R_1 enhancement (15 min), R_1 was measured (○), then EDTA (1 mM) was added to remove external Mn, and R_1 was measured (●).

shows the results of several typical experiments with various illumination periods. R_1 was measured before (open circles) and after (closed circles) addition of EDTA; the fractional drop in the R_1 enhancement relative to the baseline before addition of NH_2OH provides a measure of the externally chelatable fraction of manganese. This fraction exceeds 90% after 2 min of illumination. The results shown are typical of those obtained from several chloroplast preparations, but the kinetics and ultimate extent of reorientation occasionally varied substantially.

Discussion

Mn(II) liberated by NH_2OH or Tris can be monitored both by NMR relaxation enhancements and by the characteristic 6-line ESR spectrum of Mn-$(H_2O)_6^{2+}$. An important difference between these experiments is that NMR R_1 monitors membrane-bound Mn(II), while ESR R_1 monitors the uncomplexed aqueous Mn^{2+}. The Mn(II) released by NH_2OH or Tris produces substantial enhancements of both R_1 and of the 6-line $Mn(H_2O)_6^{2+}$ ESR signal (Ref. 25–27 and Robinson, H.H., Sharp, R.R. and Yocum, C.F., unpublished observations), evidently indicating that this manganese is in equilibrium between the aqueous medium and bound sites on the thylakoid membrane. Such an equilibrium is also suggested by the effectiveness of Chelex in removing, quantitatively, exposed Mn(II) from the external thylakoid surface, since it is difficult to imagine direct, physical access of the resin to metal-binding sites, especially those in the appressed region.

Theg and Sayre [34] have shown that most of the Mn^{2+} released by Tris is membrane-bound and ESR-silent. We have confirmed this finding for Mn^{2+} liberated by NH_2OH (Robinson, H.H., Sharp, R.R. and Yocum, C.F., unpublished observations). Preliminary data, to be presented elsewhere, show a linear, quantitative correlation between decreases of the R_1 enhancement caused by Chelex, A23187 and EDTA and the removal of 4 Mn per 400 Chl from thylakoid membranes after NH_2OH treatment. These data, as well as our previous kinetic studies [27], suggest that measurements of R_1 provide a direct, unambiguous means for detecting Mn(II) associated with the water-oxidizing center.

Wydrzynski et al. [35] and Khanna [36] give an alternate interpretation of NH_2OH-induced relaxation enhancements, namely that these arise from functional manganese that is reduced in the intact water-oxidizing center. We have shown [27,31], however, that the R_1 enhancements reflect Mn(II) that is exposed to the solvent only after destruction of oxygen-evolving centers.

The data of Fig. 1 clearly identify the existence of two topologically distinct pools of Mn(II) exposed to the solvent after NH_2OH inactivation of the water-oxidizing complex. The smaller of these pools is evidently exposed to the external surface of the thylakoid membrane, and its removal by Chelex shows that this pool equilibrates with the external medium. The second, much larger pool is Chelex-inaccessible; this pool is removed from the thylakoid membranes by addition of the divalent cation-specific ionophore A23187 in the presence of Chelex (or of EDTA, Fig. 3). This finding, that Mn(II) associated with the water-oxidizing complex is located predominantly on the lumen side of the thylakoid membrane, is in accord with previous topological assessments conducted by other techniques [8–14,24].

Further support for the topological interpretation of the two Mn pools is contained in the data of Figs.

5 and 6. Here, Tricine, a membrane-impermeant buffer which binds Mn(II) and other divalent metal ions [33] causes a depression of the R_1 enhancement associated specifically with external Mn(II). This is true both for Mn(II) added as $MnCl_2$ and for the Chelex/EDTA-accessible fraction of Mn(II) liberated by NH_2OH. The internal Mn pool (i.e., the Chelex/EDTA-inaccessible pool) is unaffected by Tricine.

The data in Figs. 2–4, 7 and 8 show that the topology of Mn(II) released from the water-oxidizing center by NH_2OH is not static. Mild treatments of these membranes produce substantial reorientations of Mn(II) towards Chelex-accessible sites on the outer surface of the membrane. In our experiments, topological changes were strongly inhibited at 0–5°C, and in fact very little topological reorientation was observed in freshly prepared thylakoid membranes maintained in the dark at 0°C over a period of many hours.

One alternative interpretation of the data of Figs. 7 and 8 is that Mn is released inside the thylakoid and subsequently diffuses (or is transported) to the external medium. In these experiments, however, the release of Mn and its subsequent removal using Chelex and A23187 were conducted under conditions that would appear to preclude transmembranal Mn transport (0°C, dark, in the presence of uncouplers). Under these conditions, the internal Mn pool is sequestered from Chelex or EDTA for many hours (e.g., Fig. 4). Although the various environmental influences studied (incubation at 25°C, illumination, freezing in 0.4 M sucrose) could conceivably promote Mn translocation, these treatments were terminated prior to the addition of NH_2OH. Furthermore, all of these treatments appear to be benign with respect to O_2 evolution activity and osmotic integrity of the thylakoids on a time-scale in which quite large topological changes occur. For example, frozen thylakoids that routinely release between 40–60% of the manganese to the external medium (values up to 75% have occasionally been observed) showed $P/2e^-$ ratios near 1.16 (range 1.10–1.20 (Robinson, H.H., unpublished results)), and O_2 evolution activities are quite comparable to those of freshly prepared chloroplasts. We therefore conclude that, as a result of these pretreatments, a large (at least approx. 50%) variable portion of the Mn liberated by NH_2OH is released to the external medium even in thylakoids that are osmotically tight and which, prior to addition of NH_2OH, show high rates of O_2 evolution activity. These observations may in part explain a number of previous topological experiments [15–24] utilizing other techniques which indicate that at least some portion of the water-oxidizing complex is externally localized.

The physiological significance of these topological changes is not clear at present, but our experiments raise the possibility that proton release from water oxidation may also exhibit variable topology. This could, for example, reflect a mechanism that limits internal pH changes under conditions of strong illumination. If such variations occur, they should be evident in $P/2e^-$ measurements or in experiments that monitor light-induced pH changes in the external medium. In comparisons of $P/2e^-$ ratios of fresh vs. frozen chloroplasts, we have found little systematic difference (at most approx. 10%) (Robinson, H.H., unpublished results). More extensive studies comparing the topologies of manganese and proton release are now in progress.

Acknowledgment

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